Hop acids as natural antibacterials can efficiently replace antibiotics in ethanol production

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Abstract

This study was carried out at the Fermentation Technology Institute of the University of Hohenheim. The aim of the study was to evaluate the potential of hop acids as natural antibacterials in distillery mashes for alcoholic fermentation. LactoStab™ and IsoStab™ were investigated for their effect on growth and lactic acid production by two selected strains of lactobacilli. The potential of improving ethanol yields by use of LactoStab™ and IsoStab™ during alcoholic fermentation of wheat mash and sugar beet molasses mash was monitored. Penicillin G and Virginiamycin were used as referential substances for comparison of antibacterial activity. LactoStab™ and IsoStab™ prevented growth of lactobacilli and production of lactic acid at ppm levels. Production of lactic acid and acetic acid was blocked at inhibitory concentrations of LactoStab™ and IsoStab™. Both substances acted bactericidal when inhibitory concentrations were doubled. Of all tested antibacterials, Virginiamycin was most effective and acted bactericidal at a concentration of only 0.5 ppm. However, ethanol yields obtained at inhibitory/ bactericidal concentrations of hop acids were comparable to those obtained with inhibitory/ bactericidal concentrations of antibiotics. Penicillin G, at a concentration of only 0.25 ppm, inhibited bacteria growth in most trials, but induced resistance in *L. fermentum* in wheat mash fermentation. IsoStab™ and LactoStab™ are available to the ethanol industry through BetaTec Hop Products GmbH. They consist of components contained in beer and spent hops and bear no risk for human or animal health . Thus, they provide an excellent, safe alternative to control bacteria in ethanol fermentations.

Introduction

It is common knowledge that bacterial contamination in alcoholic fermentation leads to formation of undesirable side products and causes losses in ethanol yield. Lactic acid bacteria are the most troublesome bacterial contaminant in ethanol production because they develop fast in the carbon dioxide atmosphere of fermentation mashes, at fermentation temperatures between 30 and 40°C, and at low pH values. Contamination can originate from various infection sources like raw material, enzymes, and dead legs in the production facilities. Especially during yeast propagation and yeast cell recycling bacteria are given time to proliferate. High bacteria numbers lead to decrease of yeast growth and yeast metabolism due to competition for available nutrients and the excretion of toxic metabolites like lactic acid (de Oliva Neto et al., 1994). Consequently it is essential to take measures to control bacteria in ethanol fermentation. Besides recognised factors such as good maintenance and cleaning, a wide range of antibacterials are available for disinfection of mash. Nevertheless, all conventional antibacterials in ethanol production show certain weaknesses with respect to spectrum of antibacterial activity, selectivity and yeast health, chemical stability under fermentation conditions, or environmental safety regarding animal and human health. Mounting evidence suggests that widespread overuse of technical antibiotics may contribute to the emergence of resistant pathogenic germs. The antibacterial properties of the hop plant *Humulus lupulus* have long been known and used in the brewing of beer by breweries for over 1,000 years (Jährig et al., 1981).The aim of the study was to investigate the inhibitory potential of hop acids as a natural alternative to control bacteria in ethanol fermentation.

Materials and methods

Lactobacilli for infection of mash

Two different strains of the genus *Lactobacillus* were used for inoculation of fermentation mashes: *L. brevis* LTH 5290 and *L. fermentum* LTH 5298. Preliminary tests proved that both strains were capable of growing in sugar beet molasses wort and wheat mash containing ethanol up to 9% v/v. Both strains belong to the family of heterofermentative lactobacilli and are able to ferment sucrose besides glucose. Their glucose-metabolism produces DL lactic acid besides acetic acid or ethanol, and CO_2. The optimum growth temperature is 30°C for *L. brevis* and 37°C for *L. fermentum*. Bacteria were grown in sterile MRS broth under anaerobic conditions. The pH was adjusted to pH5.2 to allow for the bacteria to adapt to the conditions at the start of fermentation.

Preparation of hop acids

Hop extract containing various hop acids was tested for antibacterial activity. The CO_2 hop extracts were diluted in demineralised water to give a concentration of 1,000 ppm hop acid. Appropriate quantities of the dilutions were added to mash to give a concentration ranging from 1 to 30 ppm hop acid.

Assay of MIC (Minimal Inhibitory Concentration)

The MIC for each hop acid was determined by tube dilution technique in MRS broth adjusted to pH 5.2. The test inoculum was prepared by aseptically harvesting the cells of a log phase culture by centrifugation at 10,200 x g for 15 minutes. The bacteria pellets were washed twice and resuspended in sterile peptone water before adding 10^6 CFU/ ml to the test tubes containing different concentrations of hop acids. The bacteria were incubated in anaerobic jars at their optimum growth temperature of either 30°C or 37°C for 60 hours. Bacterial growth was determined by turbidity measurement.

Preparation of antibiotics as conventional antibacterial reference

It has been reported (Hynes et al., 1997) that a concentration of 0.5 mg/ kg Virginiamycin in mash was sufficient to control lactic acid bacteria. 0.25 mg of Virginiamycin was disssolved in 100 ml demineralised sterile water. 1 ml of this dilution was added to 500 g of mash to give a concentration of 0.5 ppm in mash. Penicillin G Sodium for technical use was used according to manufacturer's recommendation at a concentration of 0.25 ppm.

Reduction of yeast inoculum

The number of viable cells per gram of active dry yeast (Schlienzmann Brennereihefe forte) was determined by enumeration of yeast cells on YPD medium. Enumeration resulted in approximately 10^9 viable yeast cells per gram of active dry yeast. Fermentation time was monitored subject to content of fermentable sugars, fermentation temperature and yeast dosage in order to minimize yeast dosage and create a worst-case scenario to reveal the effects of bacterial contamination. It had been reported before (Hynes et al., 1997) that high yeast numbers could mask the effect of bacterial contamination if yeast outgrows bacteria. For each fermentation sample of 500g mash a yeast inoculum of 0.6 g was dispersed in 10 ml of tap water and incubated at 30°C for the minutes on a shaker. Yeast inoculum corresponds to an initial viable number of 1.2 x 10^6 *Saccharomyces cerevisiae*/ ml.

Preparation and inoculation of sugar beet molasses wort

The content of sucrose in beet molasses was determined polarimetrically after clarification with lead acetate. For fermentation beet molasses was diluted with distilled water to obtain a wort containing 130 g/l of sucrose. The wort was heated to 80°C for 30 min at pH 5.2 in order to pasteurise the wort and invert sucrose. After cooling to 30°C or 37°C various concentration of the inhibitory substances (hop acids or conventional antibacterials) were added diluted in distilled water. The samples were then inoculated with bacteria in the log phase to give an initial viable cell number of around 10^6 CFU/ ml. Bacteria cell numbers were determined by turbidity measurement using a Beck photometer. Bacteria were transferred in used up MRS broth to replace yeast nutrient supplement and monitor the effect of high lactic acid content on fermentation. After yeast addition the wort was transferred to 1 litre fermentation flasks and filled up with tap water to 500 g. Fermentation trials were carried out at particular optimum temperature for each contaminant. Fermentation was completed after 96 hours when incubating flasks at 30°C and after 72 hours when incubating flasks at 37°C.

Preparation and inoculation of wheat mash

The amount of fermentable substance (maltose, glucose and fructose) contained in commercial winter wheat was determined by HPLC method (Senn, 1988). The wheat containing 60% fermentable substance was ground on a 0.5 mm disk mill. 80 grams of ground wheat were diluted in 300 ml tap water and transferred to a MA-3/E mash bath before adding high temperature bacterial alpha-amylase. Gelatinisation and liquefaction were carried out during a liquefaction rest of 30 minutes at 65°C. The saccharification mash was cooled to 52°C and pH was adjusted to pH 5.2 before adding *Aspergillus niger* glucoamylase. After a saccharification rest of 30 minutes, mash was cooled to 30°C or 37°C and various concentrations of the inhibitory substances (hop acids or conventional antibacterials) were added diluted in distilled water. The samples were then inoculated with bacteria in the log phase to give an initial viable cell number of around 10^7 CFU/ ml. Bacteria cell numbers were harvested by centrifuging at 10,200 x g for 15 minutes, washed twice to wash out lactic acid and re-suspended in sterile peptone water. Cell numbers were determined by turbidity measurement using a Beck photometer. After yeast addition the mash was transferred to 1 litre fermentation flasks, filled up with tap water to 500 g. Fermentation trials were carried out at particular optimum temperature for each contaminant. Fermentation was completed after 96 hours when incubating flasks at 30°C and after 72 hours when incubating flasks at 37°C.

Viable count of bacteria cells

Viable cell count after fermentation was monitored by a rapid method of streak plate technique (Baumgart, 1994). MRS-agar plates were divided into six pie chart pieces. Dilution series were made from each sample of fermented mash and a 50 µl each dilution were transferred into a pie chart section. After incubation in anaerobic jars for 48 hours at 30°C or 37°C the sections containing between 5 and 50 colonies were taken for enumeration. The number of colony forming units per ml wort was calculated as a weighed average:

$$CFU/\ ml = [\sum C/ (n_1 \times 1 + n_2 \times 0.1)] \times d$$

n_1 = number of plates at lowest numerable dilution
n_2 = number of plates at highest numerable dilution
d = 1/ lowest numerable dilution

HPLC analysis

Content of residue sugars (raffinose, sucrose, maltose, glucose and fructose), content of organic acids (lactic acid and acetic acid) and ethanol concentration in fermented mash were determined by HPLC analysis using a ProntoSIL 120-3-C18 AQ column maintained at 50°C after calibration with standards of analytical grade.

Determination of ethanol yield in fermented mash

Distillation was carried out using an automatic vapour distillation equipment for sample distillation. 50 g mash was transferred into a distillation flask and adjusted to pH 7 in order toprevent carry over of organic acids. The distillate was caught in a 100 ml graduated flask, topped up to the 100 ml mark with distilled water and tempered to 20°C for determination of ethanol yield in a digital density meter.

Results and discussion

Determination of the MIC for LactoStab™ and IsoStab™

The MIC for LactoStab™ and IsoStab™ was determined by tube dilution technique in MRS broth adjusted to pH 5.2 as described above. LactoStab™ and IsoStab™ stopped growth of bacteria at concentrations between 18 and 48 ppm. *L. fermentum* was more sensitive to the antibacterial action of hop acids than *L. brevis*. The MICs for LactoStab™ and IsoStab™ are given in Table 1.

Table 1. MIC of LactoStab™ and IsoStab™ against different lactobacilli after 60 hours in sterile MRS broth.

MIC	*L. brevis*	*L. fermentum*
IsoStab™	48 ppm	24 ppm
LactoStab™	30 ppm	20 ppm

LactoStab™ and IsoStab™ as antibacterials in beet molasses fermentation

As described above, sugar beet molasses wort for fermentation was inoculated with 10^6 lactobacilli/ ml in spent MRS-broth. This technique was responsible for a high initial concentration of lactic acid in wort. High lactic acid content together with reduced yeast dosage of 1.2 x 10^6 viable yeast cells/ ml and helped to visualise the inhibitory effect of bacterial metabolism on yeast performance. Acetic acid and lactic acid concentrations given in diagrams, table and text of this article refer to metabolites produced during fermentation. In 1997, Hynes et al. found that bacteria must create biomass quickly in order to create enough metabolic potential to compete with yeast cells for sugar and create ethanol-reducing levels of lactic acid prior to termination of fermentation. The toxic effect of lactic acid on yeast in molasses fermentation has been described by Ngang et al, 1989. Yeast performance and consequently ethanol yields are affected when bacteria numbers exceed 10,000 cells/ ml, depending on sugar content and osmotic stress in fermentation (Ngang et al, 1990).

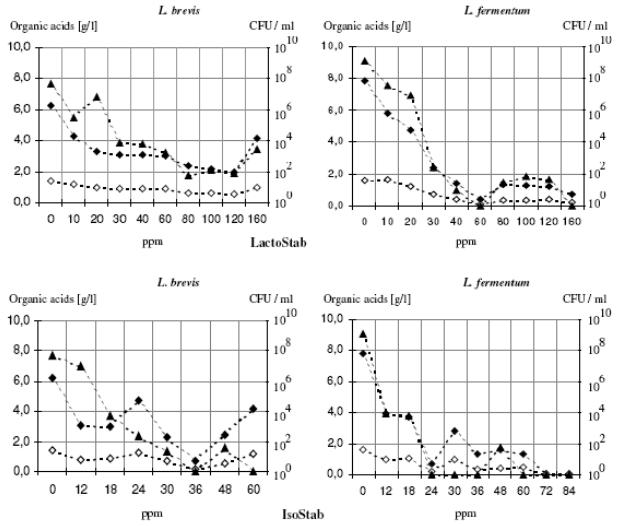
The antibacterial mode of action of hop acids was discovered by Simpson in 1993. He described hop acids as true ionophores. They dissipate the bacterial transmembrane pH gradient by transport and accumulation of protons inside bacteria thus inhibiting uptake of nutrients and, consequently, excretion of metabolites (Simpson, 1993).

Bacterial count in fermented molasses mash decreased with increasing concentration of hop acids. Along with the decrease in bacteria numbers a decrease in the concentration of lactic acid and acetic acid in fermented mash was observed. Fig 1. shows the decrease of viable cell numbers and bacterial metabolites for LactoStab™ and IsoStab™.

In control samples without inhibitory disinfectant, bacteria numbers of *L. brevis* increased from 10^6 CFU/ ml close to 10^8 CFU/ ml and bacteria numbers of *L. fermentum* increased by almost three decimal powers to over 10^9 CFU/ ml. Samples without growth inhibitor contained around 1.5 g/l acetic acid regardless of the contamination strain. Control samples infected with *L. brevis* contained an average of 6.2 g lactic acid and control samples infected with *L. fermentum* contained an average of 7.9 g lactic acid/ per litre. Generally *L. fermentum* grew faster, produced more metabolites and caused higher losses in ethanol yield compared to *L. brevis* in beet molasses fermentation. On the other hand, *L. brevis* was more tolerant to hop acids and higher concentrations were required to reduce bacteria numbers.

Fig 1. Concurrent decrease of bacteria numbers, lactic acid and acetic acid content in fermented mash at increasing concentrations of LactoStab™ and IsoStab™.

---▲--- Colony Forming Units/ ml, ---♦--- lactic acid concentration [g/l],



---◊--- acetic acid concentration [g/l].

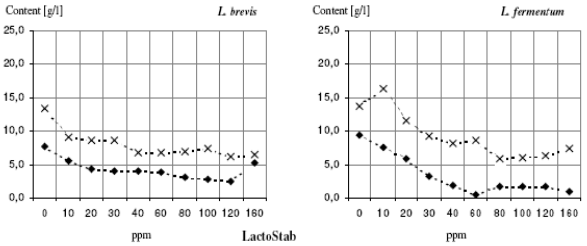
As shown in table 2. lactic acid concentrations in fermented mash could be reduced to half the amount contained in control samples by applying inhibitory concentrations for reduction of viable count to 10,000 CFU/ ml.

Table 2. Reduction of lactic acid concentration in fermented mash at inhibitory concentration of various hop acid derivatives .

Lactic acid [g/l]	*L. brevis*	*L. fermentum*
Control	6.2	7.9
IsoStab™	3.0	3.9
LactoStab™	3.0	2.4

Along with the decrease in organic acid content (lactic acid and acetic acid) a concurrent residue sugar reduction in fermented mash was observed. This is an indication for better sugar utilisation by yeast. Fig 2. shows the reduction of residue sugar content in fermented mash, concurrent to the decrease of bacterial metabolites for LactoStab™.

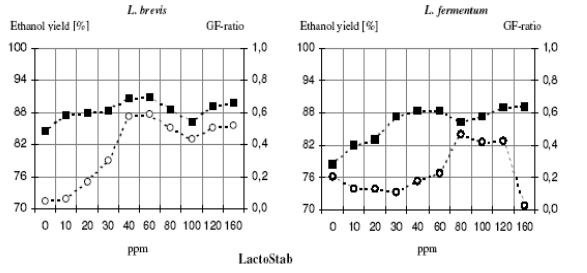
Fig 2 Decrease of residue sugar content concurrent with decrease of bacterial metabolites in fermented molasses mash at increasing concentrations of LactoStab™.



---x--- Lactic acid and acetic acid content [g/l], ---♦--- Content of residue sugars consisting of raffinose, sucrose, maltose, glucose, and fructose [g/l].

Sugar utilisation by yeast is dependent on the glucose-fructose ratio in the substrate. A glucose-fructose ratio of less than 0.2 may constrain yeast fermentation abililty (Gafner, 2001). Concurrent to the decrease in bacterial metabolites at inhibitory hop acid concentrations, the glucose-fructose ratio in residue sugar improved. Fig 3. gives an example for the correlation between ethanol yield and glucose-fructose ratio in residue sugar of fermented mash for LactoStab™. Sucrose and raffinose content in residue sugar remained stable in all samples and was not significantly influenced by the level of infection.

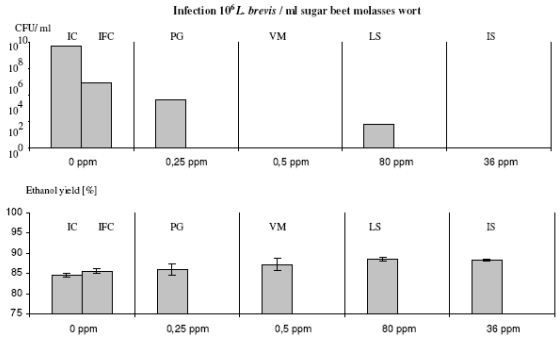
Fig 3. Correlation between improvement of ethanol yield and glucose-fructose ratio in fermented molasses mash at increasing concentrations of LactoStab™.



---■--- Ethanol yield [%], ---○--- Glucose-Fructose ratio

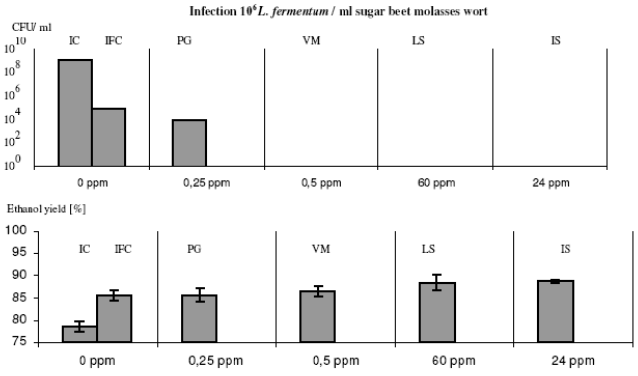
Penicillin G and Virginiamycin were used to compare the inhibitory efficiency of hop acids as natural antibacterials to conventional antibiotics. Penicillin G was added according to the manufacturer's recommendation at a concentration of only 0.25 ppm. This was sufficient to inhibit bacteria in molasses mash and reduce viable cell count to 10^4 CFU/ ml. Ethanol yields obtained by use of Penicillin G were lower than those obtained from infected control samples. Virgiamycin added at a concentration of 0.5 ppm proved to be most effective against bacteria and acted bactericidal. Ethanol yields could be improved by 2% of theoretical yield compared to infected control samples by use of Virginiamycin. Best ethanol yields were obtained at bactericidal concentrations of LactoStab™ and IsoStab™. Fig 4 and 5 show ethanol yields obtained by use of antibiotics and by use of bactericidal concentrations of hop acids. Numbers given for ethanol yield represent percentage of theoretical yield on the basis of fermentable substance. Non-infected control samples contained an average of 8 x 10^4 bacteria/ ml at the end of fermentation.

Fig 4. Reduction of *L. brevis* and corresponding ethanol yields obtained by use of antibiotics and by use of bactericidal concentrations of hop acids in molasses wort.



IC = Infected Control, IFC = Infection free Control, PG = Penicillin G, VM = Virginiamycin, LS = LactoStab™, IS = IsoStab™

Fig 5. Reduction of *L. fermentum* and corresponding ethanol yields obtained by use of antibiotics and by use of bactericidal concentrations of hop acids in molasses wort.



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IsoStab™ and LactoStab™ as antibacterials in fermentation of wheat mash

Prior to fermentation, wheat mash was inoculated with 10^7 CFU/ ml of each strain suspended in peptone water as described above. Hop acids were added after cooling to fermentation temperature of 30°C in samples containing *L. brevis* and 37°C in samples containing *L. fermentum.* Viable cell count of *L. brevis* in control samples increased to 5 x 10^7 CFU/ ml, and viable cell counts of *L. fermentum* in control samples increased to 10^8 CFU/ ml. In wheat mash, *L. brevis* produced more metabolites than *L. fermentum*. Content of bacterial metabolites in fermented mash was significantly lower compared to beet molasses fermentation due to the fact that bacteria cells had been washed before inoculation of mash. Control samples infected with *L. brevis* contained an average of 0.8 g/l acetic acid and 2 g/ l lactic acid, control samples infected with *L. fermentum* contained an average of 0.5 g/ l acetic acid and 1.9 g/ l lactic acid in fermented wheat mash. Higher concentrations of hop acids were required to control bacteria in wheat mash fermentation due to higher initial bacteria numbers and higher content of solids in mash. A reduction of viable cell count by one decimal power below 10^6 CFU/ ml was sufficient to very efficiently block bacteria metabolism, reduce content of bacterial metabolites in mash and improve ethanol yields. Table 3. shows the concurrent reduction of lactic acid at inhibitory concentrations.

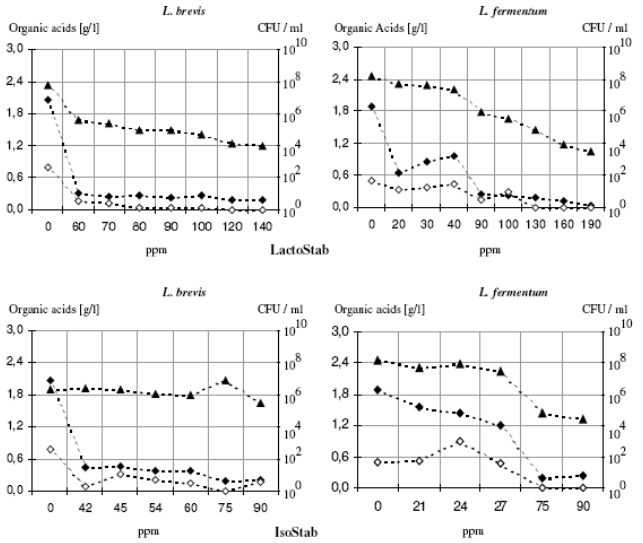
Table 3. Reduction of lactic acid concentration in fermented mash at inhibitory concentration of LactoStab™ and IsoStab™ in wheat mash.

Lactic acid [g/l]	*L. brevis*	*L. fermentum*
Control	2.00	1.90
IsoStab™	0.20	0.19
LactoStab™	0.32	0.25

The amount of residue sugar increased when bacteria numbers were reduced. This was different from observations made in sugar beet molasses fermentation trials. It indicates that yeast was not seriously affected by the small amount of bacterial metabolites in wheat mash and ethanol yield losses in infected control samples were probably caused by competition for sugars with bacteria. Fig 6. shows the decrease of viable cell numbers and bacterial metabolites in wheat mash at increasing concentrations of LactoStab™ and IsoStab™. Fig. 7. shows improvement of ethanol yield concurrent with reduction of viable cell count.

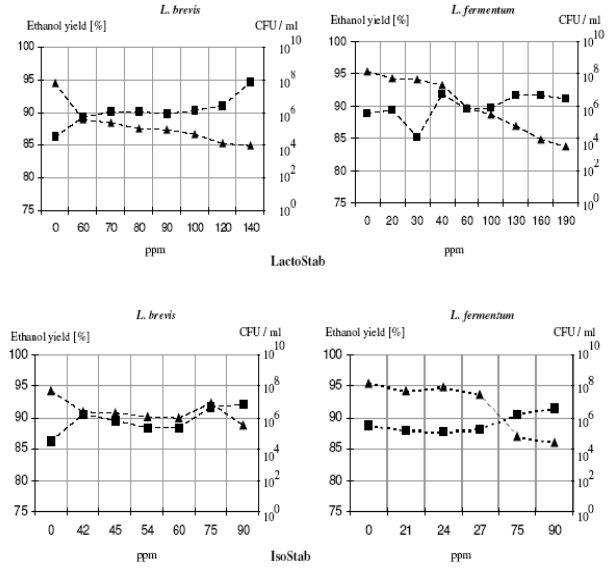
For comparison with conventional antibiotics, Penicillin G was added according to the manufacturer's recommendation at a concentration of only 0.25 ppm and Virginiamycin was added at a concentration of 0.5 ppm. Virginiamycin was most active against bacteria and reduced viable cell count from 10^7 CFU/ ml to 1000 CFU/ ml and below. However, ethanol yields obtained by use of Virginiamycin in wheat mash were sometimes even lower than ethanol yields obtained by inhibitory concentrations of hop acids and did not necessarily correspond to the observed reduction of bacteria numbers.

Fig 6. Concurrent decrease of bacteria numbers, lactic acid and acetic acid content in fermented mash at increasing concentrations of LactoStab™ and IsoStab™.



---▲--- Colony Forming Units/ ml, ---♦--- lactic acid concentration [g/l], ---◊--- acetic acid concentration [g/l].

Fig 7. Concurrent decrease of bacteria numbers and increasing ethanol yields in fermented mash at increasing concentrations of LactoStab™ and IsoStab™.



---▲--- Colony Forming Units/ ml, ---■--- theoretical ethanol yield [%].

Penicillin G significantly reduced average viable cell count of *L. brevis* in wheat mash. Nevertheless, high deviations indicate that a concentration of 0.25 ppm might not be sufficient to control bacteria due to the instability of Penicillin G at low pH values or bear the risk of

inducing resistance development. Resistance development against Penicillin G was observed in wheat mash fermentation trials using *L. fermentum* as contamination organism.

Optimum ethanol yields were obtained using LactoStab™ and IsoStab™ at a concentration that reduced viable count to 10^4 CFU/ ml or below. Non-infected control samples contained an average of 350 bacteria/ ml at the end of fermentation (technically infection free). In breweries, hop acids are known to slow down yeast flocculation at the end of fermentation. It is assumed that the adhesion of hop acids to yeast cell walls was responsible for improvement of ethanol yields above ethanol yields obtained from infection free samples. Fig 8. and 9. show ethanol yields obtained by use of antibiotics and by use of hop acids at a concentration to reduce viable cell count from 10^7 CFU/ ml to 10,000 CFU/ ml. The numbers for ethanol represent percentage of theoretical yield on the basis of fermentable substance.

Fig 8. Reduction of *L. brevis* and corresponding ethanol yields obtained by use of antibiotics and by use of hop acids in wheat mash.

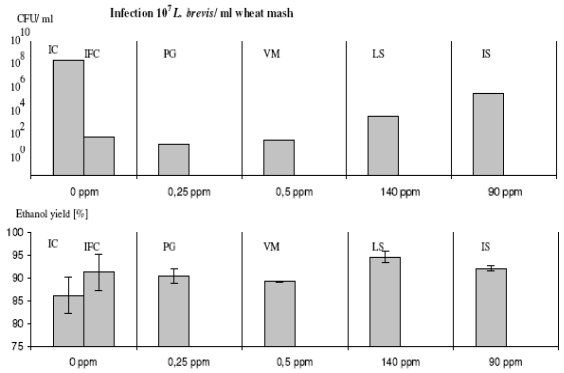


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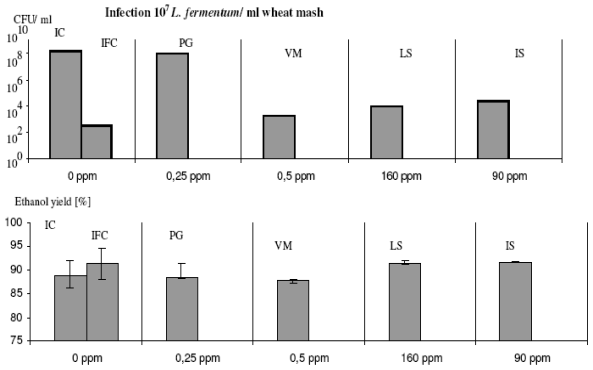
Fig 9. Reduction of *L. fermentum* and corresponding ethanol yields obtained by use of antibiotics and by use of hop acids in wheat mash.



IC = Infected Control, IFC = Infection free Control, PG = Penicillin G, VM = Virginiamycin, LS = LactoStab™, IS = IsoStab™

Conclusions

LactoStab™and IsoStab™ proved to be very efficient at controlling lactic acid bacteria in ethanol production. Efficiency of hop acids depends on solubility in substrate and is dependent on initial bacteria numbers, pH value, and amount of solids contained in mash. Higher concentrations of LactoStab™ or IsoStab™ are required for viscous mashes based on starchy materials than for mashes obtained from sugar-based materials. Under a worst case scenario with heavy initial infection > 10^6 bacteria/ ml, low yeast count and fermentation temperatures exceeding 37°C, LactoStab™ and IsoStab™ acted bactericidal at concentrations between 30 and 80 ppm in sugar beet molasses wort and considerably reduced viable count at concentrations between 90 and 160 ppm in wheat mash. Bacteria metabolism was blocked at inhibitory concentrations and an concurrent increase in ethanol yield was observed. Conventional antibiotics are active against bacteria at very low concentrations, but ethanol yields obtained by use of the natural products LactoStab™ and IsoStab™ at bactericidal concentrations were similar to those obtained by use of antibiotics, if not better. In fact, LactoStab™ and IsoStab™ helped to improve ethanol yields over ethanol yields obtained from infection free control samples in some cases.

Safety with respect to human and veterinary medication must be considered the big advantage using LactoStab™ and IsoStab™ as natural antibacterials. Penicillin G used at recommended dosage rate of 0.25 ppm induced resistance in *L. fermentum* in wheat mash fermentation trials. Residues of antibiotics in DDGS to be used as cattle feed could cause residues of antibiotics in milk and cause severe disfunction when using lactic acid bacteria as starter cultures for dairy products like cheese and yoghurt. When used as a fertilizer, solid residues containing residual antibiotics from alcoholic fermentation may induce resistance in bacteria living on crops. Even worse, resistant bacteria in animal feed for livestock production could transfer resistance genes to animals and contaminate food. Due to these concerns the European Union banned antibiotics as growth promoting compound in animal feed. LactoStab™ and IsoStab™ may be considered a safe and efficient natural alternative for controlling bacteria in ethanol production.

Acknowledgement

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