ENVIROTECH

EVALUATION STUDY

Efficacy of Peroxyacetic Acid (PAA) Against *Lactobacillus plantarum*

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Background

The yeast Saccharomyces cerevisae is used during fermentation in the production of ethanol. Contamination by *Lactobacillus* spp. in the corn mash mixture during fermentation causing loss of ethanol yield and a reduction in yeast growth. Traditionally, antibiotics such as Virginiamycin are routinely applied to corn fermentation tanks in order to control the proliferation of these undesirable Lactobaccilus spp. bacteria. However, there are growing concerns about the use of antibiotics because the antibiotic residues remain in the distiller's grain by-product used for animal feed, and the cost of the antibiotic regimen can be quite expensive.

This report explores an alternate means of controlling *Lactobacillus* spp. through the use of low concentrations (< 5ppm) of peroxyacetic acid (PAA). In particular, this study is a follow-up on the report entitled "The Efficacy of Peroxyacetic Acid (PAA) Against Saccharomyces cerevisae," where it was shown that PAA at less than 5ppm had negligible adverse impact upon the survival and growth of Saccharomyces cerevisae. The study challenges *Lactobacillus plantarum* against low concentrations of PAA to determine whether the bacteria will be mitigated using levels of PAA that are ineffective on the Saccharomyces cerevisae yeast.

There were a few obstacles to overcome before successfully culturing *Lactobacillus plantarum* for this testing. Some of these included adjustment to the correct pH and temperature, the use of anaerobic culturing conditions, and selection of the optimum growth medium.

Methods

A stock solution of *Lactobacillus plantarum* (ATCC#14917) was incubated at 37 degrees C for three days anaerobically in Criterion MRS Broth. The broth and bacteria mixture were centrifuged leaving the *Lactobacillus plantarum* to be re-suspended in 500mL Modesto city water. The pH of the *Lactobacillus*-city water solution was then adjusted to 3.97 (mimicking the pH of the corn mixture used in ethanol production) using hydrochloric acid and split into five of the following test solutions:

a) 100mL to serve as the control (no PAA added).
b) 100mL to be dosed to 1ppm PAA (from MP-2).
c) 100mL to be dosed to 2ppm PAA (from MP-2).
d) 100mL to be dosed to 5ppm PAA (from MP-2).
e) 100mL extra.

The PAA (from Perasan® MP-2) used in this experiment tested at 5.36% Hydrogen Peroxide and 15.55% PAA. A 1000ppm stock solution of PAA was made to dose the test solutions to 1-5ppm.

The control was serially diluted and plated at 102, 103 and 104 on previously prepared agar plates made from Criterion Lactobacilli Selective Agar. Three of the other test solutions were dosed to 1ppm PAA, 2ppm PAA and 5ppm PAA, respectively.

The PAA dosed to each solution was immediately measured using the modified DPD colorimetric method. Additional readings were taken at 30 minutes and one-hour intervals when the solutions were also serially diluted and plated at 102, 103 and 104 using prepared agar plates made from Criterion Lactobacilli Selective Agar. A final pH was taken for each test solution.

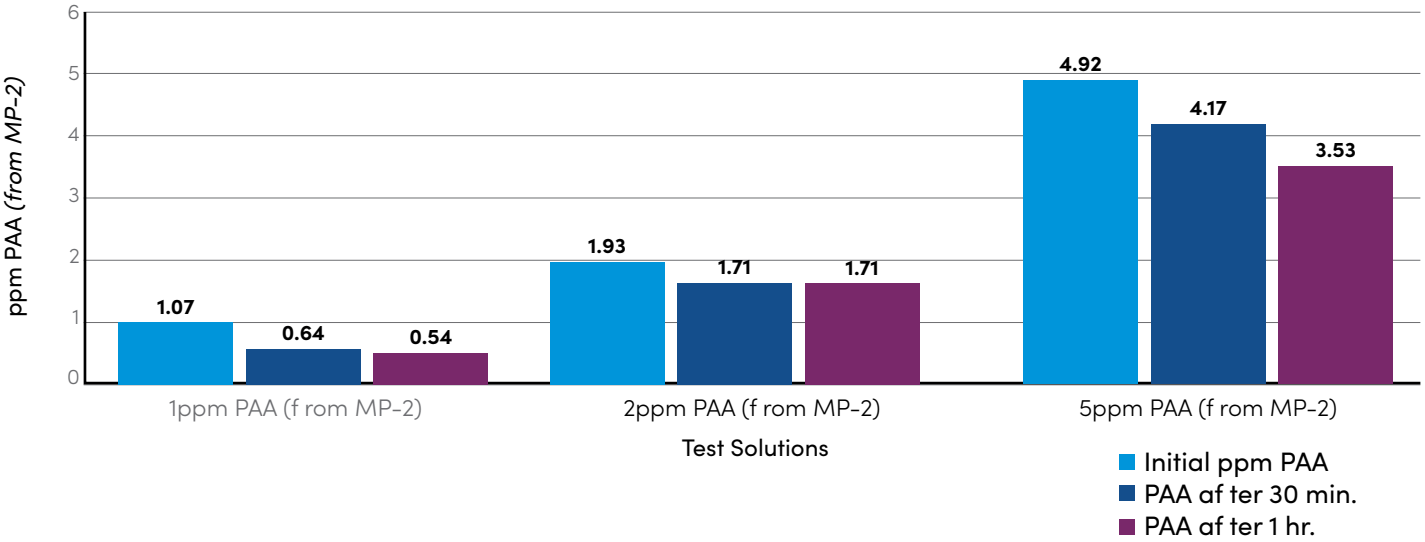
Results and Discussion

The actual PAA concentration of each test solution immediately after dosing with the 1000ppm stock solution of PAA (from MP-2), can be see in Table 1. This ensures that the test solutions used for bacterial plating were actually at the target PAA concentrations. The table also shows the recovered concentration of PAA (from MP-2) after 30 minutes and one hour for each test solution as well as the pH. The recovered PAA concentrations for the test solutions are reported in Table 1 and charted in Figure 1.

TABLE 1

Date	Description/ Challenge	Recovered PPM PAA	PPM PAA *(added)*	Initial pH	Final pH
1/18/2008	To 1ppm	1.07	1ppm	3.97	
	After 30 min.	0.64			
	After 1 hour	0.54			4.05
1/18/2008	To 2ppm	1.93	2ppm	3.97	
	After 30 min.	1.71			
	After 1 hour	1.71			3.9
1/18/2008	To 5ppm	4.92	5ppm	3.97	
	After 30 min.	4.17			
	After 1 hour	3.53			3.94

FIGURE 1 PAA Concentrations for Test Solutions (in ppm)



Efficacy of Peroxyacetic Acid (PAA) Against *Lactobacillus plantarum*

The media used in this experiment and the previous experiment performed with Saccharomyces does exert some PAA demand but in neither case is it significant enough to impact the results.

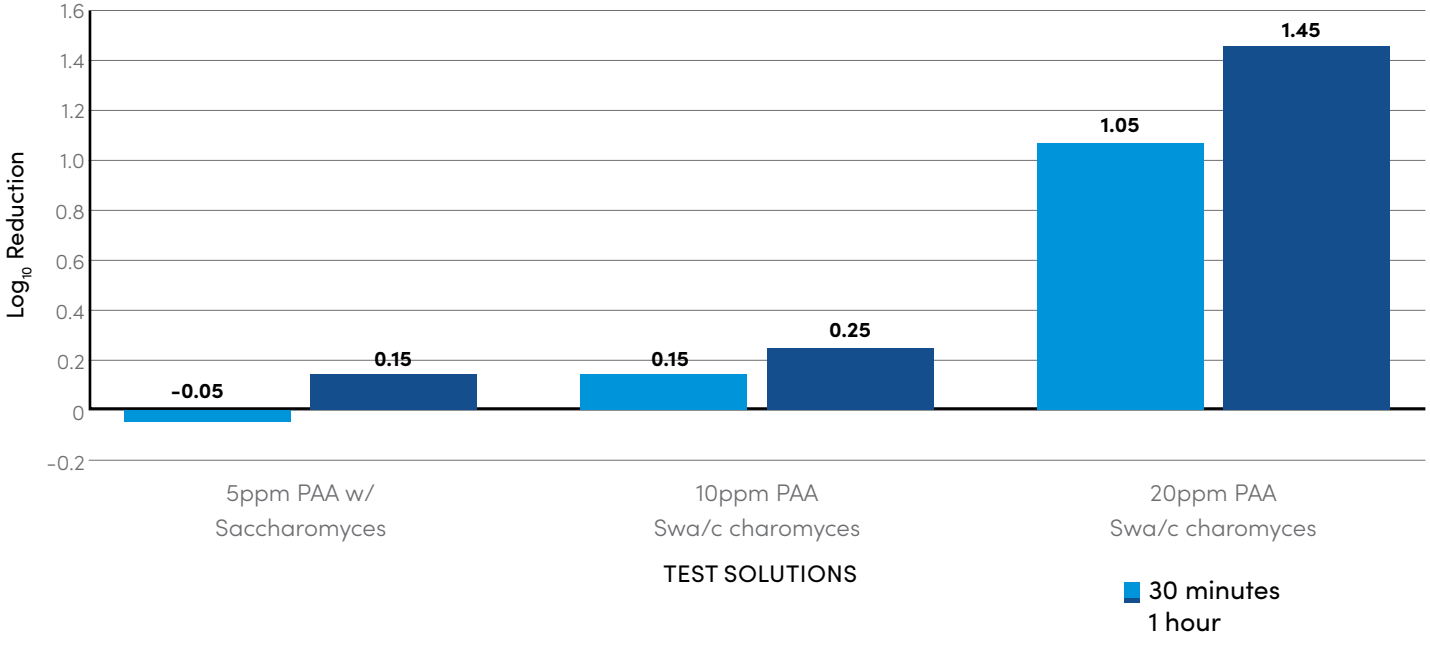
Table 2 demonstrates the log10 reduction for *Lactobacillus plantarum* which was 1.63 CFU/mL (97.7%) after 30 minutes when dosed to 1ppm PAA (from MP-2). There was no bacterial growth for the test solutions containing *Lactobacillus* and 2ppm PAA (from MP-2) and 5ppm PAA (from MP-2). This indicates a greater that 97.7% reduction in bacterial numbers. This shows that PAA is extremely effective against *Lactobacillus* spp. at low concentrations.

TABLE 2 Lactobacillus Challenge

Date	Description	Log_{10} CFU/mL	Log_{10} Reduction	Log_{10} CFU/mL	Log_{10} Reduction
1/18/2008	Control	3.63	N/A	N/A	N/A
1/18/2008	1ppm	2	1.63 (97.7%)	<2	>1.63 (>97.7%)
1/18/2008	2ppm	<2	>1.63 (>97.7%)	<2	>1.63 (>97.7%)
1/18/2008	5ppm	<2	>1.63 (>97.7%)	<2	>1.63 (>97.7%)

Figure 2 shows the effect of PAA on the organism Saccharomyces cerevisae. It can be seen that there is a log10 reduction of 1.45 CFU/mL when the Saccharomyces yeast cells are challenged with 20ppm PAA for one hour which is detrimental to their survival. Figure 2 also shows that the low concentrations, 5ppm PAA and 10ppm PAA, are ineffective against Saccharomyces cerevisae but compared to table 2, the same concentration is clearly very effective in controlling Lactobacillus. The log10 reduction data for Saccharomyces stated in this report and shown in Figure 2 came from the report "The Efficacy of Peroxyacetic Acid (PAA) Against Saccharomyces cerevisae."

FIGURE 2 Log_{10} Reduction ofS acchaormyces Test Solutions



Conclusions

- The yeast Saccharomyces cerevisae is used during ethanol fermentation. Contamination by the bacteria *Lactobacillus* in the corn mixture during fermentation has been a problem in the ethanol industry. An alternate means of controlling *Lactobacillus* spp. is needed in Ethanol production because of concerns about the use of antibiotics.
- This study is a follow up on the report called "The Efficacy of Peroxyacetic Acid (PAA) Against Saccharomyces cerevisae," where it was shown that PAA at less than 5ppm had negligible adverse impact upon the survival of Saccharomyces cerevisae. This report challenges *Lactobacillus plantarum* against low concentrations of PAA to demonstrate that the bacteria will be controlled with concentrations of PAA where Saccharomyces cerevisae is unaffected.
- The $\log_{10}$ reduction for *Lactobacillus plantarum* was 1.63 CFU/mL after 30 minutes when dosed to 1ppm PAA (from MP-2). There was no bacterial growth for the test solutions containing *Lactobacillus* and 2ppm PAA (from MP-2) and 5ppm PAA (from MP-2). This shows that PAA is extremely effective against *Lactobacillus* spp. at low concentrations but ineffective against Saccharomyces cerevisae at the same concentrations. Based on the results of this study, an acceptable level of PAA to control *Lactobacillus plantarum* would be between 1-3ppm. It is recommended that a 1-3ppm PAA dose to fermentation tanks in ethanol production would control *Lactobacillus* spp. and leave the Saccharomyces yeast largely unaffected.

Addendum to Report 7/29/08

- A contact in the bioethanol industry gave me the following figures which I have crunched as if it were the Altra Biofuels plant in Goshen, CA:
- Capacity 31.5 million gallons/year (from Altra Biofuels website).
- 2.7 gallons ethanol is produced per bushel of corn,
- 31.5/2.7 = 11.7 million bushels of corn per year
- The plant operates 357 days per year.
- 11.7/357 = 0.0326 million bushels of corn per day.
- There are 56 lb of corn in a bushel
- 0.0326 x 56 = 1.83 million lb corn per day.
- The mash is 40% corn.
- 1.83/0.4 = 4.6 million lb total mash weight per day.
- This 4.6 million lb total mash weight is dosed to 80 mg PAA/kg to overcome the demand and establish a residual.
- Assuming Perasan MP-2 is 15% PAA, and has a density of 9.25 lb/gallon, I calculate that 265.5 gallons of Perasan MP-2 would be required per day. At $14.00/gallon, the total daily cost is $3,717.00

This is significantly different to the 34 gallons per day of MP-2 product that was estimated for the Goshen plant using the numbers they supplied us with. Altra Biofuels indicated the flow rate of water into the plant was only 50 gpm. Say there are 2.77 million lb of water in the mash per day. This corresponds to a flow rate of 230 gallons per minute, so it looks as if Goshen was operating at 4.6 times lower than nameplate capacity. When the 34 gallons of MP-2 (original estimate) is multiplied by 4.6 we get 156.4 gallons which is closer to the calculated value of 265.5 gallons of MP-2 required per day.

Now let's look at the cost of using 2 mg/L of Virginiamycin in the same 4.6 million lb total mash weight per day. This is a typical application dose (www.lactrol.com). It can be calculated only 9.2 lb of Virginiamycin would be required. At $90.00 per lb the daily cost is $828.00 (see also www.lactrol.com) which is close to the $300,000 per anum cost that Goshen claimed to have been spending on antibiotics. Of course, both sets of calculations assume that the entire flow of water to the plant is treated with either Perasan MP-2 or Virginiamycin. We know that once the PAA demand had been satisfied it was envisaged that the PAA feed would have been terminated and that each batch tank would have been treated just once and perhaps twice during the 45-hour fermentation, but we also do not know if Virginiamycin is used in this fashion also.

However, if the calculations are correct, there is a large cost differential between treating the mash tanks with PAA or Virginiamycin. It seems that Altra Biofuels fed us bogus information on which we went forward with this endeavor.