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ORIGINAL PAPER

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Effect of yeast inoculation rate on the metabolism of contaminating lactobacilli during fermentation of corn mash

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Abstract Two separate 4 (bacterial concentrations)×6 (yeast concentrations) full factorial experiments were conducted in an attempt to identify a novel approach to minimize the effects caused by bacterial contamination during industrial production of ethanol from corn. *Lactobacillus plantarum* and *Lactobacillus paracasei*, commonly occurring bacterial contaminants in ethanol plants, were used in separate fermentation experiments conducted in duplicate using an industrial strain of *Saccharomyces cerevisiae*, Allyeast Superstart. Bacterial concentrations were 0, 1×10^6, 1×10^7 and 1×10^8 cells/ml mash. Yeast concentrations were 0, 1×10^6, 1×10^7, 2×10^7, 3×10^7, and 4×10^7 cells/ml mash. An increased yeast inoculation rate of 3×10^7 cells/ml resulted in a greater than 80% decrease ($P<0.001$) and a greater than 55% decrease ($P<0.001$) in lactic acid production by *L. plantarum* and *L. paracasei*, respectively, when mash was infected with 1×10^8 lactobacilli/ml. No differences ($P>0.25$) were observed in the final ethanol concentration produced by yeast at any of the inoculation rates studied, in the absence of lactobacilli. However, when the mash was infected with 1×10^7 or 1×10^8 lactobacilli/ml, a reduction of 0.7–0.9% v/v ($P<0.005$) and a reduction of 0.4–0.6% v/v ($P<0.005$) in the final ethanol produced was observed in mashes inoculated with 1×10^6 and 1×10^7 yeast cells/ml, respectively. At higher yeast inoculation rates of 3×10^7 or 4×10^7 cells/ml, no differences ($P>0.35$) were observed in the final ethanol produced even when the mash was infected with 1×10^8 lactobacilli/ml. The increase in ethanol corresponded to the reduction in lactic acid production by lactobacilli. This suggests that using an inoculation rate of 3×10^7 yeast cells/ml reduces the growth and metabolism of contaminating lactic bacteria significantly, which results in reduced lactic acid production and a concomitant increase in ethanol production by yeast.

Keywords *Saccharomyces cerevisiae* · *Lactobacillus* · Inoculation rate · Ethanol · Lactic acid

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Introduction

Lactic acid bacterial contamination is the major cause for reduced ethanol yield during fermentation of starch-based feedstock by *Saccharomyces cerevisiae*. These Gram-positive, rod-shaped bacteria can tolerate high temperature and low pH [10], and are able to survive and grow rapidly under ethanol production conditions. Predominant contaminants isolated from distilleries and fuel alcohol plants belong to the genus *Lactobacillus*. Lactobacilli ferment carbohydrates for growth and energy production, with the latter leading to the production of lactic acid and small amounts of acetic acid. In addition to reducing ethanol yield, the presence of bacterial metabolites in the fermentation medium inhibits yeast growth [3, 5, 12, 14]. Methods used in the beverage alcohol industry to control bacteria include stringent cleaning and sanitation, and acid washing of yeast destined for reuse.

In the fuel ethanol industry, control of bacterial contamination is achieved by using antibiotics such as penicillin G, streptomycin, tetracycline [1, 4], virginiamycin [7, 9], monensin [17], or mixtures thereof. However, the concept of antibiotic use in an industrial process is controversial. Studies by Islam et al. [9] on the stabilities of virginiamycin and penicillin G at 25 or 35°C during alcohol fermentation at pH 4.8 revealed that the biological half-life of penicillin G was 24 and 4 h at 25 and 35°C, respectively, whereas the concentration of virginiamycin remained unaltered for 72 h at 35°C. Virginiamycin, when used at concentrations over 2 ppm, was found to suppress fermentation rates [6]. This latter study also found that the stillage contained

about 13.2% of the original virginiamycin level. Further heating of the stillage (30 min at 100°C) reduced virginiamycin levels to 2.6% of the original value.

Despite the large number of available methods of control, lactic acid bacterial contamination does occur during industrial scale ethanol production. Moreover, inappropriate use of antibiotics contributes to the buildup of reservoirs of antibiotic-resistant bacteria [11]. Decreasing the incidence of antibiotic resistance will require improved systems for monitoring outbreaks of antibiotic-resistant bacteria and more judicious use of antibiotics. Unawareness of, or inadequate surveillance for, resistance results in misuse or overuse of antibiotics. Therefore, strategies to minimize the effects of contaminating bacteria without using antibiotics will result in the prevention of the outbreak of antibiotic resistance in bacteria.

Preliminary laboratory work demonstrated that the use of increased yeast inoculation rates help yeast to outcompete bacteria. In brewing, higher yeast inoculation rates cause attenuation to begin more rapidly, and reduce viability losses that occur immediately after pitching [2]. Increased yeast inoculation rates also alleviate the effects of nitrogen limitation in high gravity brewing [15] and in grape juice fermentations [8].

In this paper, the effects of yeast inoculation rates on the metabolism of contaminating lactic acid bacteria were examined. Ethanol production by yeast was evaluated at different yeast inoculation rates in corn mashes contaminated with levels of lactic acid bacteria pre-determined to be detrimental to ethanol yield [14].

Materials and methods

Microorganisms

The strain of *S. cerevisiae* used in these studies is an industrial strain common in the fuel and beverage alcohol industries (Allyeast Superstart™; Alltech, Nicholasville, Ky.). *Lactobacillus plantarum* and *Lactobacillus paracasei* were isolated from contaminated mashes in fuel ethanol plants and identified both by API CHL50 and Biolog Microlog™ microbial identification systems.

Preparation of corn mash and fermentation

Corn (US#1, from a local supplier; Thompson & Shearer, Nicholasville, Ky.) was ground using a hammer mill (Model#9506TF, Bliss Industries, Ponca City, Okla.) with a #4 screen to get the appropriate grind size. To make 5 l of 25% dry solids mash, 1.436 kg corn was slurried in 3.564 l tap water (since the corn had ~13% moisture). Tap water (3.564 l) was placed in a pot, which was then placed in heated water bath. The whole set-up was placed under a Silverson L4RT laboratory mixer (Silverson Machines, Waterside, Chesham, Bucks, UK). Ground corn was slowly added to the heated water. Once a few scoops of ground corn were added, one-third of the α-amylase (high TDS—145,000 amylase units/ml; Alltech) was added to reduce viscosity, thereby preventing starch retrogradation. The dose of α-amylase is 1 ml/100 g grain. All the ground corn was then added and continuously mixed. The pot was covered with aluminum foil to prevent loss of moisture due to evaporation. Once the temperature of the mixture reached 85°C, it was held for 15 min (for gelatinization of starch). The mixture was then autoclaved at 121°C for 15 min. After autoclaving, the mixture (mash) was cooled to 85°C and placed in a water bath at 85°C for 1 h (with continuous stirring) with the rest of the α-amylase added (for liquefaction of starch). After 1 h, the mash was cooled to the fermentation temperature (30°C), and 200-g quantities were dispensed into 500-ml sterile Erlenmeyer flasks for fermentation. The mash pH was not adjusted. At the start of fermentation, the pH of the mash was 5.5.

Experimental design

A 4 (bacterial concentrations) ×6 (yeast concentrations) full factorial experiment was conducted. Bacterial concentrations were 0, 1×10^6, 1×10^7, and 1×10^8 cells/ml mash, and yeast concentrations were 0, 1×10^6, 1×10^7, 2×10^7, 3×10^7, and 4×10^7 cells/ml.

In following the normal recommended dose for normal gravity mashes [14], urea (8 mM) was added to all the flasks. Saccharification of dextrins to glucose was carried out by adding filter-sterilized glucoamylase (Allcoholase II L400; Alltech) at 0.08% by weight of grain. The flasks were incubated at 30°C and the fermentation was carried out for 72 h, after which samples were withdrawn and analyzed for ethanol and lactic acid using high-performance liquid chromatography (HPLC).

HPLC analysis

Sugar and lactic acid concentrations were determined by HPLC analysis. A 20-μl aliquot from a suitably diluted fermentation sample was analyzed using an HPX-87H column (Bio-Rad, Hercules, Calif.)—which analyzes sugars, alcohols, and organic acids—maintained at 65°C. Sulfuric acid (2 mM) was used as the mobile phase at a flow rate of 0.6 ml/min. Standard samples were prepared using known concentrations (in % w/v) of all components of interest, such as maltodextrins, maltotriose, maltose, glucose, lactic acid, glycerol, acetic acid, and ethanol. Components were detected with a differential refractometer (Model 2410; Waters, Milford, Mass.). Data were processed using the Millennium[32] computer program (Waters).

Results

Lactic acid production by lactobacilli

Lactic acid production by both *L. plantarum* and *L. paracasei* was reduced significantly as yeast inoculation rates increased to 30×10^6 and 40×10^6 cells/ml mash (Figs. 1, 2). An 82% reduction in lactic acid was observed with *L. plantarum*, whereas a 56% reduction in lactic acid was observed with *L. paracasei*. The lactic acid produced by these lactobacilli serves as a good indicator for the numbers of bacteria since there is a direct relationship between the number of bacterial cells and the final amount of lactic acid produced [14]. This suggests use of a higher yeast inoculation rate as one

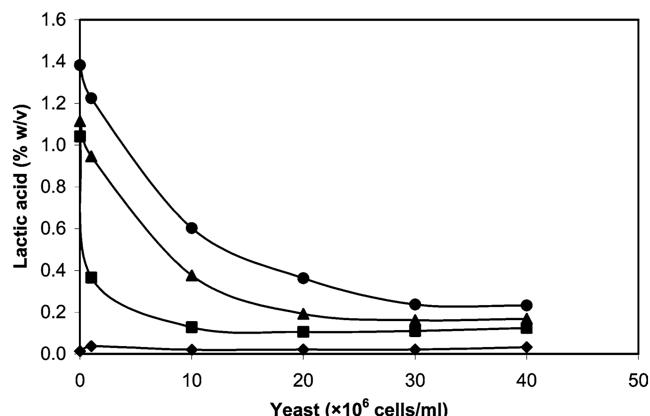

Fig. 1 Lactic acid (% w/v) produced by *Lactobacillus plantarum* at 30°C inoculated at various levels to fermenting corn mashes with different yeast inoculation rates. Values are means of duplicate fermentations. The coefficient of variation was <5%. *Diamonds* No bacteria, *squares* 1×10^6 cells/ml, *triangles* 1×10^7 cells/ml, *circles* 1×10^8 cells/ml

Table 1 Ethanol (% v/v) produced after 72 h of fermentation of corn mash (25% dissolved solids, infected with *Lactobacillus plantarum* at different levels) at 30°C by yeast inoculated at various levels. Values are means of duplicate fermentations. The ethanol levels in each column are compared; means with the same letter are not significantly different ($P > 0.05$) from each other. *SEM* Standard error of the mean, *LSD* least significant difference

Bacteria (cells/ml)	Ethanol (%v/v)				
	1×10^6 yeast cells/ml	1×10^7 yeast cells/ml	2×10^7 yeast cells/ml	3×10^7 yeast cells/ml	4×10^7 yeast cells/ml
0	14.09^a	13.96^a	14.18^a	14.32^a	14.04^a
10^6	14.02^a	13.95^a	14.14^a	14.16^a	14.00^a
10^7	13.42^b	13.61^b	13.99^a	14.10^a	13.98^a
10^8	13.17^c	13.27^c	13.64^b	14.09^a	13.95^a
SEM	0.06	0.05	0.08	0.10	0.07
LSD	0.23	0.18	0.31	0.40	0.26

Table 2 Ethanol (% v/v) produced after 72 h of fermentation of corn mash (25% dissolved solids, infected with *Lactobacillus paracasei* at different levels) at 30°C by yeast inoculated at various levels. Values are means of duplicate fermentations. The ethanol levels in each column are compared; means with the same letter are not significantly different ($P > 0.05$) from each other

Bacteria (cells/ml)	Ethanol (%v/v)				
	1×10^6 yeast cells/ml	1×10^7 yeast cells/ml	2×10^7 yeast cells/ml	3×10^7 yeast cells/ml	4×10^7 yeast cells/ml
0	14.20^a	14.18^a	14.16^a	14.11^a	14.09^a
10^6	13.75^{ab}	13.85^{ab}	13.98^{ab}	13.99^a	14.02^a
10^7	13.57^b	13.74^b	13.92^b	13.98^a	14.01^a
10^8	13.50^b	13.66^c	13.76^b	13.92^a	13.94^a
SEM	0.12	0.10	0.06	0.07	0.06
LSD	0.45	0.39	0.23	0.26	0.23

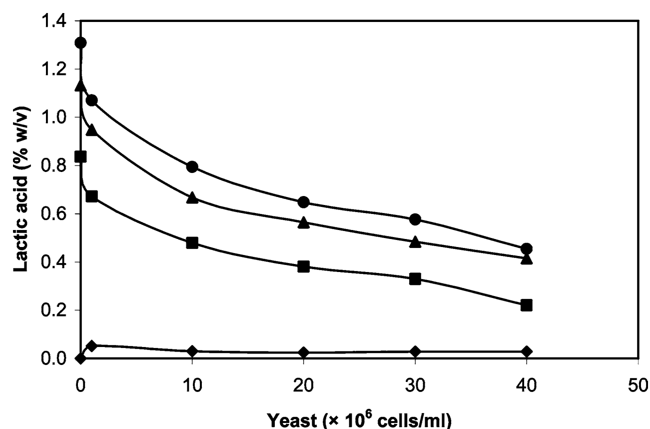

Fig. 2 Lactic acid (% w/v) produced by *Lactobacillus paracasei* at 30°C inoculated at various levels to fermenting corn mashes with different yeast inoculation rates. Values are means of duplicate fermentations. The coefficient of variation was <5%. *Diamonds* No bacteria, *squares* 1×10^6 cells/ml, *triangles* 1×10^7 cells/ml, *circles* 1×10^8 cells/ml

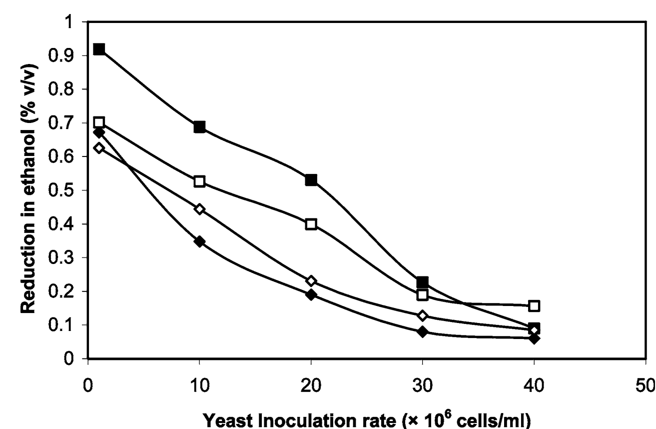

Fig. 3 Reduction in final ethanol concentration (in a corn mash fermentation) due to bacterial contamination as influenced by yeast inoculation rate. *Filled symbols* Mash contaminated with *L. plantarum* at various levels, *open symbols* mash contaminated with *L. paracasei* at various levels. *Diamonds* 1×10^7 cells/ml, *squares* 1×10^8 cells/ml

way to stress the contaminating lactic bacteria so that the growth and metabolism of the latter are inhibited.

Effects on final ethanol concentration

No significant differences were observed in the final ethanol produced by the yeast at any of the inoculation rates tested in the contaminant-free mashes (Tables 1, 2). However, in the presence of bacteria, no significant differences in final ethanol concentrations were observed when the yeast inoculation rates were over 20×10^6 cells/ml. Similar observations were made for the two strains of lactobacilli tested even when the bacteria were inoculated at a level of 1×10^8 cells/ml. This relates well with the lactic acid levels, i.e., at higher yeast inoculation rates, the lactobacilli are stressed and their growth and metabolism inhibited. This results in lower lactic acid production and a higher final ethanol concentration in the fermentation. The reduction in ethanol produced by the yeast (even when mash is infected with 1×10^8 lactobacilli/ml) becomes insignificant when higher yeast inoculation rates such as 30 or 40×10^6 cells/ml are used (Fig. 3). The fermentation supernatants were tested for antibacterial activity against the test bacteria as described by Oliva-Neto et al. [16]. No antibacterial activity was observed, indicating that the yeast strain used did not produce any antibacterial compounds to outcompete the test bacteria. Similar experiments performed in mashes with 30% dry solids yielded very similar results (data not shown).

Discussion

Bacterial contamination in an industrial-scale ethanol production process is unavoidable. Occurrence of 10×10^6 lactobacilli/ml mash results in approximately 1% v/v reduction in the final ethanol produced by the yeast, depending on the strain of the contaminant bacteria [14]. This 1% reduction in ethanol yield is quite significant to distillers of fuel alcohol since their profit margins are very narrow [13]. Antibiotics are used for the control of these bacteria in fuel ethanol plants, but use of antibiotics in beverage ethanol production is not permitted. Moreover, if antibiotics are not administered correctly, the development of antibiotic resistant strains can become a reality. Considering the growing concern surrounding antibiotic resistance development in bacteria, the strategy of using higher yeast inoculation rate discussed in this paper provides a means to minimize the effects caused by contaminant lactobacilli, thereby avoiding the use of antibiotics during ethanol production. The results presented demonstrate that a high yeast inoculum at the start of fermentation allows the yeast to outgrow the contaminant bacteria.

The standard recommendation in the ethanol industry is to inoculate or "pitch" yeast at 1×10^6 cells/ml per percent dry solids (i.e., 25×10^6 cells/ml for a 25% solids mash), and this recommendation may indeed be followed for contaminant-free mashes. From the data presented, it can be concluded that by using a yeast inoculation rate of 30–40×10^6 cells/ml mash, the growth and metabolism of contaminating lactobacilli are significantly inhibited (due, presumably, to competitive exclusion). This results in lower lactic acid production and ultimately increases the final ethanol produced. Use of higher than normal yeast inoculum (a comparatively cheap input) can increase ethanol yields significantly even if the mash is infected by bacteria.

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