Penicillin as Contaminant Control in Alcohol Fermentation Near Economic Reality

CONTAMINATION INHIBITION

Antibiotics as Contamination-Control Agents In Grain Alcohol Fermentations

W. H. DAY, W. C. SERJAK[1], J. R. STRATTON, and LEONARD STONE
Research Department, Hiram Walker & Sons, Inc., Peoria, Ill.

Bacteria in fermentations of distillery grain mash can be responsible for the production of excessive amounts of lactic acid with a reduction in yield of alcohol and under certain conditions may produce minor fermentation products which affect the quality of alcoholic distillates. Investigations were made to determine whether the development of the bacteria present in grain mash fermentations could be controlled by the use of antibiotics. In laboratory fermentation tests, 0.75 to 2.0 units of penicillin were found to inhibit bacterial growth; Aureomycin, bacitracin, Chloromycetin, and Terramycin proved somewhat less effective; tyrothricin and streptomycin were ineffective and polymixin B was ineffective when used at levels to 10 γ per ml., the maximum tried. In addition to the effect upon bacterial growth, other beneficial properties were exhibited by the antibiotics. Plant scale fermentations using penicillin in bourbon, rye, and spirit mashes were favorable. Because of the decreased cost of penicillin and the low concentration required in mashes, its use approaches an economic reality at the present time.

FOREIGN MICROORGANISMS IN INDUSTRIAL FERMENTATIONS cause changes in the fermentation which are variable in nature and in most cases are undesirable. Generally speaking, these changes affect the quality of the product and the economics of the fermentation process.

The grain alcohol fermentation process in which barley malt is used for saccharification is more complicated than pure culture fermentations. Because saccharification temperatures above 65.6° C. (150° F.) are destructive to the malt enzymes, it is not possible to heat-sterilize the fermentation medium (cooked and saccharified mash) without destroying the enzymes, which in turn would limit saccharification and result in a low alcohol yield. For a good grain mash fermentation, these enzymes, particularly α-amylase, must survive the saccharification process and continue to be active for about 48 hours of the fermentation period. Incomplete saccharification, therefore, precludes the use of heat treatment at the time of or subsequent to the saccharification period.

Barley malt, which supplies the saccharifying enzyme system, is heavily laden with bacteria. The bacterial population of commercial distiller's barley malt ranges from 1,000,000 to 10,000,000 per gram. A small percentage of these bacteria, in spite of improved mashing and sanitation procedures employed by the distiller, survive saccharification temperatures and contaminate each fermentation even before the pure culture of yeast is introduced. The distiller has been able by the use of controlled fermentation conditions to retard the development of bacteria and favor yeast growth and rapid fermentation and has minimized but not completely eliminated the effects of bacterial contamination. No practical method has been found for the sterilization of barley malt for subsequent use in a grain distillery. The problem is more involved than merely removing bacteria by physical or chemical methods. The sterilization must not destroy the enzymes, must not leave toxic materials that would impair or destroy the yeast in the grain mash fermentation, and must not impart off-flavors or toxicity to either the distilled beverage or the by-product distiller's feeds. The present study and report represent a step in controlling bacterial contamination in the distillery and reducing its effect upon the product and upon alcohol and by-product yields.

Investigations of bacterial contaminants in alcoholic fermentation and control measures began with the work of Pasteur. Gayon and Dupetit (*6*) at the turn of the century used a so-called bacterial antiferment—i.e., bismuth nitrate—in distillery fermentation mashes. They claimed that this compound prevented the pronounced effects of secondary fermentations and, accordingly, increased the alcohol yield, decreased the acid products, and generally avoided the production of undesirable flavors and products. Since Effront's (*4*) studies in 1891, the use of fluorides in grain mash fermentations has been common practice. Brewers have used hops for centuries in the manufacture of beer. Methods have been designed (*1*) for the treatment of barley malt with formaldehyde prior to use in the distillery mashing process. Sulfur dioxide has been used for the treatment of barley malt and in the fermentation of wines. A French patent (*2*) was granted in 1945 for a process for maintaining the purity of biological reactions. This process suggests the use of antibiotics in biological reactions, employing microorganisms, for protection against infecting organisms. Hesseltine and Bohonos (*7*) have prepared yeasts for fermentation by using broad spectrum antibiotics to inhibit the growth of infectious bacteria during the yeast propagation or storage period.

Previous studies in this laboratory (*5*, *8*) have shown the bacterial contaminants associated with grain mashes

[1] Present address, Hughes Aircraft Corp., Tuscon, Ariz.

undergoing alcoholic fermentation to be largely of the *Lactobacillus* type. These studies and plant experience have shown that when these bacteria are not properly controlled, not only may alcohol yield be lowered, but under certain conditions the bacterial contaminants may produce minor fermentation products such as acrolein which affect the quality of the alcohol distillates.

A large number of compounds having bactericidal and/or bacteriostatic properties have been evaluated in this laboratory for use in alcoholic grain mash fermentations. Of the materials studied (fluorides, surface active agents, quaternary ammonium compounds, antibiotics, formaldehyde, and many others), only certain antibiotics were found to meet the requirements previously set forth. The results of investigations in which antibiotics were used are presented here in two parts; the first is concerned with laboratory studies of various antibiotics and the second with penicillin in plant scale fermentations.

Antibiotics were evaluated in milo—i.e., grain sorghum—corn, and rye mashes. Bacterial development varies with the grain mashed and normally is more rapid in rye mashes than in corn or milo mashes. The maximum bacterial populations in corn and milo are about equal, whereas in rye the number of bacteria is generally much higher. For example, at 72 hours' fermentation time in milo and corn mashes the bacterial population rarely exceeds 100,000,000 bacteria per ml., whereas in rye mashes the number of bacteria may reach or exceed 300,000,000 bacteria per ml.

Methods

Bacterial populations were determined by the tomato juice agar tube method of Garey *et al.* (*5*). Yeast population was determined by the conventional plate-counting method using Difco wort agar. Acrolein was determined by the method of Circle *et al.* (*3*).

Mashes for laboratory scale fermentations were prepared by adding 77.7 grams of corn or milo meal to 450 grams of 60° C. (140° F.) water along with 0.7 gram of premalt. The mash was cooked in an autoclave at 115° C. (240° F.) for 45 minutes, then cooled and saccharified with 5.5 grams of barley malt meal in a water bath at 64° C. (147° F.) with agitation. After saccharification for 30 minutes the mash was cooled to 23.9° C. (75° F.). In the case of rye mashes 77.7 grams of rye meal and 6.2 grams of barley malt meal were added to 450 grams of 60° C. (140° F.) water. The mixture was then cooked at 63.5° to 64.5° C. (146° to 148° F.) for 45 minutes and cooled to 23.9° C. (75° F.). The cooled mash was adjusted to pH 4.8 with lactic acid and inoculated with a pure culture of distiller's yeast. The bacterial contaminants, if used, were added at this time.

In laboratory tests, heavy bacterial contamination was used for the purpose of screening compounds—i.e., in addition to the bacterial flora of the barley malt that survived the saccharification temperatures of laboratory mashing. Two types of bacterial contaminants were used. One was a mixed flora (as whole fermented mash) from 72-hour commercial scale fermentations. The second was a pure culture of *Lactobacillus* 208 from 72-hour fermented mash, which under certain conditions produces acrolein. This organism is a representative of the Group H organisms isolated from distillery mashes (*8*). These contaminants are largely, if not exclusively, gram-positive.

In the plant scale tests, the bacterial contamination was limited to the bacteria normally developing from the barley malt and from miscellaneous sources of admission in the usual plant scale fermentations.

Alcohol yields are reported in terms of proof gallons per 56 pounds of grain. Alcohol was determined on distillates by pycnometer.

Residual amylase was determined colorimetrically and is reported as the milliliters of 1% starch solution dextrinized at 28° C. in 1 hour by 1 ml. of fermenting mash. Acid production was measured by titration with phenolphthalein as the indicator. Values are reported as per cent lactic acid.

All antibiotics except penicillin were C.P. or pharmaceutical grade. Both pharmaceutical grade and crude penicillin were used; the crude penicillin assayed 1401 to 1499 units per milligram. The antibiotics were added to the grain mashes at the times specified.

In addition to the effectiveness of antibiotics against bacterial contaminants, their effect upon the alcohol fermentation also was studied. In this connection yeast populations, acid production, acrolein production, alcohol yield, and, in some instances, the amount of residual amylase in the fermenting mashes were followed. In the plant scale tests, whisky distillates were examined chemically and organoleptically at the time of manufacture and periodically during aging.

Table II. Aureomycin in Corn Mash Fermentations

Contaminant Added[a]	Aureomycin, γ/Ml. of Mash	Hours of Fermentation 0	24	48	72	Final Fermenter Data: Acidity as lactic, %	pH	Proof gal. alcohol/56 lb. grain
		Bacteria, Millions/Ml.						
−	..	0.0002	0.05	0.08	0.05	0.23	4.20	5.28
+	..	0.12	28.0	30.0	28.0	0.47	3.55	4.76
+	0.9	0.12	6.0	6.0	10.0	0.33	3.88	5.09
+	1.8	0.12	2.0	6.0	6.0	0.29	3.90	5.25
+	3.5	0.12	1.8	1.2	3.0	0.26	4.05	5.33
+	5.2	0.12	0.5	0.5	0.9	0.23	4.15	5.35
+	7.0	0.12	0.4	0.5	0.8	0.23	4.20	5.36
+	14.0	0.12	0.4	0.2	0.15	0.23	4.20	5.28

[a] Mixed flora from normal distillery beer.

Table I. Streptomycin in Milo Mash Fermentations

Contaminant Added[a]	Streptomycin, γ/Ml. of Mash	Hours of Fermentation 0	24	48	72	Final Fermenter Data: Acidity as lactic, %	pH	Proof gal. alcohol/56 lb. grain
		Bacteria, Millions/Ml.						
−	..	<0.0001	0.0002	0.002	0.01	0.29	4.23	5.25
+	..	0.19	20	20	19	0.48	3.63	4.56
+	0.5	0.19	18	18	19	0.47	3.65	4.63
+	1.0	0.19	18	19	20	0.49	3.63	4.63
+	1.5	0.19	22	16	17	0.51	3.65	4.64
+	2.0	0.19	20	16	19	0.51	3.69	4.65
+	5.0	0.19	20	18	20	0.51	3.67	4.68
+	10.0	0.19	20	16	18	0.51	3.70	4.67

[a] Mixed flora from normal distillery beer.

Laboratory Fermentation Tests

Table I shows typical data obtained with an antibiotic—i.e., streptomycin—which is not effective in grain (milo) mash against the bacterial contaminants characteristically found in grain alcohol fermentations. Streptomycin at levels of 0.5 to 10.0 γ per ml. had no inhibitory effect upon bacterial numbers. Like

Table III. Effect of Marginal Level of Penicillin on Production of Acrolein by *Lactobacillus* 208 in Milo Mashes

Contaminant Lactobacillus 208 Added to Mash, Millions/Ml.	Penicillin, Units/Ml. in Mash	Fermentation Time, Hours	Bacteria, Millions/Ml.	pH	Acrolein in Alcohol Distillates, P.P.M. (Five Separate Fermentations)				
0.0012	..	72	50	..	T[a]	0	T	2	5
		96	75	4.17	3	T	2	6	6
		120	100	..	1	T	1	1	1
		144	65	4.09	T	T	T	T	T
0.0110	..	72	95	..	3	2	4	4	4
		96	125	4.15	6	4	5	3	3
		120	125	..	1	1	1	1	1
		144	110	4.11	1	1	1	1	1
1.19	..	72	30	..	T	T	T	1	0
		96	50	3.91	0	T	2	T	T
		120	65	..	1	1	1	1	0
		144	85	3.89	2	1	1	T	0
0.0012	0.75	72	0.0001	..	0	0	0	0	0
		96	0.002	4.68	0	0	0	0	0
		120	24	..	0	0	0	0	0
		144	75	4.41	30	40	60	40	40
0.0110	0.75	72	0.0001	..	0	0	0	0	0
		96	0.03	4.68	0	0	0	0	0
		120	60	..	6	1	6	T	6
		144	95	4.41	30	60	50	60	60
1.19	0.75	72	0.002	..	0	0	0	0	0
		96	50	4.43	20	20	20	20	20
		120	80	..	20	50	50	50	50
		144	65	4.32	10	30	40	40	40

[a] T indicates a trace; see text for description.

wise, acid production was not restricted and alcohol yields were not improved in the contaminated mashes. Waksman *et al.* (*9*, *10*) have shown that the activity of streptomycin is greatly affected by certain components of the medium. Although streptomycin is effective against both gram-positive and gram-negative bacteria, it is possible that the complex nature of the grain mash rendered it ineffective.

Table II shows typical results obtained with an antibiotic that is effective against typical distillery contaminants—viz., Aureomycin (trade-mark of the American Cyanamid Co. for the antibiotic, chlortetracycline). When added at levels ranging from 0.9 to 14.0 γ per ml. of mash, Aureomycin was inhibitory to bacterial development. In all the concentrations shown in this table, Aureomycin either partially or completely inhibited bacterial growth. Between 0.9 and 5.2 γ per ml. of mash, its effectivity increased as the concentration was raised; thereafter, the benefits of higher levels were not pronounced.

Data in Table II show that in spite of the heavy added bacterial contamination of 120,000 bacteria per ml., Aureomycin restricted bacterial growth and acid production, and alcohol yields were raised to or slightly above that found in the uncontaminated control. Although bacterial populations were not lowered to those of the uncontaminated control, bacterial growth and activity were inhibited to the extent that the effect of the contaminant was prevented. The bacterial population in the control fermentation never exceeded 80,000 per ml., whereas in the contaminated control the number of bacteria reached 375 times that of the uncontaminated control. Although the initial concentration of bacteria in the contaminated fermentations was 120,000 per ml., the addition of 7.0 γ of Aureomycin per ml. allowed the bacterial population to reach only 10 times that of the uncontaminated control. Likewise the contaminated control had a final acidity (as lactic) of 2 times the uncontaminated control, a 90% alcohol yield, and a low pH value. The use of 7.0 γ of Aureomycin per ml. kept the final acidity down, equal to the uncontaminated control, and increased the alcohol yield to slightly above and a pH value equal to the uncontaminated control.

Although the effect of the antibiotic manifests itself in the bacterial populations at 24 hours, it was noted that 72-hour bacterial populations provide a better means of evaluating the effectiveness of an antibiotic. Therefore, instead of presenting complete fermentation progress data on all of the antibiotics surveyed, a comparison of antibiotics is given on the basis of the bacterial populations of the mashes at 72 hours' fermentation.

These 72-hour data are summarized in Figure 1. The quantities of each antibiotic used are expressed in micrograms per milliliter of mash with the exception of penicillin, which is expressed in units of penicillin. All data shown in this figure were taken from corn or milo mashes contaminated with a mixed flora of plant bacteria, so that the initial bacterial populations (start of fermentation) ranged from 100,000 to 180,000 bacteria per milliliter of mash. This is roughly 100 times the normal bacterial population of plant scale mashes when seeded with yeast.

The curves show, on the basis of the amount of antibiotic required, that penicillin was most effective; Aureomycin, bacitracin, Chloromycetin (trade-mark of Parke, Davis & Co. for the antibiotic chloramphenicol), and Terramycin (trade-mark of Chas. Pfizer & Co. for the antibiotic oxytetracycline) were less effective, while tyrothricin and streptomycin were relatively ineffective. Experimental data from a single series of fermentations indicated polymyxin B (sulfate) up to 10 γ per ml. of mash was also ineffective.

Marginal Use Of Antibiotics In all previous experiments, the contaminants used were a mixed flora from a final fermented grain mash. This mixed flora represents bacteria that affect acidity, pH values, and alcohol yields, and may affect quality of the alcoholic distillates. From this mixed flora a particular organism (*Lactobacillus* 208) which produces acrolein from glycerol was chosen for a series of studies. *Lactobacillus* 208 in grain mashes normally does not produce large quantities of acid nor does it affect alcohol yields to a great extent, but does, under

Table IV. Effect of Terramycin on Production of Acrolein by *Lactobacillus* 208 in Pure Rye Mashes

(Terramycin added at start of fermentation)

Contaminant Lactobacillus 208 Added to Mash	Terramycin, γ/Ml. of Mash	Hours of Fermentation				Acrolein in Alcohol Distillate, Av. P.P.M.
		0	24	48	72	
		Bacteria, Millions/Ml.				
−	..	<0.0001	0.014	3	54	0
+	..	0.012	0.039	145	324	100
+	2.5	0.012	0.048	188	242	330
+	5.0	0.012	0.015	15	193	750
+	7.5	0.012	0.007	9	164	625
+	10.0	0.012	0.005	35	98	650
+	20.0	0.012	0.001	0.42	45	50
+	40.0	0.012	0.0004	0.007	0.14	0

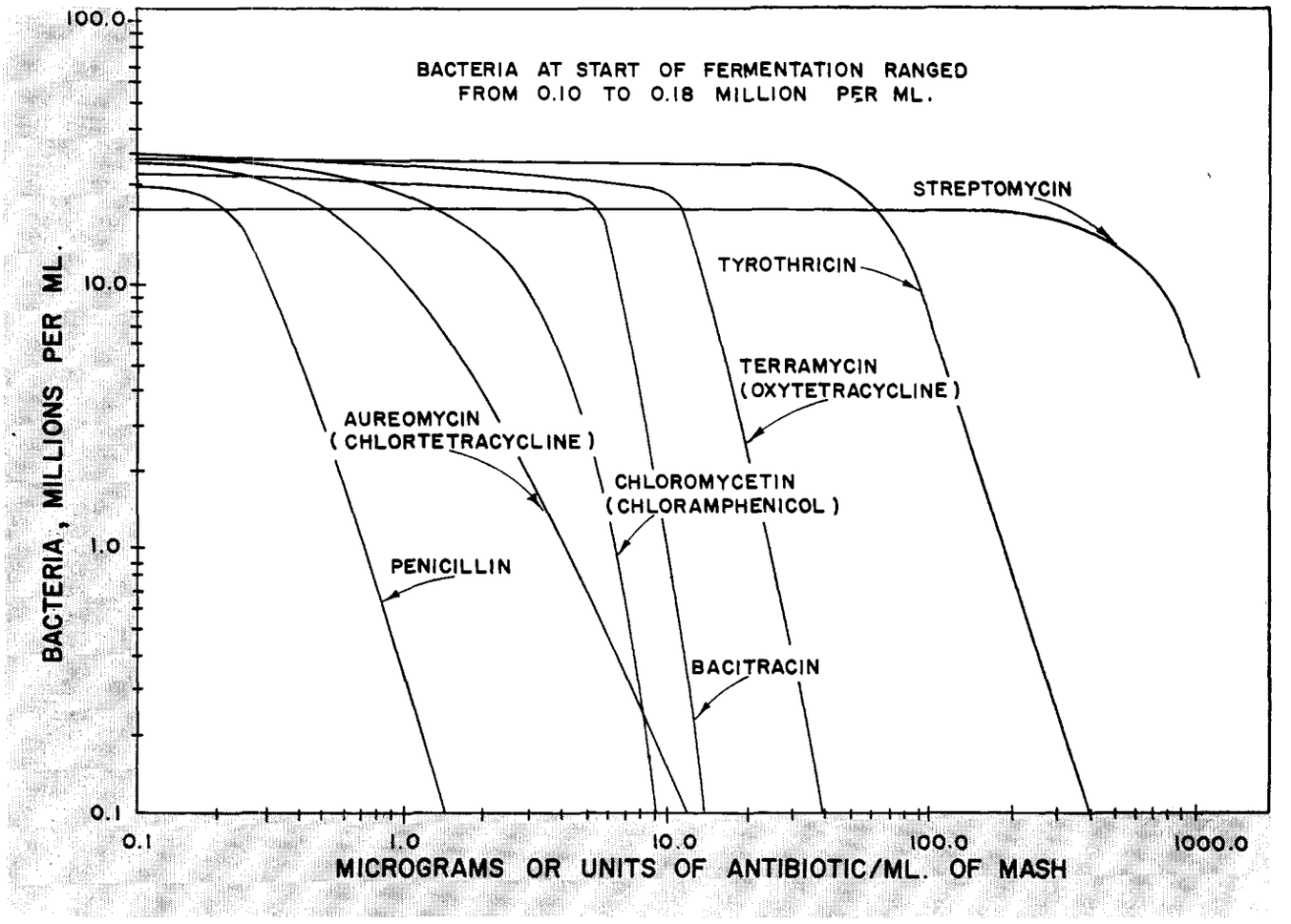


Figure 1. Effect of antibiotics added at start of fermentation on bacterial population

After 72 hours of fermentation in contaminated corn or milo mashes

certain conditions, produce acrolein which affects the quality of alcoholic distillates.

Studies using *Lactobacillus* 208 have shown that the antibiotics which are effective against the mixed flora are in general effective against strain 208. However, if the antibiotic level is insufficient to inhibit bacterial growth completely, acrolein is often produced. This suggests that bacteria of the 208 type are more resistant to certain antibiotics than the general flora.

Table III shows the effect of a marginal treatment with penicillin on the production of acrolein in milo mashes. Three-fourths unit of penicillin per ml. was used and various amounts of *Lactobacillus* 208 were added. The normal bacterial flora present in grain mashes was the same in all samples.

The bacterial populations show the break-through of contamination in the samples to which penicillin was added. The time of break-through is dependent upon the amount of contaminant 208 added. The amount of acrolein produced is shown in parts per million of acrolein in the alcohol distillate. It will be noted that acrolein was produced in 91% of the mashes receiving no penicillin. The amount of acrolein produced ranged from a trace to 6 p.p.m. In mashes receiving 0.75 unit of penicillin per ml., no acrolein was produced until after 72 hours. In the case of the highest level of contaminant, acrolein was produced in 96 hours, in the next lower quantity in 120 hours, and in the lowest level in 144 hours. Of particular interest was the observation that in penicillin-treated mashes, the acrolein ranged from 10 to 60 times that found in untreated mashes. Laboratory evaluations indicate this may be due to one of two causes. Serjak *et al.* (*8*) in this laboratory showed that a mixed flora added with *Lactobacillus* 208 inhibited acrolein

Table V. Effect of Terramycin on Production of Acrolein by *Lactobacillus* 208 in Pure Rye Mashes

(Terramycin added at times indicated)

Contaminant Lactobacillus 208 Added to Mash	Hours of Fermentation			Hours of Fermentation				Acrolein in Alcohol Distillates, Av. P.P.M.
	0	24	48	0	24	48	72	
	Terramycin Added, γ/Ml. of Mash			Bacteria, Millions/Ml.				
−	..	..	..	<0.0001	0.014	3	54	0
+	..	..	..	0.012	0.039	145	324	100
+	2.5	..	..	0.012	0.048	188	242	330
+	2.5	2.5	..	0.012	0.012	60	600	500
+	2.5	2.5	2.5	0.012	0.004	49	286	350
+	5.0	7.5	..	0.012	0.006	30	200	300
+	5.0	7.5	5.0	0.012	0.001	40	100	70
+	7.5	7.5	7.5	0.012	0.002	0.75	63	25
+	10.0	5.0	5.0	0.012	0.005	0.78	18	0
+	10.0	10.0	10.0	0.012	0.005	0.23	4	0

Table VI. Plant Scale Evaluation of Penicillin in Pure Rye Mash

	Set Data		24-Hour Data		48-Hour Data		62-Hour Data	
	Penicillin[a]	Control	Penicillin	Control	Penicillin	Control	Penicillin	Control
Balling, °	11.6	11.6	4.4	4.4	3.2	2.4	2.2	2.3
Acidity as lactic, %	0.22	0.21	0.25	0.27	0.25	0.65	0.32	0.75
pH	4.95	4.98	4.76	4.56	4.88	3.93	4.50	3.92
α-Amylase, ml. of 1% starch	18.0	16.0	18.0	16.0	18.0	1.0	14.0	0
Bacteria, millions/g.	0.003	0.002	0.02	18.0	0.06	180.0	0.006	125.0
Yeast, millions/g.	2.3	2.4	81.0	78.0	46.5	0.16	17.0	0.005
Acrolein in distillate, p.p.m.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Proof gal. alcohol per 56 lb. grain (dry basis)	....	....	...	...	...	....	5.28	5.11

[a] 1 unit penicillin/ml. of fermenter mash.

production. It was also shown that a small amount of this organism added as inoculum produced a higher concentration of acrolein than a large amount of inoculum (due to late development in the fermentation). The use of a marginal dosage of penicillin would favor either or both of these conditions.

Table IV shows the effect of Terramycin on the production of acrolein by *Lactobacillus* 208 in pure rye mashes. In this study the amount of infection was kept constant and the amount of Terramycin was varied from 2.5 to 40 γ per ml. Results show that 2.5 γ of Terramycin per ml. had no effect upon the bacterial population attained during the fermentation period; 5.0 γ per ml. had a slight effect early in fermentation. As the amount of Terramycin was increased, the inhibitory effect on bacterial growth increased. At 20 γ per ml. bacterial growth was controlled until after 48 hours. At 40 γ per ml. bacterial growth was restricted until 72 hours. The maximum amount of acrolein was produced at Terramycin levels of 5.0, 7.5, and 10 γ per ml., where bacterial growth was delayed until 48 hours. At the 20 γ level some acrolein was produced; however, at 40 γ per ml., bacterial growth was controlled and no acrolein was produced in 72 hours.

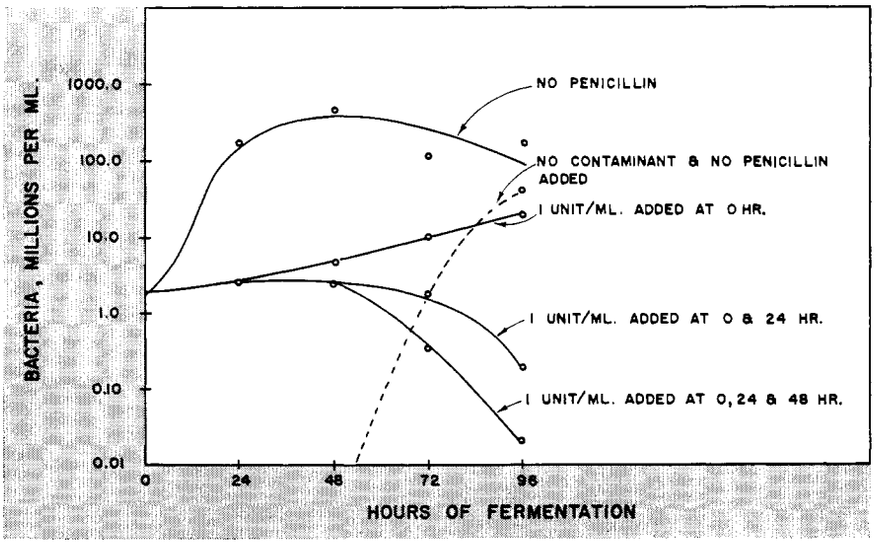
The possibility of controlling the production of acrolein was next investigated by the periodic addition of lower levels of Terramycin at 24-hour intervals after the start of fermentation. Terramycin was added to each series in increments ranging from 2.5 to 10 γ per ml. Data in Table V show the effect of Terramycin on bacterial growth at 72 hours. Acrolein was produced in all the contaminated flasks except those receiving 10 γ per ml. initially, followed with increments of 5 or 10 γ per ml. each 24 hours. Again the maximum acrolein was produced at levels totaling 2.5 to 12.5 γ of Terramycin per ml. regardless of the interval of addition. These results and those found previously with Terramycin and penicillin show marginal treatment with an antibiotic to be dangerous.

Antibiotics in Grossly Contaminated Fermentations

In experiments thus far described, the contamination at the start of the fermentations has been 10 to 100 times that experienced in normal plant scale fermentations. Some of the antibiotics, however, were studied under conditions of gross contamination in which bacterial population was varied from 1000 to 10,000 times normal numbers. Data in Figure 2 show the effect of penicillin upon these severely contaminated corn mashes. Penicillin was added initially at a level of 1 unit per ml. and at intervals to give a total of up to 3 units per ml. The bacterial population in the contaminated samples without penicillin reached a viable cell count of 600,000,000 in 48 hours from the initial population of 2,000,000 per ml. The contaminated samples with 1 unit of penicillin added at the start of fermentation increased slowly in bacterial population until a maximum of 20,000,000 per ml. was attained in 96 hours. The samples to which 1 unit per ml. was added at the start of fermentation and again at 24 hours for a total of 2 units per ml. slowly decreased in population to 200,000 per ml. in 96 hours. The samples to which 1 unit of penicillin was added at 0, 24, and 48 hours for a total of 3 units per ml. decreased in bacterial population to 28,000 in 96 hours. The samples receiving no contaminant and no penicillin had an initial population of 100 per ml. and reached a maximum bacterial population of 40,000,000 in 96 hours. This is shown on Figure 2 by the dotted line.

Figure 2. Effect of penicillin added to grossly contaminated corn mash fermentations

Contamination composed of mixed flora from normal distillery beer



Effect of Antibiotics On Viability of Yeasts

The effect of penicillin upon the viability of yeast cells in contaminated corn mashes is shown in Figure 3. Viable yeast numbers were determined every 12 hours up to 120 hours of fermentation. The initial bacterial population of contaminated mashes was 2,000,000 bacteria per ml. The number of viable yeast cells in contaminated mash with no penicillin decreases rapidly. At 108 hours, less than 100 viable yeast cells per ml. were found present. In mashes with normal contamination present from the barley malt less than 1000 viable yeast cells per ml. were present after 96 hours. The addition of 1 unit of penicillin per ml. at the start of fermentation helps substantially in the

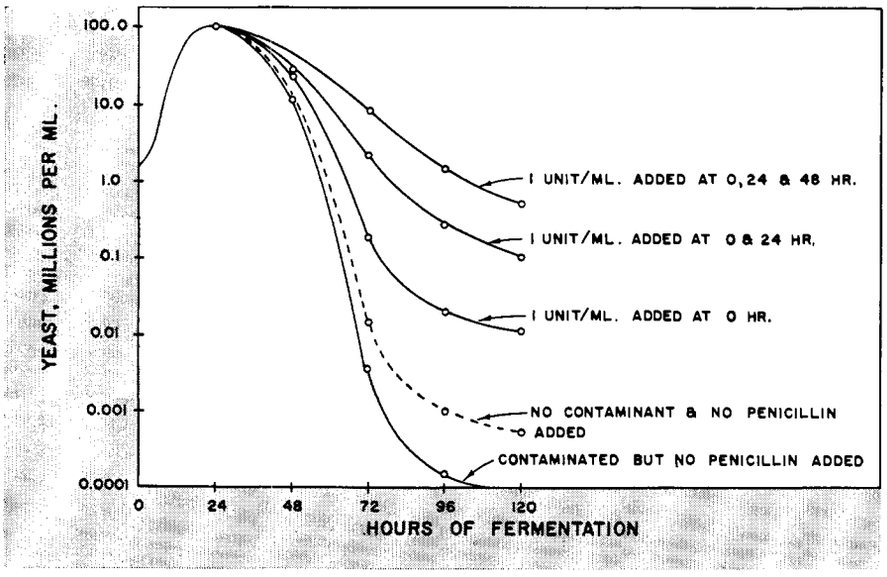


Figure 3. Effect of penicillin on viability of yeast in grossly contaminated corn mash fermentations

Initial bacterial contaminotion, 2,000,000 per ml. of normal distillery mash flora

preservation of viable yeast. The addition of 1 unit per ml. at the start and at 24 hours or a total of 2 units of penicillin per ml. was even more beneficial with a viable yeast population of 100,000 at 120 hours of fermentation. In the case of 1 unit added at start, 24 hours, and 48 hours—i.e., a total of 3 units of penicillin per ml.—the viable yeast population was 700,000 per ml. of mash at 120 hours of fermentation.

Plant Scale Fermentation Tests

The favorable results obtained with antibiotics in laboratory tests warranted plant scale trials. A total of 22 plant fermentors each containing 100,000 gallons of mash was run and evaluated. Data representative of these fermentations are shown in Tables VI and VII. Data from a plant scale trial with penicillin in a pure rye mash are shown in Table VI. In one fermentor, 1 unit of penicillin was added per ml. of mash at the start of fermentation. No penicillin was added to the control. The data show that penicillin decreased acid production and correspondingly maintained the pH at a higher level. The beneficial effect upon residual amylase, also, is readily apparent. In the fermentation with penicillin 14.0 units of residual amylase were available at the end of 62 hours, whereas none was left in the control fermentation. The bacterial numbers are similar to those found in laboratory studies, the final bacterial population in the control fermentor being 125,000,000 per ml. compared to 6000 per ml. in the fermentor to which 1 unit per ml. of penicillin had been added. Again, a high viable yeast population was maintained in the presence of penicillin. At 62 hours there were 5000 yeasts per ml. in the control and 17,000,000 per ml. in the penicillin-treated fermentor. No acrolein was produced and the alcohol yield was increased significantly.

Data in Table VII show the effect of penicillin in bourbon mashes. Although the analytical values are somewhat different from those for pure rye mashes, the over-all beneficial effects are similar.

Rye and bourbon whiskies produced from mashes with and without penicillin were analyzed chemically and examined organoleptically. Unaged whiskies and whiskies periodically examined during aging were found to be no different chemically and organoleptically from the control samples. Organoleptic examination by an expert quality panel showed that all whiskies were of good quality and standard character and that whiskies from penicillin mashes were indistinguishable from control samples.

Summary

Penicillin, Aureomycin, Chloromycetin, Terramycin, streptomycin, tyrothricin, bacitracin, and polymyxin B exhibited some inhibitory effect on the contaminants characteristic of grain alcohol fermentation, but the various antibiotics varied appreciably in effectiveness. Based upon the amount of antibiotic required, penicillin was found most effective; Aureomycin, bacitracin, Chloromycetin, and Terramycin less effective; tyrothricin and streptomycin ineffective and polymyxin B ineffective at levels up to 10 γ per ml. The antibiotics, particularly penicillin, prevented growth of bacteria in fermentation mashes, prevented destruction of α-amylase with a reduction in alcohol yield, inhibited acid production, and prevented acrolein production. More viable yeast cells were found in the mashes at the end of fermentation.

Marginal treatment of a grain mash fermentation with an antibiotic was found dangerous. In the presence of an acrolein-producing *Lactobacillus*, the marginal use of an antibiotic increased the quantity of acrolein produced.

The following antibiotic ranges were found effective in grain mash fermentations: penicillin 0.75 to 2.0 units per ml. of fermentation mash; Aureomycin 2.0 to 10.0 γ per ml.; Chloromycetin 7 to 20 γ per ml.; bacitracin 10 to 20 γ per ml.; Terramycin 20 to 40 γ per ml.; tyrothricin 300 to 500 γ per ml., and streptomycin little or no effect at the maximum concentration tried, 1000 γ per ml.

Table VII. Plant Scale Evaluation of Penicillin in Bourbon Mash

	Set Data		*24-Hour Data*		*48-Hour Data*		*72-Hour Data*	
	Penicillin[a]	*Control*	*Penicillin*	*Control*	*Penicillin*	*Control*	*Penicillin*	*Control*
Balling, °	13.0	13.0	1.60	1.98	0.92	1.35	0.40	0.42
Acidity as lactic, %	0.25	0.25	0.31	0.32	0.31	0.47	0.37	0.61
pH	4.70	4.69	4.30	4.29	4.46	4.12	4.38	3.82
Alcohol, proof	....	....	12.58	12.34	13.73	13.47	13.85	13.53
α-Amylase, ml. 1% starch	14	18	8	12	5	4	5	0.3
Bacteria, millions/g.	0.045	0.063	0.061	6.50	0.051	87.0	0.85	57.5
Yeast, millions/g.	7.4	7.1	100.5	98.5	18.5	0.22	4.0	<0.0001
Acrolein in distillate, p.p.m.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Proof gal. alcohol per 56 lb. grain (dry basis)	....	....	.....	...	....	...	5.98	5.88

[a] 1 unit penicillin/ml. of fermenter mash.

Literature Cited

(1) Atwood, H., U. S. Patent 2,222,306 (Nov. 19, 1940).
(2) Biosyn, G., French Patent 905,075 (Nov. 3, 1945).
(3) Circle, S. J., Stone, L., and Boruff, C. S., *Ind. Eng. Chem., Anal. Ed.*, **17**, 259 (1945).
(4) Effront, J., "Microorganisms and Fermentation," by Alfred Jorgensen, 5th ed., p. 27, London, England, Charles Griffin & Co., Ltd., 1925; U. S. Patent 620,022 (Feb. 21, 1899).
(5) Garey, J. C., Rittschof, L. A., Stone, L., and Boruff, C. S., *J. Bacteriol.*, **49**, 307 (1945).
(6) Gayon, U., and Dupetit, "Manufacture of Whiskey and Spirit," by J. A. Nettleton, p. 350, Aberdeen, Scotland, G. Cornwall & Sons, 1913.
(7) Hesseltine, C. W., and Bohonos, Nestor, Abstract of Application 229,296 U. S. Patent Office; *Patent Gazette*, p. 1368 (Sept. 29, 1953).
(8) Serjak, W. C., Day, W. H., Van Lanen, J. M., and Boruff, C. S., *J. Appl. Microbiol.*, **2**, 14 (1954).
(9) Waksman, S. A., and Green, S. R., *Proc. Soc. Exptl. Biol. Med.*, **67**, 281 (1948).
(10) Waksman, S. A., Iverson, W. P., and Green, S. R., *Ibid.*, **67**, 285 (1948).

Received for review December 3, 1953. Accepted February 8, 1954. Presented before the Division of Agricultural and Food Chemistry, Fermentation Subdivision, Symposium on Fermentation Process Control, at the 124th Meeting of the AMERICAN CHEMICAL SOCIETY, *Chicago, Ill.*

Heat-Stable Toxic Principle in Tung Meal Requires Consideration

OILSEED PROCESSING

Detoxification and Toxicological Studies Of Tung Meal

G. E. MANN, W. H. HOFFMAN, Jr., Southern Regional Research Laboratory, New Orleans, La., and ANTHONY M. AMBROSE, Pharmacology Division, Western Regional Research Laboratory, Albany, Calif.

Use of tung meal as an animal feed is infeasible because of its toxic nature; hence experiments were performed to resolve some of the apparent contradictory statements on the detoxification of this material. Employing rats as test animals it was found that a tung meal, prepared by hexane extraction of tung kernels, could not be detoxified completely by a combination of autoclaving and extraction with ethyl alcohol. Tung press cake (obtained by a commercial expeller process involving exposure to high temperatures) was detoxified almost completely by extraction with ethyl alcohol. The application of heat alone does not appear to effect complete detoxification of tung meal. The occurrence of a heat-stable toxic principle seems to require consideration in any proposed commercial detoxification process.

THE FRUIT OF THE TUNG TREE (*Aleurites fordii*) has long been used as a source of the valuable tung or China wood oil of commerce. Introduced into the United States in 1902, this tree has been cultivated in the Gulf Coast area to give rise to an important southern industry. An exhaustive bibliography on the chemistry and technology of tung products has been published recently by Planck, Pack, and Skau (*19*).

To obtain commercial tung oil, the seeds (or nuts) from the mature fruit commonly are crushed in an expeller press; the cake of meal obtained in this process is sometimes solvent-extracted to remove the residual oil. Analyses of meals left after petroleum ether extraction of both seed kernels and whole seeds have indicated that both materials should be valuable as animal feedstuffs, as they are fairly rich in protein and carbohydrate (*7, 13*).

This valuable outlet for the oil-free tung meal, however, is closed because of its long-recognized toxic nature, the first scientific report on toxicity of tung seeds appearing to be that of Mutschler and Krauch in 1879 (*18*).

At present the meal is being used as a fertilizer only; this represents a relatively low-priced outlet. Tung meal has been used as an insecticide (*14, 21*), but it does not appear to be so employed to any great extent. The possible use of meal protein as a plastic has been investigated (*16*), but did not result in a quantity outlet for the meal. Undoubtedly a reliable procedure for the detoxification of tung meal on a large scale would be attractive, thus converting it from a low-priced fertilizer into a more valuable animal feed. The present publication deals mainly with the results of a series of experiments, devised on the