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Abstract

Ozonation of uncooked corn mash from the POET BPX process was investigated as a potential disinfection method for reducing bacterial contamination prior to ethanol fermentation. Corn mash (200 g) was prepared from POET ground corn and POET corn slurry and was ozonated in 250 mL polypropylene bottles. Lactic and acetic acid levels were monitored daily during the fermentation of ozonated, aerated, and nontreated corn mash samples to evaluate bacterial activity. Glycerol and ethanol contents of fermentation samples were checked daily to assess yeast activity. No yeast supplementation, no addition of other antimicrobial agents (such as antibiotics), and spiking with a common lactic acid bacterium found in corn ethanol plants,*Lactobacillus plantarum*, amplified the treatment effects. The laboratory-scale ozone dosages ranged from 26–188 mg/L, with very low estimated costs of $0.0008–0.006/gal ($0.21–1.6/m3) of ethanol. Ozonation was found to decrease the initial pH of ground corn mash samples, which could reduce the sulfuric acid required to adjust the pH prior to ethanol fermentation. Lactic and acetic acid levels tended to be lower for samples subjected to increasing ozone dosages, indicating less bacterial activity. The lower ozone dosages in the range applied achieved higher ethanol yields. Preliminary experiments on ozonating POET corn slurry at low ozone dosages were not as effective as using POET ground corn, possibly because corn slurry samples contained recycled antimicrobials from the backset. The data suggest additional dissolved and suspended organic materials from the backset consumed the ozone or shielded the bacteria.

Keywords

Ozonation, No-cook, BPX process, Corn mash, Fuel ethanol, Bacteria, Dry-grind ethanol, Lactic actid

Comments

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Reducing Bacterial Contamination in Fuel Ethanol Fermentations by Ozone Treatment of Uncooked Corn Mash

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ABSTRACT: Ozonation of uncooked corn mash from the POET BPX process was investigated as a potential disinfection method for reducing bacterial contamination prior to ethanol fermentation. Corn mash (200 g) was prepared from POET ground corn and POET corn slurry and was ozonated in 250 mL polypropylene bottles. Lactic and acetic acid levels were monitored daily during the fermentation of ozonated, aerated, and nontreated corn mash samples to evaluate bacterial activity. Glycerol and ethanol contents of fermentation samples were checked daily to assess yeast activity. No yeast supplementation, no addition of other antimicrobial agents (such as antibiotics), and spiking with a common lactic acid bacterium found in corn ethanol plants, *Lactobacillus plantarum*, amplified the treatment effects. The laboratory-scale ozone dosages ranged from 26−188 mg/L, with very low estimated costs of $0.0008−0.006/gal ($0.21−1.6/m^3) of ethanol. Ozonation was found to decrease the initial pH of ground corn mash samples, which could reduce the sulfuric acid required to adjust the pH prior to ethanol fermentation. Lactic and acetic acid levels tended to be lower for samples subjected to increasing ozone dosages, indicating less bacterial activity. The lower ozone dosages in the range applied achieved higher ethanol yields. Preliminary experiments on ozonating POET corn slurry at low ozone dosages were not as effective as using POET ground corn, possibly because corn slurry samples contained recycled antimicrobials from the backset. The data suggest additional dissolved and suspended organic materials from the backset consumed the ozone or shielded the bacteria.

KEYWORDS: *ozonation, no-cook, BPX process, corn mash, fuel ethanol, bacteria, dry-grind ethanol, lactic acid*

INTRODUCTION

POET leads the fuel ethanol industry in streamlining corn dry milling to improve ethanol production efficiency, as well as with their feed coproduct, Dakota Gold BPX distiller grains. The efficient BPX, or no-cook, process involves raw starch hydrolysis that, like the conventional process, adds enzymes to convert starch to sugars and adds yeast to ferment sugars to ethanol but without the cooking step.[1] The BPX process is now used by 24 of the 27 POET biorefineries. POET produces over 1.5×10^9 gal (5.7×10^6 m^3) of ethanol annually in a network spanning seven Midwestern states.

The POET BPX process for dry-grind corn ethanol production has important advantages over the conventional process. The energy-intensive liquefaction and cooking steps are omitted, thus reducing plant energy requirements by 8−15%.[1] The BPX process achieves higher ethanol yields by improving starch accessibility, lowers volatile organic carbon (VOC) emissions, cuts cooling water needs, and enhances the nutrient quality, flowability, and anticaking properties of the distillers dried grains with solubles (DDGS) coproduct. Amino acid digestibility in DDGS samples varies considerably among conventional ethanol facilities, attributable to heat damage from corn mash cooking and DDGS drying.[2−5] Heat damage is responsible for lower amino acid digestibility in DDGS than in corn grain.[4,6] Removing the cooking step, therefore, improves the feed quality of Dakota Gold BPX DDGS over conventional DDGS by reducing heat damage of the protein.[7]

Unlike most beverage alcohols, fuel ethanol fermentations are not conducted under pure culture conditions.[8] High-temperature jet cooking (90−165 °C) in the conventional process not only gelatinizes the corn starch but also helps reduce contamination and yield losses caused by unwanted lactic acid bacteria (LAB).[9] Contamination in fuel ethanol fermentations originates from bacteria present on the corn from the fields.[10] Bacteria compete with yeast for nutrients—glucose, trace minerals, vitamins, and free amino nitrogen—and lower ethanol yields by diverting glucose to lactic and acetic acids, which are inhibitory compounds to yeast.[11−13] In addition to yield losses by chronic LAB contamination, acute infections arise unpredictably and can result in plant shutdowns for cleaning, resulting in expensive losses in productivity.[8,13,14]

Gram-positive and Gram-negative bacterial isolates from fuel ethanol production include species of *Pediococcus*, *Enterococcus*, *Acetobacter*, *Gluconobacter*, *Clostridium*, and *Lactobacillus*.[8,15] *Lactobacillus* sp. are the most prevalent in the industry and are ubiquitous in nature. In a study of two commercial dry-grind ethanol plants by Skinner and Leathers,[8] the genus *Lactobacillus* was isolated most frequently, representing 38% and 77% of total bacterial isolates from the first and second plants,

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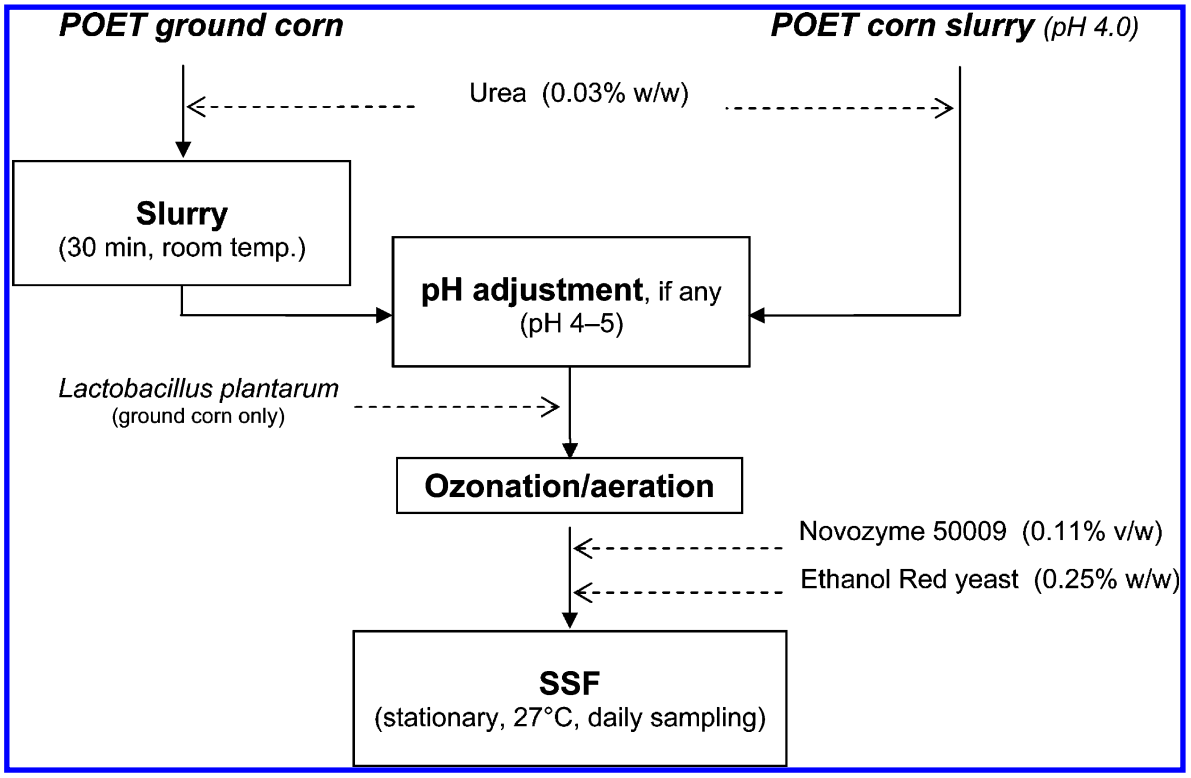

Figure 1. Corn mash preparation, ozonation/aeration, and fermentation with POET ground corn and POET corn slurry.

respectively. Gram-positive LAB are ethanol-tolerant, grow faster than yeast, and produce organic acids that inhibit yeast.

During this research, bacterial growth was controlled at POET biorefineries potentially by dosing with antibiotic and antimicrobial agents and by operation at lower pH levels (pH∼4.2). Conventional ethanol plants, despite jet cooking, also add antibiotics to fight chronic or acute bacterial infections.[8,16−18] If no antibiotics are used, it is common for a facility to lose 1−5% of its potential ethanol yield.[10] Rising concerns about the use of antibiotics and the emergence of antibiotic-resistant bacteria are creating a demand for alternative methods of controlling bacteria.

Cleaning and sanitation are important microbial control measures in the corn ethanol industry. Infections in process tanks and continuous yeast propagators, as well as resistant biofilms within the process train, can continually reintroduce bacterial contaminants into the fermentors.[8,19] Large yeast inocula (≥2% v/v) help control contamination during fermentations; however, yeast growth and fermentation rates are still reduced by high lactobacilli concentrations.[20]

Penicillin and virginiamycin are the antibiotics available commercially for fuel ethanol production.[15,21] Virginiamycin, produced by *Streptomyces virginiae*, has had limited use in human medicine but extensive use as a growth-promoting additive in animal feed.[22] The recommended dose of virginiamycin in fuel ethanol fermentations is 0.25−2.0 ppm.[23] The use of antibiotics has limitations. Commercial antibiotic applications are essentially selection experiments for resistant microorganisms. Hynes et al.[23] observed reductions in the effectiveness of virginiamycin after extended fermentations, possibly resulting from its breakdown by lactobacilli.[24] *Lactobacillus* sp. with higher tolerance to virginiamycin have been isolated from fuel ethanol plants that dose with virginiamycin. Moreover, bacterial isolates have emerged with resistance to both virginiamycin and penicillin.[15,25]

Hop acids offer a natural alternative to antibiotics for fuel ethanol production.[10] Many of the organic acids found in hops exhibit antimicrobial activities. Hop compounds inhibit growth by disrupting the transmembrane pH gradient of microbes.[26,27] Hops have long been used in the brewing industry to contribute to the flavor of beer, by adding bitterness and aroma, and to improve product shelf life. IsoStab, a liquid hop extract, is a commercially available product for fuel ethanol applications. U.S. producers using IsoStab have obtained higher ethanol yields with less fluctuation.

A variety of disinfectants have been investigated on a laboratory scale for fuel ethanol fermentations, including hydrogen peroxide, chlorine dioxide, potassium metabisulfite, 3,4,4′-trichlorocarbanilide, and lactate with a lactate-tolerant yeast strain.[8,28−33] Bacteria were inhibited over yeast by these agents, but the effectiveness depended on the bacterial strain.[8] Chlorine dioxide and ozonation are common alternatives to chlorination for disinfection of drinking water. Meneghin et al.[30] evaluated the effect of chlorine dioxide against bacteria prevalent in ethanol fermentations (e.g., *Lactobacillus plantarum* and *Lactobacillus fermentum*). The minimum inhibitory concentrations (MIC) for chlorine dioxide on the bacteria and on the yeast inoculum were determined in nutrient media; the effectiveness in nutrient media is likely different from that in the high suspended solids corn mash at fuel ethanol plants. The MICs ranged from 10 to 125 ppm for the bacterial strains tested; *L. plantarum* had the highest MIC of 125 ppm. Yeast growth was also affected at chlorine dioxide concentrations over 50 ppm.

Ozone is a microbial inhibitor that is considered an alternative to chlorine and hydrogen peroxide in food applications.[34] The rate of microbial inactivation is affected

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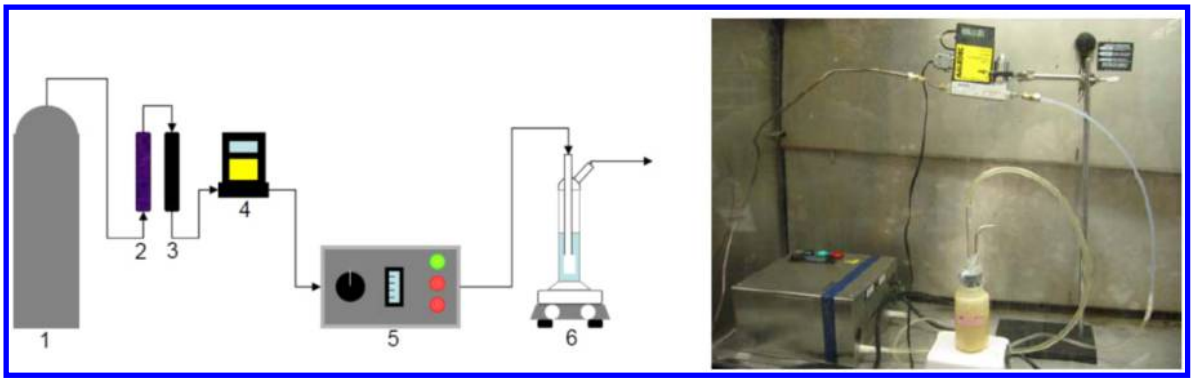

Figure 2. Setup and photo of ozonation of corn mash: (1) compressed air tank, (2) moisture trap, (3) hydrocarbon trap, (4) mass flow controller, (5) corona-discharge ozone generator, and (6) corn mash sample (200 g) in 250 mL PP bottle.

by several environmental factors (concentration of organic compounds, physiological state of the microorganism, pH of the medium, temperature).[35] Thus, ozone has had mixed success in the food industry. García-Cubero et al.[36] illustrated the benefit of pretreatment of straw with ozone, resulting in a reduction in acid-insoluble lignin (Klason lignin) and improved enzymatic hydrolysis of cellulose and hemicelluloses for biofuel production. Freitas-Silva and Venâncio[37] demonstrated mycotoxin degradation by ozone use in corn on the cob, corn kernels, and corn and rice powder. Miller et al.[38] reviewed the advantages and disadvantages of ozonation use for fruits and vegetables preservation, while Gerrity and Snyder[39] review its use in water reuse applications. Thus, the application of ozone in dry-grind corn ethanol production is needed.

Research Objectives. In this research, ozone was used in place of antibiotics (e.g., Lactrol and IsoStab) for control of bacterial contamination in ethanol (yeast) fermentations. Preliminary experiments were subsequently performed on corn slurry to evaluate the effectiveness of ozonation for reducing bacterial contamination under conditions more similar to plant operation, including the contributions of recycled water sources.

MATERIALS AND METHODS

Corn Mash Preparation. Ground corn and corn slurry were obtained from POET Biorefining in Jewell, IA, in the fall and spring of 2007 and 2008. The corn slurry obtained from POET contains the ground corn in a slurry with recycled water streams, such as backset. Figure1 illustrates corn mash preparation, ozone treatment, and simultaneous saccharification and fermentation (SSF) procedures. Corn mash was prepared in 250 mL polypropylene (PP) bottles with 35% (w/w) ground corn (200 g of mash/bottle) and deionized water or with 200 g of corn slurry/bottle. Urea was added (0.03% w/w) as a N source. The final corn mash volume was 186 mL/bottle. Bottles were shaken for 30 min at 250 rpm and room temperature to slurry contents. The pH was adjusted to 5.0 in select experiments. In preliminary work (pH 4.2), all controls with and without Lactrol and/or IsoStab added had similar values for lactic and acetic acids, glycerol, and ethanol, which indicated minimal bacterial contamination even without ozonation (results not shown). Therefore, no antibiotics or other antimicrobials (i.e., Lactrol or IsoStab) were added in these experiments to exemplify treatment effects of ozone. The POET corn slurry experiments, however, potentially contained recycled antimicrobials contributed in the backset from previous fermentations at the plant. Select samples were also LAB-spiked and/or not yeast-supplemented to amplify the differences in treatment effects.

Microorganisms. Select corn mash samples were spiked with *Lactobacillus plantarum*, a lactic acid bacterium, obtained from the American Type Culture Collection (ATCC 14917, Rockville, MD) prior to ozone treatment. Stock cultures of *L. plantarum* were freeze-dried and stored previously in sterile skim milk at 4 °C. Fermentis Ethanol Red (Lesaffre Yeast Corp., Milwaukee, WI), containing the yeast *Saccharomyces cerevisiae*, was added (0.5 g) after ozone treatment for ethanol fermentation.

Ozonation. The laboratory-scale installation used to apply the ozone treatment is shown in Figure 2. Ozone gas was generated from oxygen supplied by a compressed-air gas cylinder by use of a corona-discharge ozone generator (TOG C2B, Trigon, Glasgow, Scotland). Moisture and hydrocarbon traps (Restek, State College, PA) were placed between the ozone generator and compressed-air cylinder to remove impurities in the feed gas. A mass flow controller (GLC17, Aalborg, Orangeburg, NY) was used to control the gas flow rate.

Ozone gas was bubbled into the corn mash samples (200 g) in 250 mL PP bottles at a flow rate of 500 mL/min. The ozone concentration in the gas phase was measured by the potassium iodide titration method.[40] The ozone dose, or consumption, was calculated on the basis of ozone concentrations in the influent and effluent gas from the bottle over time. Corn mash samples were ozonated and residual ozone in off-gas was measured by titration after varying ozonation times. This setup was used to determine the effects of different ozone dosages on ethanol fermentation of corn mash samples prepared from ground corn or corn slurry. Aeration treatment, as a control, was also performed with this equipment. The aeration and ozonation procedures were the same (e.g., similar gas flow rate), but with the ozone generator turned off.

Ethanol Fermentation. Following ozonation, raw starch hydrolyzing enzyme (Novozyme 50009, 0.11% v/w) and Ethanol Red (0.25% w/w) were added to ozonated, aerated, and nontreated corn mash (Figure 1). Bottles were shaken for 2 min at 250 rpm to mix contents and incubated without shaking at 27 °C (POET incubation temperature) until ethanol levels reached a plateau, up to 168 h. SSF samples were HPLC-analyzed on a daily basis.

Sample Analyses. The ethanol, glycerol, lactic acid, and acetic acid contents of corn mash and SSF samples were quantified on a Waters high-pressure liquid chromatograph (HPLC) (Millipore Corp., Milford, MA). The HPLC system was equipped with a Waters model 401 refractive index detector, column heater, automatic sampler, and computer controller. The Aminex HPX-8711 column (300 × 7.8 mm, Bio-Rad Chemical Division, Richmond, CA) separated sample constituents with a mobile phase of 0.012 N sulfuric acid, flow rate of 0.8 mL/min, injection volume of 20 μL, and column temperature of 65 °C.[41]

Experiment Sets. A pH experiment was conducted prior to fermentation. Aeration treatment was included to confirm that ozonation, rather than aeration and mixing, lowered the initial corn mash pH prior to fermentation (0 h).

The ethanol fermentation experiments reported in this publication are divided into three sets, two with POET ground corn and one with POET corn slurry, as outlined in Table 1.

POET Ground Corn (Experiment Sets 1 and 2). Two sets of fermentation experiments with ozone-treated corn mash prepared

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Table 1. Experiments Performed with Corn Mash Prepared from POET Ground Corn and POET Corn Slurry[a]

corn mash	treatment	initial pH[b]	LAB spiked	yeast added
	Experiment Set 1			
ground corn	ozone (0−188 mg/L)	5.0	yes	no
ground corn	ozone (0−152 mg/L)	5.0	yes	yes
	Experiment Set 2			
ground corn	none	4.7	no	yes
ground corn	air (60 min)	4.7	no	yes
ground corn	ozone (60 min)	4.7	no	yes
ground corn	none	4.7	yes	yes
ground corn	air (60 min)	4.7	yes	yes
ground corn	ozone (60 min)	4.7	yes	yes
	Experiment Set 3			
corn slurry	none	4.0	no	yes
corn slurry	none	4.0	no	no
corn slurry	none	5.0	no	yes
corn slurry	ozone (60 min)	5.0	no	yes

[a]No antimicrobials were added in these experiments; however, corn slurry obtained from POET contained backset, which potentially has antimicrobials from previous fermentations. [b]Initial pH refers to the pH after adjustment and before treatment. Without adjustment, the pH of corn mash prepared from ground corn was 4.7 and from corn slurry was 4.0.

from ground corn were performed. The first set included spiking with *L. plantarum* before ozone treatment (0−188 mg/L) of corn mash; no yeast and no other antimicrobial agents were added to select bottles to further amplify the treatment effects. The second set included ozonation and aeration treatments of corn mash prior to ethanol fermentation. Select bottles were spiked with *L. plantarum*. Fermentation experiments were conducted with aeration treatment as a control to determine whether lower lactic acid contents in daily samples resulted from ozonation as a disinfect/oxidant or from possible stimulatory effects of aeration on the yeast.

POET Corn Slurry (Experiment Set 3). A preliminary set of fermentation experiments was conducted with POET corn slurry to assess the effectiveness of ozonation for reducing bacterial contamination under conditions more similar to plant operation, including contributions of recycled water streams. The recycled backset potentially contributed other antimicrobials in these experiments. Corn mash samples were prepared with/without addition of yeast, with the slurry initial pH of 4.0 and adjusted to pH 5.0, and with/without ozone treatment, as shown in Table 1.

Statistical Analysis. All treatments were performed in replicates of three. Statistical analysis was carried out with the Statistical Analysis System package (SAS computer program version 9.1.2, SAS Institute Inc., Cary, NC). Student's *t*-test analyses were performed on HPLC data sets to determine statistical significance with a p value of 0.05.

RESULTS AND DISCUSSION

Ozone Dosages. The results of ozonating corn mash samples for various treatment times in order to determine ozone consumption (i.e., ozone dosage) are illustrated in Figure 3. Ozone consumption is the difference between ozone applied to the corn mash, $f(x)$, and unused ozone exiting the headspace of the treated corn mash over time (off-gas), $g(x)$; it equals the green area in Figure 3. The following fitted equations were used to calculate ozone consumption/dosage:

$$\text{applied ozone (mg/min)} = f(x) = 0.996 \quad (1)$$

$$\text{off-gas ozone (mg/min)} = g(x) = 0.00319x + 0.000538x^2 \quad \text{for } 0 \le t \le 37.5 \text{ min} \quad (2)$$

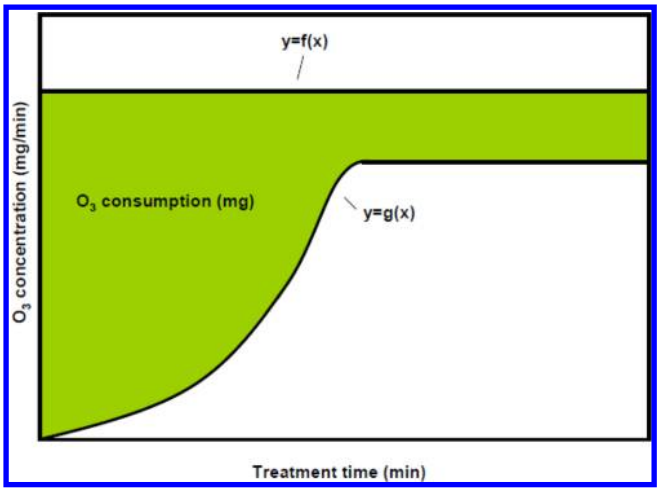

Figure 3. Ozone consumption/dose (green area) as a function of ozonation treatment time for corn mash samples. The curve was fit on the basis of effluent ozone discharge rates, $g(x)$, observed in corn mash over time. The influent ozone application rate was constant, $f(x)$, and $n = 4$.

$$\text{off-gas ozone (mg/min)} = g(x) = 0.884 \quad \text{for } 37.5 \text{ min} \le t \quad (3)$$

$$\int_0^t f(x)\,dx - \int_0^t g(x)\,dx = 0.996t - 0.00159t^2 - 0.000179t^3 \quad \text{for } 0 \le t \le 37.5 \text{ min} \quad (4)$$

$$\int_0^t f(x)\,dx - \int_0^t g(x)\,dx = 0.112(t - 37.5) + 25.7 \quad \text{for } 37.5 \text{ min} \le t \quad (5)$$

The ozone dosages computed for each treatment time are provided in Table 2. In our preliminary work, additional dosages of 26 and 75 mg O_3/L were tested. The higher dosages (127, 152, and 188 mg/L) were chosen for replicate experiments, as reported in this publication.

Table 2. Ozone Dosages Applied to Corn Mash and Estimated Costs in Our Study for Different Ozonation Times

ozonation time (min)	ozone dose[a] (mg of O_3)	ozone dose[b] (mg of O_3/L of corn mash)	est cost[c] ($/1000 gal of ethanol)
5	5	26	0.84
15	14	75	2.39
30	24	127	4.04
60	28	152	4.82
120	35	188	5.97

[a]Ozone dose/consumption was determined by ozonating corn mash samples. The resulting equations used for ozone calculations (milligrams) are $0.996t - 0.00159t^2 - 0.000179t^3$ for $0 \le t \le 37.5$ min and $0.112(t - 37.5) + 25.7$ for $t \ge 37.5$ min. [b]The volume of ozonated corn mash was 0.186 L; ozone dose (milligrams) was divided by this volume. [c]Estimated cost was calculated from ozone dose (milligrams per liter) based on the conversion factor of 3.79 L/gal, an ozone cost of $\$1.68 \times 10^{-6}$/mg, and the assumption of 20% (v/v) final ethanol content (or 1 gal of ethanol to 5 gal of corn mash).

Ozone Cost Estimates. The ozone cost estimates include operating costs to purchase electricity for the ozone generator and to prepare air for the ozonation. The costs of electricity and air are calculated as follows:

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electricity consumption of typical ozone generators

$$= 10\ \text{kW·h/kg of ozone} \quad (6)$$

$$\text{electricity} = \$0.07/\text{kW·h} \quad (7)$$

Equation 7represents the 2009 average for industrial consumers.[40]

electricity consumption for air feed drying

$$= \sim 2\ \text{kW·h/kg of ozone} \quad (8)$$

$$\text{total electricity consumption} = 12\ \text{kW·h/kg} \quad (9a)$$

$$\text{total electricity cost} = \$0.84/\text{kg} \quad (9b)$$

Capital costs: since the ozone dosage has not yet been optimized in relation to corn mash formulations in industry, including recycled water sources, the required ozone generator size for a typical plant cannot be determined. As a rough guide, the amortization costs will equal the energy costs. The cost of ozone, therefore, can be expected to amount to roughly $1.68/kg. This cost still needs to be determined to a greater degree of accuracy. Table 2 presents the estimated ozonation costs based on the ozone dosages applied in experiments to date.

Initial pH Experiment. The initial pH of corn mash prepared from ground corn, prior to fermentation (0 h), was adjusted to 4.2 or 5.0. The pH of corn mash samples tended to decrease with increasing ozone treatment times/dosages (Figure 4). The pH of ozonated samples (initial pH 5.0) started to decrease after 15 min of ozonation (75 mg of O_3/L), reaching an average pH of 4.8 in 60 min (152 mg of O_3/L). With an initial pH of 4.2, the corn mash pH was also reduced by 0.2 pH unit to 4.0 after 60 min of ozonation. Aeration with the same procedure, but no ozone generated, did not affect the corn mash pH. This finding shows that the ozone or ozone byproducts, rather than aeration and additional mixing, were responsible for the corn mash pH reductions observed in all experiments with ground corn.

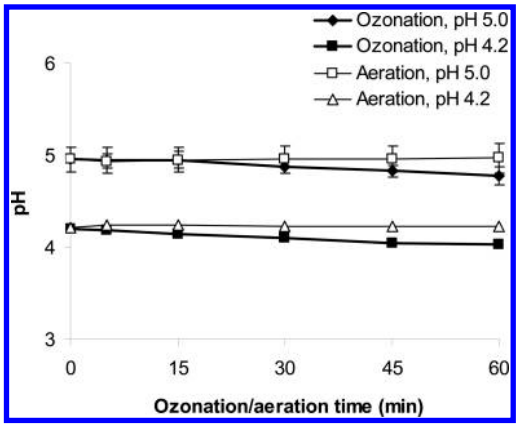

Figure 4. Initial pH experiment: changes in pH observed during ozonation and aeration of corn mash samples prepared from ground corn and adjusted to different initial pH values, 5.0 (n = 3) and 4.2 (n = 1).

Corn mash samples were analyzed by HPLC before and after treatment to detect any changes in concentrations, in particular for organic acids. No consistent changes were observed for ozonated and aerated samples. A slight increase in acetic acid of 0.35 g/L occurred in one ozonated sample but not in replicate samples. There were no changes in acetic acid in aerated samples. No lactic acid was detected in any samples before or after ozonation/aeration.

Ground Corn (Experiment Sets 1 and 2). *Effects of Ozonation on pH and Lactic Acid Contents.* In experiment set 1 (Figure 5), daily pH values of ozonated and nonozonated corn mash samples decreased for the first 24−48 h of fermentation only. The exceptions were samples with higher ozone dosages (152 and 188 mg/L) and no yeast added; the pH of these samples started decreasing after 24−48 h of fermentation and stabilized by 96−120 h. All pH trends corresponded with increases in lactic acid contents. Higher daily pH values, and less lactic acid, were observed in samples subjected to higher ozone dosages.

Statistical analysis was conducted to compare the significance ($p < 0.05$) of 96-h fermentation samples. As expected, lactic acid production in samples without yeast added was higher than in samples with yeast added. Yeast inoculum size affects the ability to compete with bacteria for the same substrate, fermentable sugars.[20] Increasing the ozone dosages from 0 to 127 mg/L and from 127 to 152 mg/L, without yeast added, resulted in significantly lower (p = 0.0004 and p = 0.007, respectively) lactic acid contents; no significant difference (p = 0.4) was observed between 152 and 188 mg ozone/L. These differences were amplified by not supplementing with yeast. With yeast added, the 152 mg/L ozone dosage resulted in significantly lower (p = 0.04) lactic acid contents than the 127 mg/L dosage, indicating less bacterial activity; there was no significant difference (p = 0.9), however, between 0 and 127 mg/L dosages.

In experiment set 2 (Figure 6), daily pH values of ozonated, aerated, and nontreated corn mash samples decreased for the first 24−48 h of fermentation only, without exception. These results agree with experiment set 1 since yeast was added to all samples in this set. Likewise, the daily pH values for all samples corresponded with increases in lactic acid contents.

Nontreated samples tended to have lower daily pH values and higher lactic acid concentrations. Ozonated samples had the highest pH values and least lactic acid throughout the fermentation period, indicating less bacterial and more yeast activity. LAB-spiked, aerated samples had trends similar to those of nontreated samples. Non-LAB-spiked, aerated samples had lower lactic acid values than nontreated samples, which suggest the indigenous LAB populations prefer a reduced oxygen environment. Thus, aeration was effective for reducing lactic acid production only in samples without LAB spiking, but ozonation was still more effective. Statistical comparisons of 96-h fermentation samples showed that all differences in lactic acid contents among ozonated, aerated, and nontreated samples were significant (p values <0.05).

Effects of Ozone on Glycerol Contents. In experiment set 1, glycerol production was lowest in corn mash treated with 127 mg/L of ozone prior to fermentation, compared to nontreated and 152 mg/L ozone-treated samples (Table 3). The 127 mg of O_3/L samples had the highest acetic acid and ethanol contents, which create osmotic stress on the yeast, triggering glycerol production;[42] lactic acid contents, however, were comparable to those of nontreated samples (Figure 5, yeast added). Reduced glycerol contents were also observed in preliminary experiments with increasing ozone dosages, indicating the yeast were less stressed compared to nonozonated samples.

Daily ethanol contents of ozonated and nonozonated corn mash samples began to plateau by 96 h of fermentation (Figure

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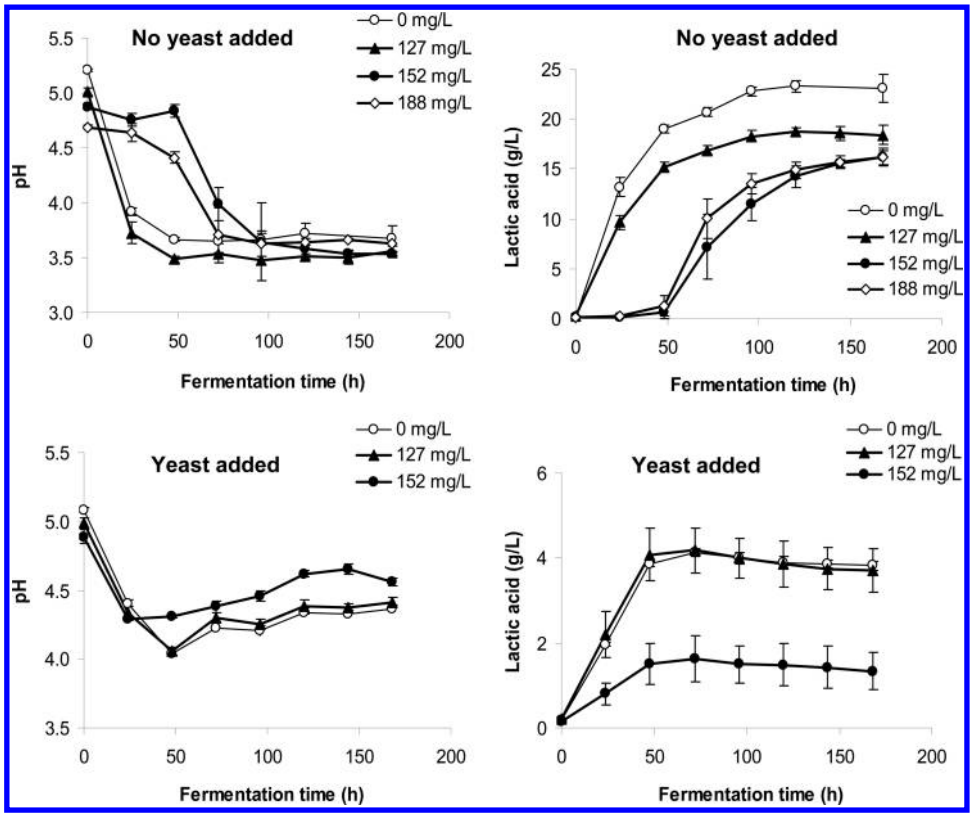

Figure 5. Experiment set 1: samples were spiked with *L. plantarum* and ozonated. Select samples had yeast added. Corn mash pH was adjusted to 5.0 prior to ozonation. No other antimicrobial agents (Lactrol or IsoStab) were added. Daily pH (left) and lactic acid contents (right) of fermentation samples of corn mash prepared from ground corn are shown (n = 3).

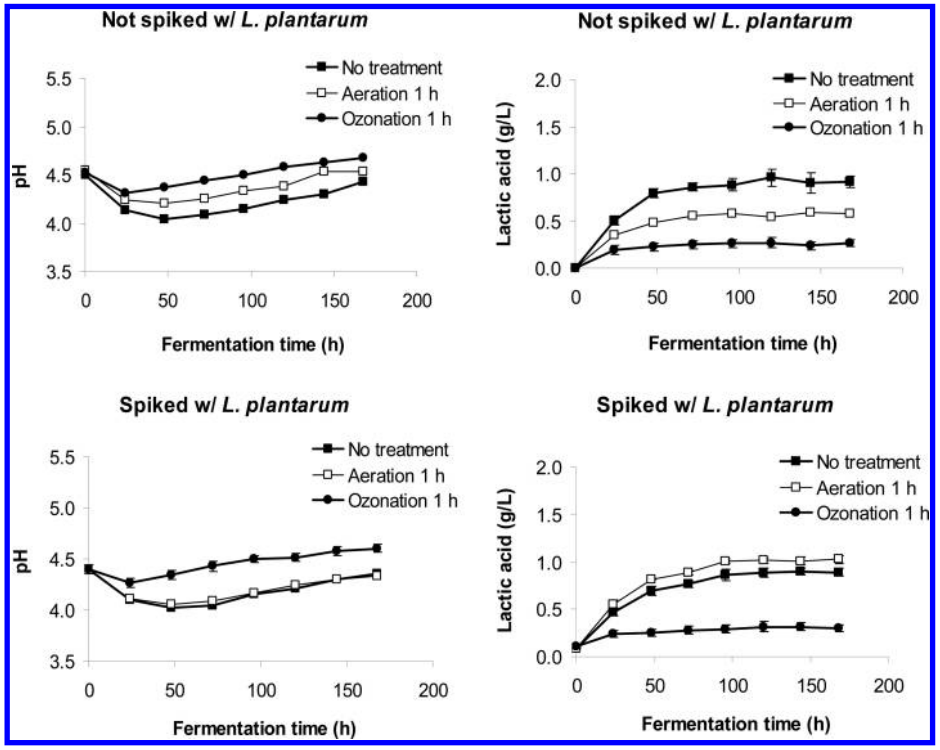

Figure 6. Experiment set 2: select samples were spiked with *L. plantarum* prior to treatment. Samples were ozonated or aerated for 60 min, except for controls with no treatment, and yeast was added. No other antimicrobial agents (Lactrol or IsoStab) were added. Daily pH (left) and lactic acid contents (right) of fermentation samples for corn mash prepared from ground corn are shown (n = 3).

7). With yeast added, the 96-h ethanol concentration ranged from 150 to 154 g/L. The ethanol concentrations in 96-h samples treated with 127 mg/L ozone dosage were significantly higher (p = 0.02) than in samples with no ozone treatment

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Table 3. Comparison of Glycerol and Ethanol Contents of 96-h Fermentation Samples from Corn Mash[a]

LAB-spiked	treatment[b]	acetic acid (g/L)	glycerol (g/L)	lactic acid (g/L)	ethanol (g/L)
		Experiment Set 1			
yes	none	0.7	10.2	4.0	150
yes	ozone (127 mg/L)	1.0	9.3	4.0	154
yes	ozone (152 mg/L, 60 min)	0.7	9.8	1.5	152
		Experiment Set 2			
no	none	0.2	10.4	0.9	147
no	aerated (60 min)	0.2	10.7	0.5	147
no	ozone (60 min)	0.4	8.6	0.3	143
yes	none	0.2	10.4	0.9	145
yes	aerated (60 min)	0.3	10.5	1.0	145
yes	ozone (60 min)	0.4	8.8	0.3	143

[a]Prepared from ground corn with yeast addition after ozone treatment (n = 3). No other antimicrobial agents (Lactrol or IsoStab) were added. [b]Initial pH values, prior to ozonation, of experiment sets 1 and 2 were 5.0 and 4.7, respectively. The initial fermentation pH values were 4.8 and 4.5, respectively.

(Table 3). Samples subjected to 152 mg/L ozone dosage had more ethanol than nonozonated samples, but the difference was not significant (p = 0.06). Ethanol production was observed in corn mash samples even without yeast added (up to 61 g/L in 96-h, nonozonated samples); the ground corn used to prepare the corn mash was obtained from POET and presumably contained yeast from exposure during storage at the ethanol biorefinery. Without supplemental yeast, ozone dosage had no significant effect (p values >0.05) on ethanol contents in 96-h samples (Figure 7).

In experiment set 2 (Table 3), daily glycerol trends in corn mash samples were not affected by spiking with *L. plantarum*. Aerated and nontreated samples had similar glycerol production throughout the fermentation period, with slightly more glycerol in the aerated samples. Ozonated samples had the least glycerol after 24 h of fermentation, which coincided with lower daily ethanol production.

The ethanol concentrations in all samples, LAB-spiked and nonspiked, began to plateau by 96 h of fermentation, as observed in experiment set 1. The 96-h ethanol concentration ranged from 143 to 147 g/L. Spiking with *L. plantarum* resulted in somewhat less ethanol than in nonspiked samples (Table 3). The ozonated samples had less ethanol and less lactic acid (Figure 6), than aerated and nontreated samples in this set of experiments, which accounts for less glycerol production. The ethanol findings suggest that higher ozone dosages (152 mg/L) may negatively impact ethanol yields, despite lowering lactic acid contents, depending on the initial pH. The starting pH (posttreatment) for fermentations in the first and second experiment sets was 4.8 and 4.5, respectively.

Corn Slurry (Experiment Set 3). *Yeast Addition.* Corn mash samples were prepared from POET corn slurry (pH 4) by adding urea, enzymes, and yeast. No yeast was added to select samples. As expected, samples without yeast added had higher levels of lactic acid within 24 h and acetic acid within 48 h of fermentation (Figure 8B,C). Higher yeast inoculum size enables the yeast to be more competitive with bacteria. Addition of yeast resulted in significantly (p = 0.002) higher ethanol contents within 24 h of fermentation (Figure 8E); 96-h samples had an average ethanol yield of 146 g/L, with yeast added, as compared to 130 g/L without yeast added.

Initial pH. The initial pH of corn mash prepared with corn slurry from POET was 4.0. This experiment was performed to investigate the effect of increasing the initial corn mash pH to 5.0 on ethanol fermentation with yeast added. Corn mash samples were prepared at slurry pH 4.0 and with pH adjusted to 5.0. The pH of corn mash samples dropped the first 24 h of fermentation (Figure 8A); corn mash with initial pH 4.0 and 5.0 decreased to pH 3.6 and 4.0 in 24 h, respectively. The pH increased gradually to pH 3.8 and 4.2 by the end of the experiment (168 h). The lactic and acetic acid contents increased for the first 24−48 h of fermentation, coinciding with decreasing pH values (Figure 8B,C). Production of lactic/acetic acids and glycerol (Figure 8D) was higher in samples at adjusted pH 5.0 than at slurry pH 4.0, which illustrates an active LAB population. Growth of lactic acid bacteria is reduced at pH less than 4.0. Ethanol production was affected by the increased levels of organic acids; this was demonstrated by higher ethanol concentrations at the slurry pH 4, indicating higher yeast activity.

Ozonation. Corn mash (adjusted pH 5) prepared with POET corn slurry was ozonated to investigate potential reductions in bacterial activity. Lactic acid production in ozonated samples was somewhat lower than in nonozonated

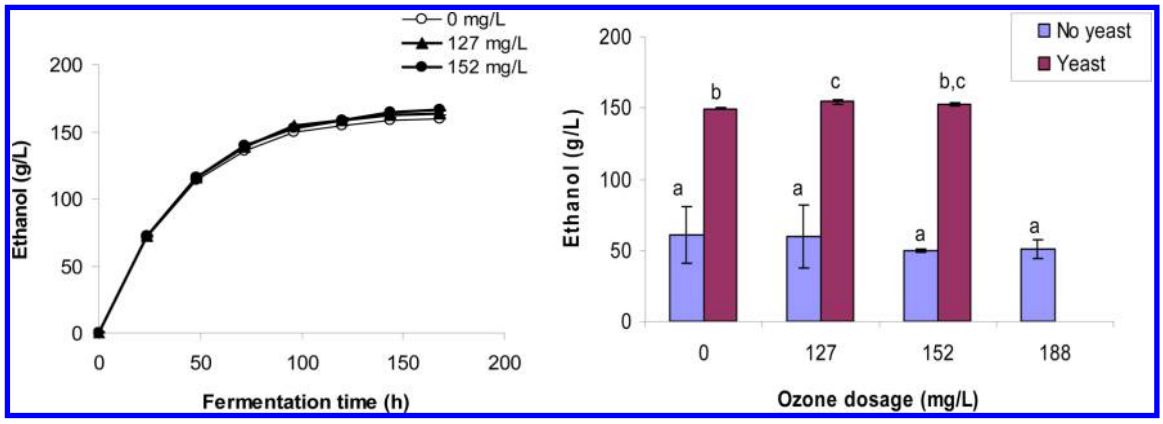

Figure 7. Experiment set 1: (Left) ethanol contents of daily fermentation samples for corn mash prepared from ground corn, spiked with *L. plantarum* and ozonated, with yeast added. No other antimicrobial agents (Lactrol or IsoStab) were added. (Right) Comparison of ethanol contents of 96-h samples (n = 3).

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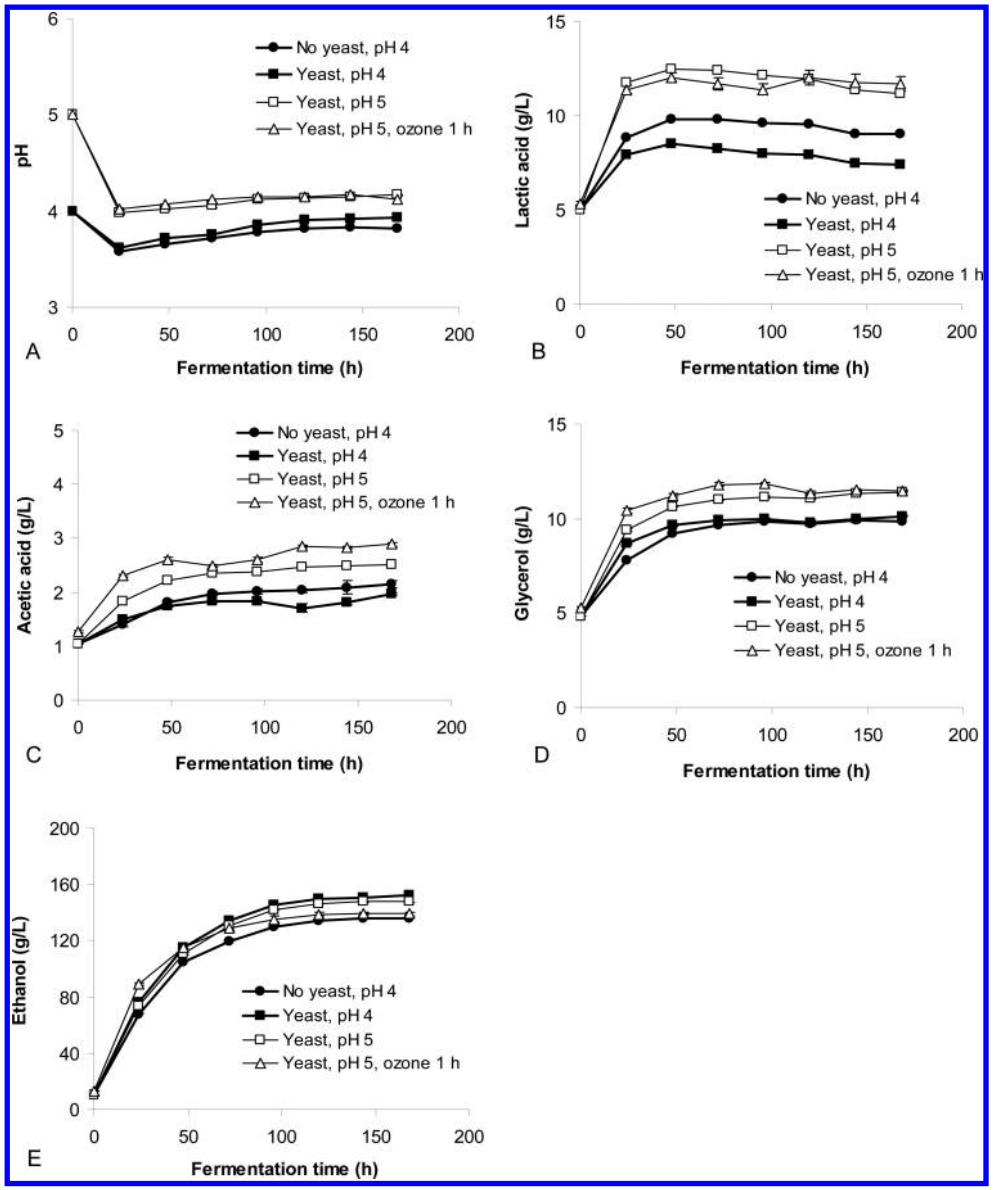

Figure 8. Corn mash prepared from POET corn slurry, which potentially contains recycled antimicrobials in the backset from previous fermentations: (A) pH, (B) lactic acid, (C) acetic acid, (D) glycerol, and (E) ethanol contents of daily fermentation samples from nonozonated/ozonated corn mash samples, with or without yeast added, and initial pH of 4 or 5 (n = 3).

samples (e.g., 12.0 versus 12.5 g/L in 48 h, respectively) up to 120 h (Figure 8B). Acetic acid contents of ozonated samples, however, were higher throughout the fermentation (e.g., 2.6 versus 2.2 g/L in 48 h) (Figure 8C). The initial acetic acid contents, after ozonation, were 0.3 g/L higher in the ozonated samples, which helps explain this finding. These results suggest that ozonation is not as effective in samples prepared from corn slurry in place of ground corn. Additional dissolved and suspended organic materials contributed from the backset may have consumed most of the ozone or shielded the bacteria. Recycled antimicrobial agents, Lactrol and IsoStab, in the corn slurry from the backset may also have reduced the effects of ozonation compared to the nonozonated controls.

Conclusions. Fermentation experiments were conducted with ozone treatment and without the addition of other antimicrobial agents, such as antibiotics. The experiments were performed with POET ground corn and corn slurry.

For POET ground corn we observed the following: (1) Ozonation could be used to lower corn mash pH and, therefore, reduce sulfuric acid requirements to adjust the pH prior to fermentation. (2) Lower lactic acid levels were observed in experiments with ground corn; on this basis, ozonation may reduce bacterial contamination, and thus antibiotic dosages, during fuel ethanol fermentations. (3) The impact of ozonation on ethanol yields, regardless of lowering lactic acid contents, depends on the ozone dosage and initial fermentation pH.

For POET corn slurry, which potentially contained some antimicrobials, we observed the following: (1) Yeast inoculum size affects the ethanol yields and enables the yeast to be more competitive than bacteria, as expected. (2) Ethanol production was higher at initial pH 4.0 than pH 5.0, indicating more yeast activity. (3) Ozone treatment, as applied to ground corn mash, is not as effective in samples prepared from corn slurry.

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Additional experiments with POET corn slurry, and the various recycled water streams at a POET ethanol biorefinery, may help clarify the effectiveness of ozonation under fermentation conditions closer to those in plant operation. Higher ozone dosages and use of only recycled water sources that do not contain antimicrobials could be investigated.

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Notes

The authors declare no competing financial interest.

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