SWITCHGRASS EXTRACTIVES INHIBIT FOODBORNE PATHOGENS

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ABSTRACT

Switchgrass 'extractives' contain compounds that inhibit biomass conversion to biofuels. Development of 'extractives' as antimicrobials, particularly for foodborne pathogens, would make switchgrass biomass a more economically competitive biofuel feedstock. In this study, we evaluated ethanol-extracted switchgrass samples for inhibition of foodborne bacterial pathogens using agar disk diffusion assays on tryptic soy agar (TSA). Compounds in the switchgrass extracts were concentrated during extraction by recycling ethanol through the reactor three times. Prior to antimicrobial assays, extractives were concentrated an additional 25X with a Thermo Scientific Reacti-Vap Evaporator. Disks were saturated with concentrated extractives; effective concentrations of disk treatments were 30X, 45X, 60X, and 75X. Control disks were treated with 95% ethanol. Treated and control disks were air-dried before use to evaporate ethanol. In separate experiments, *Escherichia coli* O157 isolates that produce shigatoxin, and *Salmonella enterica* serovars were surface spread onto TSA. Both treated and control disks were placed on inoculated TSA plates. Sterile deionized water (40 µl) was added to each disk to facilitate diffusion to TSA. After three days incubation, inhibition zone measurements from treated and control disks were examined for significance with mixed models ANOVA (PC-SAS, ver. 9.4). Growth inhibition of *E. coli* O157 isolates and *S. enterica*, serovars Enteritidis and Typhimurium, increased significantly as concentration of extractives increased.

Introduction

Panicum virgatum, a perennial native grass known as switchgrass, is a prime lignocellulosic feedstock for biofuel production. However, 5-25% of switchgrass dry weight biomass is composed of extractives (1), a non-structural fraction containing high concentrations of phenolic compounds. When not removed, extractives compounds reduce efficiency of bioconversion to biofuel (2). The overall goal of our research program is to identify potential commercial uses for extractives based on their bioactivity, thus providing high-value products with benefits for agriculture and increasing sustainability and profitability of biofuels energy production.

Objective

The objective of this study was to evaluate different concentrations of ethanol extracts of switchgrass for inhibition of foodborne pathogens. Inhibition assays included serovars of *Salmonella enterica* (Salmonella Typhimurium and Salmonella Enteritidis), and isolates of shigatoxin-producing *Escherichia coli* 0157 (isolates 0111, 045, 026, cider, and Jack in the Box).

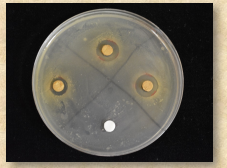

Fig. 1. Inhibition Disk Assay.

Materials and Methods

Switchgrass Extractives – Ethanol extractives of switchgrass were concentrated by recycling the ethanol with fresh switchgrass (samples CRC#25 and CRC#37 through the reactor three times). Two methods were employed to further concentrate the extractives. In Method 1, blank antibiotic disks were saturated with 250 µl of extractives and disks were dried to remove the ethanol solvent. Applications of extractives to disks were repeated to achieve final concentrations of 30X, 45X, 60X, and 75X. In Method 2, a Thermo Scientific Reacti-Vap was used to reduce the amount of solvent and concentrate extractives to 30X, 45X, 60X, and 75X with a combination of heat and exposure to a stream of filter-sterilized nitrogen gas (45-µm filters on the main gas line and line to the Reacti-Vap) until enough solvent was removed to achieve the desired concentration. Blank antibiotic disks were submerged in the concentrated extractives until disks were saturated. Treated disks were stored at -20°C until use. To remove any remaining solvent, treated disks were dried in a laminar flow hood for 30 min prior to use in inhibition assays.

Bacterial Cultures - Bacteria used for antimicrobial assays were *Escherichia coli* 0157 (isolates 0111, 026, 045, Cider, and Jack in the Box) and two serovars of *Salmonella enterica* (S. Enteritidis and S. Typhimurium).

Bacterial Inoculum - Bacteria were grown in tryptic soy broth (TSB) for 24 h at room temperature on a shaker at 150 rpm. Cultures were centrifuged to remove TSB and bacterial pellets were washed with sterile water, centrifuged, and re-suspended in phosphate buffered saline (PBS) to a turbidity of 35% using a Biolog turbidimeter. Tryptic soy agar (TSA) plates were spread with 100-µl of each bacterial-PBS solution to create bacterial lawns for inhibition assays.

Antibiotic Inhibition Disk Assay - Three disks treated with extractives and two control disks (treated with 95% ethanol and then dried to evaporate solvent; commercial gentamicin disk) were placed on TSA plates previously spread with single isolates of *E. coli*. Plates spread with *Salmonella* were treated similarly, except there was no gentamicin control (Fig. 1). To facilitate diffusion of extractives absorbed in the disks onto the bacterial lawn, 40 µl of sterile deionized water was added to each disk. Zones of inhibition that formed around each disk were measured after incubation for 3 days at room temperature. Measurements were corrected for water control values. The assay was designed as a factorial, with pathogen isolate and concentration of extractives treatment as factors. Differences in inhibition zone measurements were tested for significance with mixed models ANOVA (PC SAS, ver. 9.4).

References

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Results and Discussion

CRC#25 Extract - Method 1 (*E. coli*) – The effect of *E. coli* isolate was not significant, i.e. there were no significant differences for inhibition zone among isolates. However, the effect of extractives concentration of sample CRC#25 was significant at $P < 0.0001$ (Fig. 2). For all *E. coli* isolates combined, inhibition increased as extractives concentration increased. Although inhibition was greater at 75X than 60X, there was no significant difference in inhibition between the two high concentrations. Inhibition zones caused by extractives were not as large as those caused by commercial gentamicin disks.

CRC#25 Extract - Method 1 (*S. enterica*) – Effects of extractives concentration of CRC#25 and serovar were both significant at $P < 0.0001$. The interaction of serovar and extractives concentration was also significant ($P = 0.0017$). Inhibition zone size increased as concentration of extractives increased. However, serovar S. Typhimurium was significantly more inhibited than S. Enteritidis at higher concentrations of extractives (Fig. 3).

CRC#37 Extract - Method 2 (*S. enterica*) – The effect of *Salmonella* serovar on inhibition with CRC#37 was significant at $P = 0.0208$. As observed in previous assays, S. Typhimurium was more inhibited than S. Enteritidis (Fig. 4). Inhibition of S. Enteritidis and S. Typhimurium was greater at the highest concentration of extractives CRC#37 (Fig. 5); the effect of concentration was significant at $P = 0.0004$. The interaction of extractives concentration and pathogen serovar was not significant.

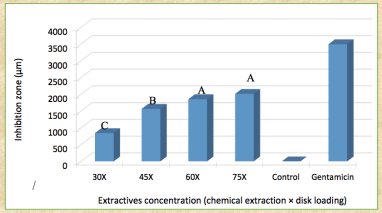

Fig. 2. Main effect of extractives (CRC#25) concentration on inhibition against five *E. coli* isolates; Method 1. Bars with the same letters are not significantly different at $P = 0.05$ (F-LSD test).

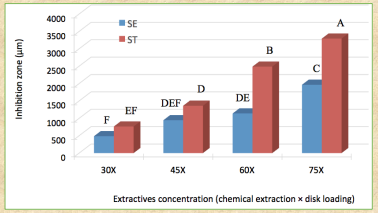

Fig. 3. Effect of the interaction of extractives (CRC#25) concentration and two *Salmonella* serovars (SE=S. Enteritidis, ST=S. Typhimurium); Method 1. Bars with the same letters are not significantly different at $P = 0.05$ (F-LSD test).

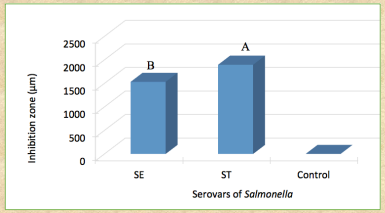

Fig. 4. Main effect of *Salmonella* serovars on inhibition after treatment with CRC#37 extractives; Method 2. Bars with the same letters are not significantly different at $P = 0.05$ (F-LSD test). SE=S. Enteritidis, ST=S. Typhimurium.

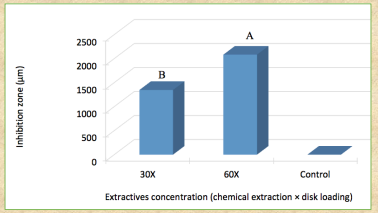

Fig. 5. Main effect of extractives (CRC#37) concentration on inhibition of two *Salmonella* serovars; Method 2. Bars with the same letters are not significantly different at $P = 0.05$ (F-LSD test).

Conclusions

Inhibition of foodborne pathogens tested with two samples of extractives at different concentrations using two methods of concentrating the extractives met with promising results. Between the two methods used to concentrate the extractives, Method 2 is 3-fold faster and reduces oxidation of sensitive active compounds in the extractives because the disks are exposed to oxygen for shorter periods of time. Extractives from CRC#25 inhibited growth of five isolates of *E. coli* when extractives were concentrated with Method 1; inhibition increased significantly as concentration increased up to 60X with no significant difference in inhibition at 75X. While none of the inhibition zones yielded measurements as large as those with gentamicin, this is the first test where we have shown activity against *E.coli* isolates with switchgrass extractives. In a very recent trial with Method 2 and *E. coli*, inhibition was also significant (data not shown). In *Salmonella enterica* assays, with extractives from CRC#25 and CRC#37, serovar S. Typhimurium was consistently inhibited more than serovar S. Enteritidis. Similar to assays with *E.coli*, inhibition increased as concentrations of CRC#25 extractives increased. However, with *Salmonella*, the differences in inhibition observed at 60X and 75X concentrations were significant. This suggests that we have not reached the upper limit of inhibition. Growth inhibition zones of *Salmonella* serovars were of similar size in tests with extractives from CRC#25 (Method 1) and CRC#37 (Method 2). This is expected since the extractions from these two samples were both prepared under comparable conditions. Future assays will focus on refining concentration techniques to obtain consistent inhibition results.

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