The Use of Ammonium Bifluoride on Yeast Propagation and Fermentation of Blackstrap Molasses[1]

Isabel M. de González and Nivia F. Murphy[2]

INTRODUCTION

It is assumed quite generally that bacterial contamination is one of the primary causes of low fermentation efficiency of molasses mashes. This assumption seems entirely consistent with the fact that blackstrap molasses is known to have a fairly large number of viable bacteria, which may attain very high values in the dilute mashes in the fermentors, whenever the alcoholic fermentation for any reason falls below normal levels. The definite deterioration caused by bacterial contamination in molasses mashes finds further support in the well-known fact that commercial blackstrap molasses is not always stored under conditions that tend to prevent microbial growth.

Examination of microorganisms in the raw material in the fermentation industry revealed the presence of different bacterial types in each plant in the same area. It thus is evident that the fermenting mash is exposed to a variety of bacterial types and that certain properties of the fermenting mash promote the subsistence of specific ones over others.

The mash in an alcoholic fermentation usually is not sterilized; the chief defense against contaminants is the adjustment of the pH to 5 or slightly below. Many contaminants will not grow readily at such pH levels. The fermentation usually is so vigorous that anaerobic conditions are quickly established and the alcohol produced tends to inhibit development of lactic or butyric acid organisms. Therefore, the types of bacteria that may grow sufficiently well during an alcoholic fermentation to produce mash contamination are limited to those growing in an anaerobic medium with a low pH, at temperatures of 25° to 30° C., when fermentation takes place normally.

Analyses of blackstrap molasses (1) have shown that it is rich in bacterial nutrients and under proper conditions, when diluted with water, should support good growth of a variety of heterotrophic bacteria. It has been shown by Sherwood (2) that the nutritional characteristic of molasses wort changes rapidly as soon as it is pitched and at the beginning of yeast

[1] Manuscript submitted to Editorial Board June 28, 1972.

[2] Assistant Chemist and Associate Bacteriologist, respectively, Rum Pilot Plant, Agricultural Experiment Station, Mayagüez Campus, University of Puerto Rico, Río Piedras, P. R.

growth and fermentation. He demonstrated however, that it is possible for the amino acids left in the molasses mash to support the growth of contaminating bacteria, or that the development of bacteria depends perhaps on certain nutrients synthesized and excreted by yeast into the fermenting mash.

A survey of the literature concerning the methods employed to control bacterial contamination indicates the effectiveness of hydrofluoric acid, or its salts, as a selective antiseptic for the protection of yeast against bacteria in diluted mashes. Effront (3) claimed that hydrofluoric acid is 10 to 20 times more inhibitory against lactic acid bacteria than hydrochloric acid. The same author also claimed that it is better to use the ammonium salt instead of the acid in concentrations ranging from 0.02 to 0.03 g. of ammonium bifluoride ($NH_4F \cdot FH$) per liter. Bokorny (4) reported that the fluorides of iron and magnesium are more powerful than those of sodium and potassium. McIntosch (5) reported that fluorides are regularly employed in beet juice distilleries in France where a concentration of as much as 0.015 g. per liter is maintained in the mashes, and claims that a larger quantity is harmful for yeast fermentation. Buchanan and Fulmer (6) reported that sodium fluoride to a concentration of 0.01 g. per liter is inhibitory to the development of lactic acid bacteria.

Webber and Taylor (7) reported the addition of sodium fluoride or sodium silicofluoride to fermenting mash in concentrations ranging from 1 p.p.m. to as much as 1,000 p.p.m. They found that the addition of any fluoridating agent in quantities up to 10 p.p.m. had practically no effect on attenuation whereas the presence of over 20 p.p.m. caused a reduction in attenuation. Klopper (8) reported that 10 p.p.m. of sodium fluoride did not disturb the fermentation of the wort.

Because yeasts generally derive nitrogen from such relatively simple substances as ammonium salts, it seems reasonable to assume that ammonium bifluoride might be superior to other fluoridating agent in this respect.

The purpose of this investigation was to evaluate the use of ammonium bifluoride as an antiseptic agent for controlling bacterial contamination in distillery fermentations.

EXPERIMENTAL PROCEDURE

Experiments were conducted first to determine the concentrations of ammonium bifluoride that inhibit development of contaminating bacteria isolated from our fermentors and second, to find out the effect of different concentrations of ammonium bifluoride in alcohol production during fermentation and, similarly, on yeast growth and viability.

Four different bacterial contaminants were isolated: one was identified

as a strain of *Leuconostoc* and three as different strains of *Lactobacilli.* The medium used for the isolation was synthetic solid medium for the cultivation of bacteria as previously described (9). Serial dilution sensitivity tests were performed in synthetic liquid and molasses media (9) and in APT commercial medium sold by Difco Laboratories. Concentrations of the $NH_4F \cdot HF$ tested ranged from 0 to 1000 mg. per liter of the medium. The test organisms were the four strains of bacteria isolated from our fermentors. Observations were made after a 72-hour incubation period at 32° C.

To determine the effect of ammonium bifluoride on alcohol production, laboratory scale batch-fermentation experiments were conducted as described previously (9). Fermentation mash at 25° Brix of blackstrap molasses was prepared and pasteurized at 170° C. When cooled, 14 liter samples were distributed in bottles of 20-liter capacity. Each 20-liter bottle served as an individual fermentor. Different concentrations of ammonium bifluoride ranging from 0 to 1000 mg. were added per liter to the laboratory-scale fermentor. The yeast strain used was grown and reactivated prior to the inoculation. The test organism used was *Saccharomyces* strain 80, which under proper conditions produces high alcohol yields with high fermentation efficiencies. Each fermentor was inoculated with 2 liters of yeast seed. Two separate experiments were conducted. After 64 hours of fermentation, the fermented mashes were analyzed for percent alcohol by volume, residual sugar content, pH and final acidity as described in (10).

Additional experiments were performed to determine the effect of ammonium bifluoride on yeast growth and viability. Again, the test organism used was *Saccharomyces* yeast strain 80. A 10 ml.-yeast culture previously agitated for 24 hours was inoculated into three Erlenmeyer flasks, each one containing 40 ml. of reactivation media, and three Erlenmeyer flasks each one containing 40 ml. of liquid molasses media: See formula given in (9). Concentrations of 0 (used as a control), 50 and 100 mg. per liter were added to both media prior to inoculation with the yeast. The flasks were then placed on a mechanical shaker and yeast cell counts were taken after an 18-hour growth period with constant shaking. Flasks were kept at room temperature for 3 days while daily viability tests were conducted. Methods employed for the hemacytometer yeast cell count and plate yeast viabilities were as previously described (9).

RESULTS AND DISCUSSION

Molasses mash was shown to contain a mixed flora which includes *Leuconostoc* and *Lactobacilli.* Table 1 shows the susceptibility of bacteria to ammonium bifluoride in different culture media. Differences in the various

TABLE 1.—*Susceptibility of bacteria to various levels of ammonium bifluoride in different culture media*

NH_4FHF mg./liter	Synthetic media				APT media	Molasses media		
	Leuconastoc	Lactobacilli I	Lactobacilli II	Lactobacilli III	Lactobacilli II	Lactobacilli I	Lactobacilli II	Lactobacilli III
0	+[1]	+	+	+	+	+	+	+
100	+	+	−[2]	+	+	+	+	+
200	+	−	−	−	+	+	+	+
300	+	−	−	−	+	−	+	+
400	−	−	−	−	+	−	+	−
500	−	−	−	−	+	−	+	−
600	−	−	−	−	+	−	+	−
700	−	−	−	−	+	−	+	−
800	−	−	−	−	−	−	−	−
900	−	−	−	−	−	−	−	−
1000	−	−	−	−	−	−	−	−

[1] + = growth.
[2] − = no growth.

TABLE 2.—*Effect of ammonium bifluoride on alcohol production*

Fermentation number		I 1-3	I 4-6	I 7-9	I 10-12	I 13-15	I 16-18	I 19-21	I 22-24
Ammonium bifluoride (mg./liter)		0	50	100	150	200	250	300	500
Yeast number		←			80				→
pH	Initial	←			4.6				→
	Final	4.4	4.5	4.4	4.4	4.4	4.3	4.4	4.4
Acidity (g./l.)	Initial	←			4.0				→
	Final	7.4	6.7	6.9	6.9	7.2	7.0	6.8	7.3
° Brix	Initial	←			28.5				→
	Final	6.8	6.6	7.0	7.7	7.9	8.2	7.0	7.3
Inv. Sugar (g./100 ml.)	Initial	←			22.7				→
	Final	1.21	1.24	1.48	1.99	2.36	2.35	1.53	1.67
Percent alcohol by volume		12.9	12.8	12.7	12.3	12.2	12.0	12.6	12.5
Fermentation time (hours)		←			64				→

media tested are observed. *Lactobacilli* are nutritionally fastidious, i.e., they have exceptionally demanding requirements for nutrients. Probably the synthetic media does not provide the bacterium with the vitamins or other growth factors they require which are provided by molasses or APT media. The results obtained from experiments conducted with *Lactobacilli* in molasses media are consequently more reliable. Besides, molasses is the medium used for alcoholic fermentation in rum production. Two strains of

TABLE 3.—*Effect of ammonium bifluoride on alcohol production*

Fermentation number		J 1-3	J 4-6	J 7-9	J 10-12	J 13-15	J 16-18	J 19-21	J 22-24
Ammonium bifluoride (mg./liter)		0	150	300	450	600	750	900	1000
Yeast number		←			80				→
pH	Initial	←			4.7				→
	Final	4.4	4.3	4.3	4.3	4.3	4.2	4.2	4.2
Acidity (g./l.)	Initial	←			4.4				→
	Final	7.4	7.8	8.2	8.3	7.9	8.5	8.5	8.6
° Brix	Initial	←			29.0				→
	Final	8.8	9.1	9.2	8.9	9.9	8.9	9.0	9.0
Inverted Sugar (g./100 ml.)	Initial	←			22.4				→
	Final	1.43	1.43	1.46	1.47	1.47	1.50	1.52	1.47
Percent alcohol by volume		12.6	12.6	12.5	12.5	12.4	12.4	12.5	12.5
Fermentation time (hours)		←			64				→

Lactobacilli are sensitive to 300 to 400 mg. of ammonium bifluoride per liter of molasses media and only one strain resisted concentrations of this antiseptic agent higher than 400 mg per liter. Thus, 400 mg. of ammonium bifluoride per liter could reduce the bacterial population of molasses mashes considerably.

The effects of ammonium bifluoride on laboratory-scale fermentation experiments are summarized in tables 2 and 3. No significant differences in alcohol production were recorded at the levels of bifluoride used. The first experiment (table 2) resulted in a slightly higher yield of alcohol in fermentors containing lower concentrations of ammonium bifluoride (0, 50, 100

mg./l.). This higher yield cannot be related to an adverse effect of the salt. In the second experiment (table 3) the alcohol yields in all fermentors were approximately the same, using concentrations of 0 to 1,000 mg. of ammonium bifluoride per liter of mash. These results indicate that am-

TABLE 4.—*Total cell count at 18 hours growth*

Media	Ammonium bifluoride mg./liter	Millions per ml.			
		Experiment I	Experiment II	Experiment III	Experiment IV
Reactivation	0	445	464	422	470
	50	—	391	226	318
	100	304	180	104	158
Molasses	0	428	435	346	374
	50	—	337	318	319
	100	376	316	302	323

TABLE 5.—*Effect of ammonium bifluoride on yeast viability*

Ammonium bifluoride mg./l.	Media	Storage time (hours)	Percent viability			
			Experiment I	Experiment II	Experiment III	Experiment IV
0	Reactivation	18	—	100	100	100
		48	93	94	98	94
		72	94	87	96	85
	Molasses	18	—	96	84	93
		48	78	81	92	95
		72	17	41	80	85
50	Reactivation	18	—	59	46	78
		48	—	5	33	45
		72	—	2	12	45
	Molasses	18	—	96	94	91
		48	—	26	38	72
		72	—	10	4	54
100	Reactivation	18	—	62	36	47
		48	1	0	4	0
		72	0	0	0	0
	Molasses	18	—	96	65	93
		48	26	14	8	51
		72	6	4	6	4

monium bifluoride at the levels of concentration tested does not affect adversely the fermentation abilities of the yeasts.

Tables 4 and 5 show the effects of ammonium bifluoride in yeast growth and viability. As the concentration of the salt increases, there is a tendency in the yeast cell count to decrease. Similarly the percent of live cells decreases as the concentration of bifluoride increases (figs. 1 and 2). Microscopic observation of the yeast cells using a phase difference microscope show damaged cells when they are grown in the presence of ammonium

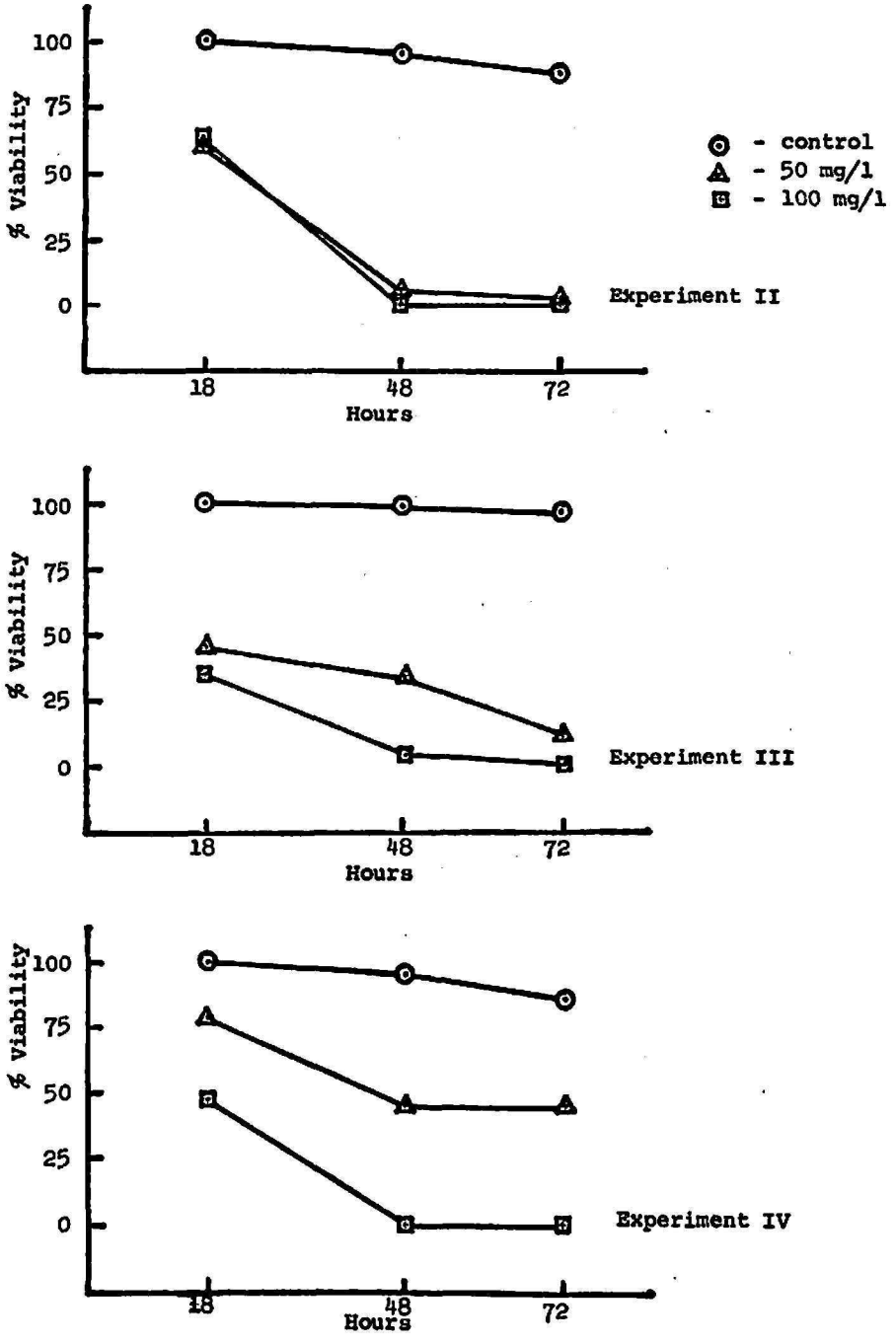

FIG. 1.—Effect of ammonium bifluoride on yeast strain 80 grown in reactivation media.

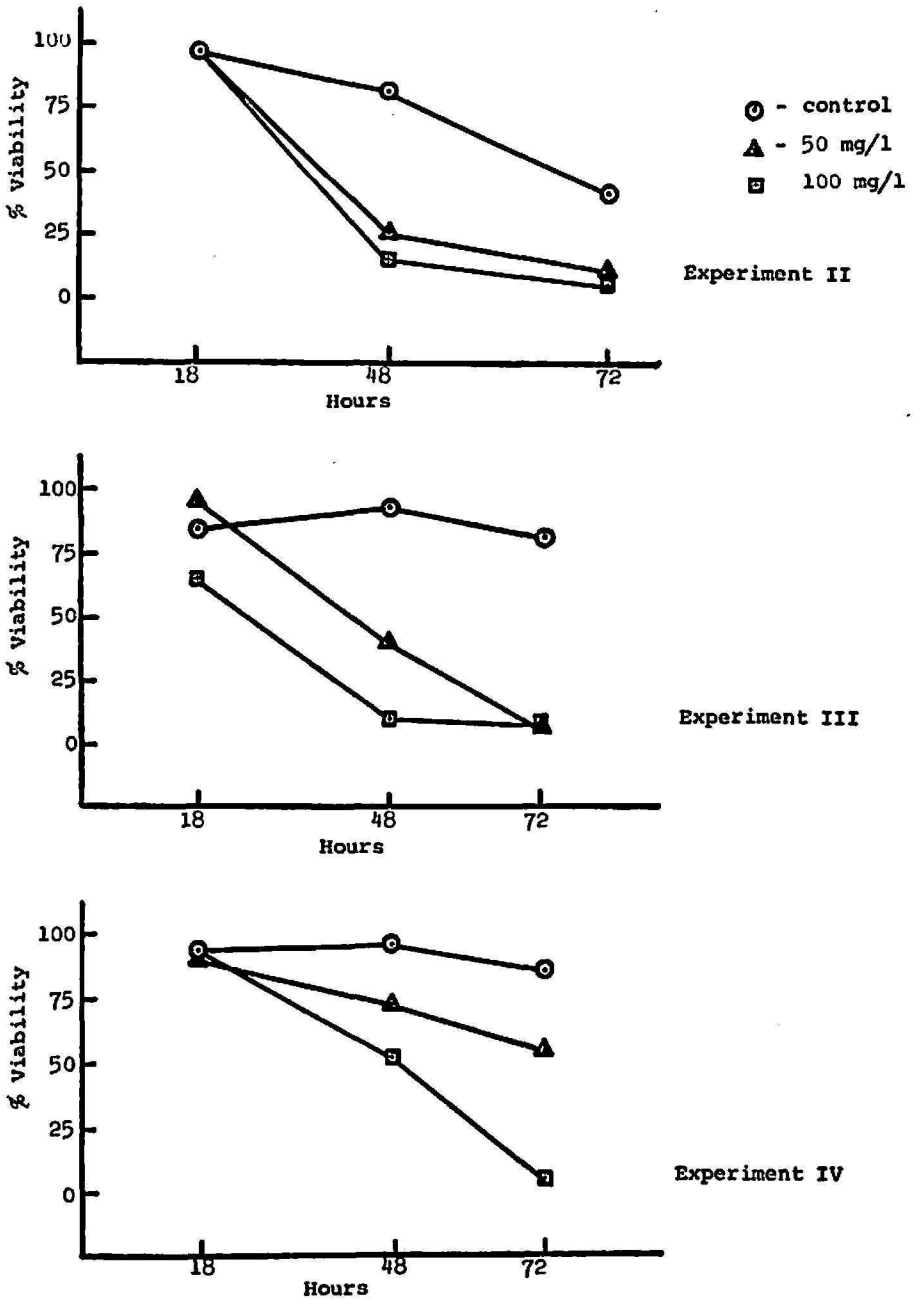

FIG. 2.—Effect of ammonium bifluoride on yeast strain 80 grown in molasses media.

bifluoride. It thus is concluded that ammonium bifluoride is highly inhibitory to yeast growth and that it adversely affects yeast viability even at concentrations as low as 50 mg. per liter. In the light of these results, the use of this antiseptic agent is not recommended for yeast propagation if high yields are required and longevity desired. High yeast yields are not required for batch fermentation experiments. This explains why ammonium bifluoride did not affect alcohol production during fermentation in spite of its adverse effect on yeast propagation.

SUMMARY

The use of fluorides for controlling bacterial contamination in commercial production of rum led to several experiments to evaluate ammonium bifluoride as a bacterial inhibitor and test its effects on yeast fermentation, yeast growth and preservation.

Sensitivity tests were performed with bacteria isolated from molasses mashes. The results indicated that with 400 mg. of ammonium bifluoride per liter, bacterial population on fermenting mashes can be reduced considerably.

Laboratory-scale batch fermentation experiments were conducted to study the effect of ammonium bifluoride on alcohol production. The results obtained indicate that ammonium bifluoride does not affect the fermentative characteristics of yeasts in concentrations of up to 1,000 mg. per liter tested.

The effect of ammonium bifluoride on yeast growth and viability during storage also was investigated. It was demonstrated that this salt is highly inhibitory to yeast propagation and adversely affects yeast viability after yeast growth terminates.

RESUMEN

En la producción comercial de ron se usan las sales de fluoruros para controlar la contaminación bacteriana. Esto dio lugar al estudio del bifluoruro de amonio ($NH_4F \cdot HF$) como agente para inhibir las bacterias, y el de su efecto sobre la fermentación alcohólica y la propagación y conservación de las levaduras.

Se llevaron a cabo pruebas de sensibilidad con las bacterias aisladas de baticiones de miel preparadas en nuestro laboratorio. Los resultados demostraron que con 400 mg. de bifluoruro de amonio por litro, se reduce considerablemente la población bacteriana en las baticiones durante la fermentación.

Se efectuaron dos experimentos de fermentación comparativa en escala de laboratorio para determinar el efecto del bifluoruro de amonio sobre el rendimiento alcohólico. Los resultados obtenidos indican que esta sal no afecta las características fermentativas de las levaduras, ni siquiera hasta niveles de concentración de 1,000 mg. por litro.

Además se estudió el efecto de esta sal sobre el crecimiento y la viabilidad de la levadura durante la preservación a temperatura ambiente. Los resultados obtenidos demostraron que el bifluoruro de amonio tiene un efecto adverso sobre la propagación y viabilidad de la levadura una vez termina ésta su crecimiento.

En fermentaciones comparativas por el método intermitente no se requieren conteos de levadura muy altos. Esto explica por qué el bifluoruro de amonio no afecta el rendimiento alcohólico durante la fermentación, a pesar del efecto adverso que tiene sobre la propagación y preservación de la levadura.

LITERATURE CITED

1. Benkley, W. W. and Walfrone, M. L., Scientific Report Series No. 15, Sugar Research Foundation, Inc., New York, N.Y., 1953.

2. Sherwood, I. R., The Colonial Sugar Refining Co., Ltd. Sydney, Austr. through J. Inst. of Brewing 62: 104, 1956.
3. Effront, J., Compt. Rend. Acad. Sci. 118: 1,420, 1893.
4. Bokorny, T., Physiology and Biochemistry of Bacteria, Vol. II, p. 396, 1930.
5. McIntosch, J. G., Industrial Alcohol, Scott, Greenwood, and Son, London, England, 1907.
6. Buchanan, R. E., and Fulmer, E. I., Physiology and Biochemistry of Bacteria, Vol. II, p. 396, 1930.
7. Webber, H. F. P., and Taylor, L., J. Inst. Brewing 60(5): 427–30, 1954.
8. Klopper, W. J., and Jongelin-Eijndhoven, J. Inst. Brewing 77(3): 259–60, 1971.
9. Manual de Métodos y Procedimientos Bacteriólogicos de la Planta Piloto de Ron (MPPR-1), Agr. Exp. Sta., Mayagüez Campus, Univ. of P.R., Río Piedras, P.R., June 1969.
10. Manual de Métodos Analíticos de la Planta Piloto de Ron (MPPR-2), Agr. Exp. Sta., Mayagüez Campus, Univ. of P.R., Río Piedras, P.R., January 1969.