

Fate of virginiamycin through the fuel ethanol production process

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Abstract Antibiotics are frequently used to prevent and treat bacterial contamination of commercial fuel ethanol fermentations, but there is concern that antibiotic residues may persist in the distillers grains coproducts. A study to evaluate the fate of virginiamycin during the ethanol production process was conducted in the pilot plant facilities at the National Corn to Ethanol Research Center, Edwardsville, IL. Three 15,000-liter fermentor runs were performed: one with no antibiotic (F1), one dosed with 2 parts per million (ppm) of a commercial virginiamycin product (F2), and one dosed at 20 ppm of virginiamycin product (F3). Fermentor samples, distillers dried grains with solubles (DDGS), and process intermediates (whole stillage, thin stillage, syrup, and wet cake) were collected from each run and analyzed for virginiamycin M and virginiamycin S using a liquid chromatography-mass spectrometry method. Virginiamycin M was detected in all process intermediates of the F3 run. On a dry-weight basis, virginiamycin M concentrations decreased approximately 97 %, from 41 µg/g in the fermentor to 1.4 µg/g in the DDGS. Using a disc plate bioassay, antibiotic activity was detected in DDGS

from both the F2 and F3 runs, with values of 0.69 µg virginiamycin equivalent/g sample and 8.9 µg/g, respectively. No antibiotic activity (<0.6 µg/g) was detected in any of the F1 samples or in the fermentor and process intermediate samples from the F2 run. These results demonstrate that low concentrations of biologically active antibiotic may persist in distillers grains coproducts produced from fermentations treated with virginiamycin.

Keywords Antibiotics · Distillers grains · Fuel ethanol · Virginiamycin

Introduction

Bacterial contamination is a continual problem in commercial fermentation cultures, particularly in fuel ethanol fermentations, which are not performed under sterile, pure-culture conditions (Narendranath 2003). Chronic bacterial contamination poses a constant drain on the sugar available for conversion to ethanol, and the bacteria scavenge essential micronutrients required for optimal yeast growth. Acute infections occur unpredictably, and bacterial byproducts such as acetic and lactic acids inhibit yeast growth and may result in “stuck” fermentations (Makanjuola et al. 1992; Narendranath et al. 1997). Although it is generally believed that lactic acid bacteria are the primary bacterial contaminants of fuel ethanol fermentations, a variety of Gram-positive and Gram-negative bacteria have been isolated from fuel ethanol fermentations including species of *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, *Acetobacter*, *Gluconobacter*, and *Clostridium* (Narendranath 2003; Skinner and Leathers 2004; Lushia and Heist 2005). A variety of antimicrobial agents have been reported to control bacterial contamination in ethanol

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fermentations, but the most common commercially available products used in the United States are based on the antibiotics virginiamycin and penicillin (Narendranath 2003).

Virginiamycin is a streptogramin antibiotic that is generally effective against Gram-positive bacteria. It is produced by *Streptomyces virginiae* as a natural mixture of two macrocyclic lactone peptolide components: factor M and factor S (Butaye et al. 2003). These components act synergistically to bind to the bacterial ribosome to form a stable complex that irreversibly inhibits protein synthesis. In the US, virginiamycin is currently approved for therapeutic use and for improved feed efficiency in food animals including swine, poultry (broilers), and cattle.

A number of studies demonstrate the effectiveness of virginiamycin in ethanol production under laboratory conditions (Hamdy et al. 1996; Hynes et al. 1997; Islam et al. 1999). Virginiamycin was stable at the pH (3.8–4.8) and temperatures (25 and 35 °C) commonly seen in industrial fermentations, retaining greater than 87 % of its initial activity levels in the wort for up to 72 h (Islam et al. 1999). Hamdy and coworkers showed that virginiamycin activity in stillage was reduced to 13.2 % of that in liquor (beer), and further heating for 30 min at 100 °C reduced activity to 2.6 % of the initial value (Hamdy et al. 1996). Since operating temperatures of dryers in ethanol plants may range from 93 to 232 °C, this data would suggest that the heat of drying the distillers grains would destroy most of the virginiamycin, and residues in distillers grains would be negligible (Shurson et al. 2003).

Published data on the presence of antibiotics in distillers grains are limited, which led the US Food and Drug Administration to initiate a nationwide survey of antibiotic residues in distillers grains (McChesney 2009). A summary report from this survey indicated that three out of 46 samples contained virginiamycin M at about 0.16 parts per million (ppm) as detected by liquid chromatography and mass spectrometry (Luther 2012). Residual biological activity of antibiotics in distillers grains was not examined. As a complement to the surveys of commercial distillers grain samples, there is a need for controlled studies that examine the fate of virginiamycin throughout the ethanol production process under operating conditions that are commonly used in the fuel-ethanol industry. The present study uses both an analytical chemistry method and a microbiological bioassay to measure virginiamycin antibacterial activity in samples of process intermediates and distillers grains produced at the pilot plant facilities located at the National Corn to Ethanol Research Center (NCERC), Edwardsville, IL.

Materials and methods

Pilot plant trials

To approximate the industrial production of fuel ethanol and DDGS, this study utilized a 1/250th scale pilot plant fermentation facility at NCERC. Similar to commercial fuel ethanol facilities, the pilot plant fermentations were not run under sterile, pure-culture conditions. For each fermentor, about 6800 kg of cleaned No 2 yellow dent corn were ground to flour, and a slurry was prepared at a dry solid concentration of 32 % (w/w). A fraction (40 %) of the total dose of α -amylase enzyme (Spezyme[®] Xtra, Genencor International, Palo Alto, CA, USA) was added to the slurry tank to control the slurry viscosity, and the remaining dose was added to the recycle line of the mash tank. The dextrose equivalents, which is an indicator of the conversion of starch to oligosaccharides, was about 7 for the slurry and 12 for the mash. Each fermentor was filled for about 21 h with flow rates that resulted in average fill masses of about 16,800 kg for each fermentor. Glucoamylase (Distillase SSF, Genencor, Palo Alto, CA, USA), urea, *Saccharomyces cerevisiae* (Ethanol Red, Fermentis, Milwaukee, WI, USA) and, when indicated, a commercial virginiamycin-based antibiotic product that is marketed for use in the ethanol industry, was added to each fermentor once the mash level was sufficient to establish mixing by recirculation. Three fermentations were run: F1 was not treated with the antibiotic product and served as a control; F2 was treated with 2 ppm (mg/l) of the antibiotic product representing a dosage within the manufacturer's recommended range; and F3 was treated with 20 ppm of the product, representing an extreme dose that is over three times the manufacturer's maximal recommended dosage of 6 ppm. Fermentation progress was monitored by measuring the concentrations of residual sugars and fermentation products using high-performance liquid chromatography (HPLC).

After fermentation was complete, the liquor was processed through distillation, where it was separated into pure ethanol and whole stillage. Whole stillage was separated into a solids fraction (wet cake) and a liquid fraction (thin stillage) by centrifugation in a decanter centrifuge. Thin stillage was pumped to an evaporator. The rate of wet cake production was measured using a weigh belt, then it was immediately transferred to the dryer. Thin stillage was pumped to a storage tank and then to the evaporator, where it was concentrated to syrup [about 35 % (w/w) dry solids] by evaporation at 63 °C. Syrup was applied to wet grains and conveyed to a Davenport rotary dryer (outlet temperature 121 °C) to produce distillers dried grains with solubles (DDGS).

Samples of the fermentor, whole stillage, thin stillage, wet cake, syrup, and DDGS were collected for each process stage. All the samples were labeled and stored frozen at $-20\text{ }^{\circ}\text{C}$ for up to 13 months until processed.

Sample extraction

Samples of the distillers grains and process intermediates were extracted with a citric acid–acetone solvent (Hamdy et al. 1996). Twenty ml of 0.1 M citric acid was added to sample (10 g) in a 50-ml conical tube, and placed in a sonicator bath for 10 min. Acetone (20 ml) was added, and the tube was shaken on a rocking platform for 1 h. Samples were cleared of solids by centrifugation ($9000\times g$, 15 min), and the supernatant liquid was decanted. Ethanol (2 ml) was added to supernatant liquid (8 ml) to produce the sample extract in a solvent that is equivalent to that used for the bioassay virginiamycin standards.

Liquid chromatography–mass spectrometry (LC–MS) method for detection of virginiamycin M and virginiamycin S

Virginiamycin M (VM) and virginiamycin S (VS) were analyzed using a Shimadzu SPD 20 HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of an autosampler, a degasser, two dual head pumps, and a thermostated column oven set at $40\text{ }^{\circ}\text{C}$. The system was connected to a triple quadrupole mass spectrometer (3200 QTrap, AB Sciex) equipped with an ESI turbo ion source. A binary solvent system was used: solvent A was 0.1 % (w/v) trifluoroacetic acid in water and solvent B was methanol. An Inertsil ODS-4 C18 column ($6\text{ mm}\times 250\text{ mm}$, $5\text{ }\mu\text{m}$) (GL sciences, Torrance, CA, USA) and a guard column ($7.1\text{ mm}\times 2.1\text{ mm}$) were used for the chromatographic separation. The chromatographic separation was performed at a flow rate of 1.0 ml min^{-1} (80 % diverted before the MS/MS) using a gradient elution program. The mass spectrometer was operated at positive mode, the MRM for virginiamycin M was 526.3/508.2 and the MRM for virginiamycin S was 826/320. The quantitation of VM and VS in samples was based on external calibration without an internal standard, and pure virginiamycin M and S standards were purchased from Sigma Chemical Co. and prepared in methanol:water (50:50) solvent. The method was validated by evaluating linearity, accuracy, and precision, and the validation results are shown in Table 1.

Bioassay for antibacterial activity

Sample extracts were analyzed for antibacterial activity using a modification of the microbiological assay described

by Hamdy et al. (1996). *Kocuria rhizophila* ATCC 9341 was embedded in Antibiotic Medium #11 agar by diluting a bacterial suspension (density of 0.5 McFarland units) 100-fold in molten agar. Six paper discs (6 mm, BD, catalog #231039) arranged in a circle were placed on the cooled surface. A 10 mg/ml stock solution of virginiamycin (a natural mixture of virginiamycin factors M and S purchased from RPI Corporation, Mt. Prospect, IL, USA) was prepared in ethanol. Virginiamycin standard solutions were prepared by diluting the stock to the indicated virginiamycin concentration (Fig. 1) in solvent containing ethanol (20 % v/v), citric acid (40 mM), and acetone (40 % v/v). Virginiamycin standard or sample extract was applied to three of the discs in an alternating pattern with a reference solution of virginiamycin (2 $\mu\text{g/ml}$). Plates were incubated at $37\text{ }^{\circ}\text{C}$ for 18–24 h. Halo diameters for standards and samples were normalized to diameters for reference discs, and a standard curve was produced by plotting normalized zone diameters versus the logarithm of virginiamycin concentration (Fig. 1). Recoveries of antibiotic activity in liquor, whole stillage, and DDGS samples spiked with virginiamycin were greater than 90 %.

Results

Fermentation performance

Ethanol concentrations in drop samples from the F1, F2, and F3 runs were 13.5 % (w/v), 13.3 %, and 13.9 %, respectively. Ethanol yields (g/g dry corn) were 0.323 (F1), 0.324 (F2), and 0.346 (F3), which is equivalent to 9.08 (2.40), 9.12 (2.41), and 9.73 (2.57) liters (gallons) per bushel, respectively. Total sugar in drop samples was less than 1 % (w/v) in all three runs.

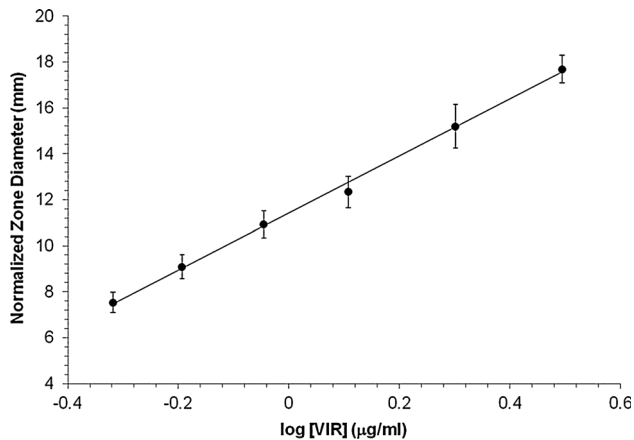
Fermentation times increased from 51 h in the F1 run, to 68 and 74 h in the F2 and F3 runs, respectively.

Chemical analysis of virginiamycin residues

Process samples from the fermentor, whole stillage, thin stillage, wetcake, syrup, and DDGS were analyzed by LC–MS methods for virginiamycin factors M and S. Generally, three representative samples from each process stream were analyzed. VM was detected in all process samples from the fermentation dosed with 20 ppm virginiamycin product. Values of the sample as received ranged from 3.8 to 8.5 $\mu\text{g/g}$ in the fermentor samples, and from undetectable to 2.7 $\mu\text{g/g}$ in the DDGS samples (Table 2). VS was also detected in most of the samples from all process points during the 20 ppm-dosed run. For the fermentation dosed with 2 ppm virginiamycin product, neither VM nor VS were detected by the LC–MS analytical method in

Table 1 Validation results for the LC–MS analytical method to quantify virginiamycin components

Validation parameters	Virginiamycin M	Virginiamycin S
Linearity (5 external standards)	0.999	0.98
Accuracy (recovery in spiked samples)	70–130 %	70–130 %
Precision–instrumental	15 % (5 injections)	15 % (5 injections)
Limit of quantitation (in sample as-received)	0.3 µg/g	0.1 µg/g

**Fig. 1** Calibration curve for virginiamycin bioassay. Shown is a representative calibration curve plotting the mean normalized zone diameter (N = 9) versus the logarithm of virginiamycin concentration

samples from any of the process points (data not shown). Table 2 also reports VM and VS concentrations normalized for moisture content. On a dry-weight basis, the VM concentration in the DDGS from the F3 run was reduced to 3.4 % of that in the fermentor samples, (1.4 and 41.1 µg/g, respectively) (Table 2).

Antibacterial activity in extracts of process samples

Using a biological activity assay, antibacterial activity was detected in all process intermediates of the F3 run, while no

activity was detected in the F1 control run and only in the DDGS sample of the F2 fermentation (Table 3.). The equivalent effective virginiamycin concentration in the F2 DDGS is about 8 % that in the F3 DDGS (0.69 and 8.9 µg/g, respectively). Extracts of DDGS samples from the F3 fermentor run inhibited growth of a virginiamycin susceptible strain of *L. fermentum* while no growth inhibition was detected against a resistant strain (Fig. 2). Extracts from the F1 control fermentor run did not have antibacterial activity against either strain.

Discussion

Based on previously published reports (Hamdy et al. 1996), we hypothesized that the majority of the virginiamycin activity would be destroyed during the ethanol and DDGS production process. The purpose of this study, however, was to evaluate the effects that downstream processing (i.e. distillation of liquor, evaporation of syrup, and drying of distillers grains) have on virginiamycin concentrations. To be reasonably certain that we would detect residual antibacterial activity at each process point, we included a fermentor run with an extreme dose (20 ppm) of virginiamycin product.

Ethanol concentrations and ethanol yields per gram corn were generally the same for all three fermentations. The only observed impact on yeast performance was an increase in fermentation time with increased antibiotic

Table 2 Virginiamycin concentration in process samples from the F3 run (20 ppm initial dose)

Process points	Range of concentration ^a (µg/g)		Concentration (dry-weight basis) ^b (µg/g)	
	VM	VS	VM	VS
Fermentor	3.8–8.5	0.1	41.1 ± 16.9	0.7 ± 0.2
Whole stillage	0.5–1.0	ND–0.2	10.6 ± 2.7	1.9 ± 0.7
Thin stillage	ND–0.5	0.1	13.0 ± 8.7	3.7 ± 0.1
Wet cake	ND–0.9	ND–0.2	1.0 ± 0.8	0.3 ± 0.2
Syrup	1.4	0.4	4.7	2.3
DDGS	ND–2.7	ND–1.1	1.4 ± 0.8	0.5 ± 0.4

^a Virginiamycin concentration was measured by LC–MS method. Data are reported as the range of concentrations in samples as received (µg virginiamycin factor per gram sample) for the samples from each process point. The number of samples from each point are as follows: fermentor, 3; whole stillage, 3; thin stillage, 3; wetcake, 3; syrup, 1; DDGS, 4. “ND” indicates no activity was detected

^b Virginiamycin concentration was measured by LC–MS method. Data are reported as the mean value for all samples from each process point on a dry weight basis (µg virginiamycin factor per gram dry weight)

Table 3 Antibacterial activity in extracts of process samples

Process points	Antibacterial activity ^a (µg/g)		
	F1 (control)	F2 (2 ppm dose)	F3 (20 ppm dose)
Fermentor	ND	ND	3.8 ± 0.7
Whole stillage	ND	ND	1.1 ± 0.1
Thin stillage	ND	ND	0.9 ± 0.1
Wet cake	ND	ND	1.3 ± 0.5
Syrup	ND	ND	4.5 ± 0.3
DDGS	ND	0.69 ± 0.1 ^b	8.9 ± 1.4 ^b

^a Antibacterial activity was measured by bioassay against *Kocuria rhizophila* versus a virginiamycin standard curve. Data are reported as the equivalent of µg virginiamycin per gram of sample. “ND” indicates no activity was detected (<0.6 µg/g)

^b Reported in Edrington et al. (2014)

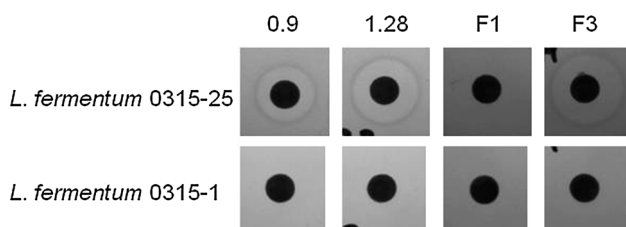


Fig. 2 Extracted antibacterial activity from DDGS does not inhibit growth of a virginiamycin resistant strain of *Lactobacillus fermentum*. The following samples (10 µl) were applied to a paper disc on agar plates inoculated with either *L. fermentum* 0315-25 (top row) or *L. fermentum* 0315-1 (bottom row): 0.9 µg/ml virginiamycin (0.9); 1.28 µg/ml virginiamycin (1.28); extract of DDGS from control fermentor (F1); extract of DDGS from fermentor dosed with 20 ppm virginiamycin product (F3). Minimum inhibitory concentrations for virginiamycin of *L. fermentum* 0315-25 and *L. fermentum* 0315-1 are ≤2 µg/ml and >16 µg/ml, respectively

dosing. A study by Hamdy and coworkers on the effects of virginiamycin on fermentation rate by yeast reported that 20 ppm virginiamycin had a pronounced inhibitory effect on ethanol fermentation (Hamdy et al. 1996). At 2.0 ppm, glucose consumption rates were slower than 0 ppm controls, but statistical analysis indicated the difference was not significant (Hamdy et al. 1996). Thus, in the present study, longer fermentation times at higher dosing of virginiamycin product are expected, although there is insufficient data to speculate as to the significance of the magnitude of the difference between untreated and treated fermentations.

Values of virginiamycin M and S in samples from the same process point, including DDGS, were highly variable, indicating heterogeneity of the distribution of the compounds among the matrix. The average value in the whole stillage (0.80 ± 0.22 µg/g) was 13.3 % that of the

fermentor samples (6.0 ± 1.7 µg/g), which compares favorably with a reduction of 13.2 % observed by Hamdy and coworkers in a fermentation of hydrolyzed corn flour (Hamdy et al. 1996). VS levels were much lower than VM, often near the limit of quantitation, which is consistent with the ratio of VM:VS in the original product (4.5:1 ppm). On a dry-weight basis, the VM concentration in the DDGS from the F3 run was reduced to 3.4 % of that in the fermentor samples, (1.4 and 41.1 µg/g, respectively) (Table 2). This is also consistent with the previous study by Hamdy and coworkers that reported heating thin stillage for 30 min at 100 °C to simulate drying reduced virginiamycin activity to 2.6 % of the original concentration (Hamdy et al. 1996).

The antibacterial activity in the F2 DDGS is about 8 % that in the F3 DDGS (0.69 µg virginiamycin equivalents/g and 8.9 µg/g, respectively), which is internally consistent with the 10-fold difference in antibiotic dosing between F2 and F3. Values from the biological assay are on the same order of magnitude as those reported from the analytical method, and changes between process points show the same trend. In general, activity values decreased from fermentor to process intermediates, but increased in the final DDGS product. This suggests that while processing reduces the total amount of biologically active antibiotic, the removal of water during the drying process concentrates the residual activity.

The virginiamycin equivalent values of antibacterial activity determined from biological assays are consistently higher than the concentrations of VM and VS determined using the analytical method. These differences could be due to the use of a different standard for each method. The analytical method used the individual purified factors VM and VS for the standards, while the bioassay used a natural mixture of the M and S factors to produce a standard curve. As the two factors act synergistically to inhibit bacterial growth, the apparent concentration of virginiamycin based on activity may be greater than the actual concentration determined by the analytical method. Impurities in the natural mixture can also account for an inflated value of virginiamycin in the bioassay standard curve. Analysis of the natural mixture used for the bioassay indicates that it is approximately 70 % virginiamycin [i.e. a 1000 µg/ml solution contains 663 µg/ml VM and 37 µg/ml VS (data not shown)]. Thus the apparent concentration of virginiamycin as determined by bioassay may be inflated by a factor of approximately 1.5.

It is important to consider that the bioassay directly measures growth inhibition, which is then correlated to antibiotic concentration using a virginiamycin standard curve. One cannot discount the possibility that the virginiamycin is broken down or converted to other inhibitory compounds that are not detected by the LC–MS method.

Thus, the higher virginiamycin equivalent values determined from the biological assay may not be due to virginiamycin, but to the presence of these compounds or other extractable factors. To test this hypothesis, we tested extracts of DDGS samples from the F3 run for antibacterial activity against two strains of *Lactobacillus fermentum*: one with a high minimum inhibitory concentration (MIC) for virginiamycin ($>16 \mu\text{g/ml}$, “resistant”) and the other with a low MIC ($\leq 2 \mu\text{g/ml}$ “susceptible”). Analysis by polymerase chain reaction indicated that the resistant *L. fermentum* 0315-1 strain possessed the *vatE* gene (data not shown), which encodes for a streptogramin acetyltransferase associated with resistance to virginiamycin (Soltani et al. 2000). Virginiamycin inhibited the growth of the susceptible strain when spotted on a disc plate assay, but it did not inhibit the resistant strain (Fig. 2). Similarly, extracts of DDGS from the F3 fermentor run, which displayed activity against *K. rhizophila* in the antibacterial bioassay, only inhibited growth of the virginiamycin susceptible strain, and no growth inhibition was detected against the resistant strain (Fig. 2). Extracts from the F1 control fermentor run did not have activity against either strain. This is consistent with the antibacterial activity being due to virginiamycin rather than another extractable factor.

Both the analytical and bioassay data from the F3 run indicate that most of the virginiamycin is destroyed or inactivated during processing. The typical dosing range for the commercial virginiamycin-based product used in the present study is 0.1–3 ppm, with a maximum of 6 ppm. The bioassay data presented here suggests that the concentration of virginiamycin residues in DDGS produced from fermentations dosed with less than 2 ppm would be below our limit of detection. Thus, the risk of finding detectable residues of antibiotics in commercial DDGS samples is presumably low. Indeed, a recent survey of commercial dried distillers grains and wet distillers grains reported 13 % of 159 samples had detectable antibiotic residues, including erythromycin, penicillin G, tetracycline, and virginiamycin (Compart et al. 2013). Only two samples tested positive for virginiamycin in a bioassay, one with a concentration of $0.6 \mu\text{g/g}$ and the other $0.5 \mu\text{g/g}$. This compares favorably with the $0.69 \mu\text{g/g}$ value reported here from the F2 fermentation dosed with 2 ppm product.

Conclusion

Under pilot plant conditions at the National Corn to Ethanol Research Center, biologically active virginiamycin was detected in the DDGS, even from the fermentation treated within the recommended dosing range for the antibiotic product. While industrial conditions for each

process point may vary in terms of temperature and residence time, the data presented here suggest the heats of distillation, evaporation, and drying of distillers grains may not be sufficient to fully inactivate virginiamycin. Producers that use virginiamycin in their fermentation could use the biological assay employed in this study to monitor coproducts for residual antibiotic activity. Since other antibiotics, including penicillin and erythromycin, are available to control bacterial contamination in the fuel ethanol industry, the results of this study also suggest that research on the fate of these other antibiotics during fuel ethanol production is warranted.

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