The Use of Calcium Carbonate on Distillery Yeast Preservation[1]

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INTRODUCTION

The preservation of yeast cells in a viable state for varying lengths of time is of special concern in the fermentation industries. This may be accomplished by several methods. The oldest and most satisfactory method is to grow the yeast on a solid medium in test tubes and after active growth has ceased, to keep cultures at 5° to 10° C. in effective airtight containers. Schulz (9) stated that yeast cultures may be satisfactorily preserved under a layer of sterile paraffin oil. Small amounts of cells may also be lyophilized. Wickerham (13) strongly recommended preservation of yeasts by this method. However, Kirsop (5) observed changes in growth requirements of freeze-dried yeasts.

A number of investigators have studied the effect of temperature and storage upon the viability of both dry and moist yeast. Felsher et al. (3) found the shelf life of either dry or moist yeast in storage lengthened as the temperature decreased. When the same yeast was kept for more than 2 years at 4.4° C. or lower it was still useful for bread baking. Adams (1) stated that moist wine yeasts can be frozen in bulk and stored at −29° C. or lower for several years without total loss of viability or essential characteristics.

In commercial rum distilleries a different problem arises because large amounts of yeast seed must be kept viable in liquid molasses media. Yeast cells die gradually if left in the medium in which they have grown. Preservation of yeasts in their growth molasses media therefore presents the problem of an accelerated loss of viability.

Because of its strong adsorptive capacity, activated charcoal has been used for purification of alcoholic beverages (4,6,7). Nikolaeva and Chernyi (7) used charcoal to remove fusel oil and aldehydes from vodka. With the aid of charcoal it was possible, therefore, to purify vodka economically. Aguiar Muxella et al. (2) evaluated the effectiveness of six different commercial charcoals in removing detrimental components and color in rum. Charcoal might be useful in reducing the death rate of yeast in a molasses

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medium or mash, because of its high adsorptive capacity for removing toxic congeners that could be the cause of death.

Calcium carbonate may also be used as an aid in controlling death rate of yeasts in growth media. This compound has been used by Rutkowski (8) for inactivating unbound bilirubin from bilirubin-albumin solutions. Kolasha (6) used $CaCO_3$ on acid soils to accelerate growth and maturation of corn. By a similar neutralization reaction $CaCO_3$ might be suitable for yeast preservation in molasses media studies. Other chemical preparations such as HCl and H_2SO_4 also have been used in yeast preservation (12).

EXPERIMENTAL PROCEDURE

The effect of several preservative agents, such as activated charcoal, $CaCO_3$, and NaOH when added to mother molasses liquid, was studied to find suitable solutions to the yeast-viability problem.

Preliminary investigation was conducted by growing *Saccharomyces* strains PPR-80 and 17 in a molasses medium. The medium was prepared by diluting blackstrap molasses with tap water to about 24° Brix and a pH value of 5.0. For nutritional purposes, ammonium sulfate was added to the diluted molasses at a concentration of 1.5 g./liter. Portions of 80 ml. were distributed in 125-ml. Erlenmeyer flasks and then sterilized at 121° C. and 15 p.s.i.

The yeasts (strains PPR-80 and 17) were grown in the molasses medium by transferring small quantities of the stock cultures to eight 125-ml. flasks (four flasks for each strain) and placed overnight on a rotary shaker. At the end of the growth period the pH reading was approximately 4.3 in all flasks. The following tests were performed with each of the strains: restored the original pH (5.0) with NaOH; restored the original pH (5.0) with $CaCO_3$; added 1 percent (0.8 g.) of activated charcoal; and a control (nothing added).

All flasks were stored at 5° to 10° C. and plate viability tests were performed periodically on the cells in each flask as previously described (11).

The same procedure was followed with seven additional yeast strains: PPR-3, 184, 191, 193, 194, 239, and 263. An additional set of flasks was included in this test in which the exposure to $CaCO_3$, NaOH, and activated charcoal was prior to the inoculation with each of the seven additional stock cultures of yeast strains. Portions of 80 ml. of the molasses medium were placed in 125-ml. flasks and treated as follows: the pH was raised from 5.0 to 5.5 with $CaCO_3$ (seven flasks); the pH was raised from 5.0 to 5.5 with NaOH (seven flasks); 0.8 g. of activated charcoal was added to the samples (seven flasks); and a control was kept for each yeast strain (seven flasks).

All flasks were autoclaved, cooled to room temperature and finally

inoculated with the seven yeast strains. The pH of the medium was lowered by the action of the yeasts to approximately 5.0 for $CaCO_3$ and NaOH, and to a still lower value for charcoal and the controls.

The following tests, similar to those described in the preliminary investigation, were performed additionally on another group of flasks with the same seven yeast strains at the end of their growth period: the pH was raised to 5 with NaOH (seven flasks); the pH was raised to 5 with $CaCO_3$ (seven flasks); and 0.8 g. of activated charcoal was added to each flask (seven flasks).

All flasks were stored at 5° to 10° C. and plate viability tests were performed periodically on the cells in each flask as previously described (11).

TABLE 1.—*Toxic absorption in growth media*

Treatment	Percent viability—Yeast 80									
	1 Week	3 Weeks	8 Weeks	11 Weeks	14 Weeks	16 Weeks	17 Weeks	18 Weeks	20 Weeks	31 Weeks
Control	90	85	97	44	36	8	—	3	0	0
NaOH	89	95	96	94	93	18	41	37	41	0
$CaCO_3$	99	98	99	95	92	96	87	89	65	45
Activated charcoal	99	96	98	87	66	224	22	23	8	0

Treatment	Percent viability—Yeast 17								
	1 Week	6 Weeks	11 Weeks	14 Weeks	16 Weeks	17 Weeks	18 Weeks	20 Weeks	31 Weeks
Control	98	86	93	51	42	54	24	51	0
NaOH	93	80	86	77	77	74	22	72	12
$CaCO_3$	96	93	94	87	83	77	62	92	60
Activated charcoal	100	93	95	65	82	—	2	39	8

Samples of the different strains were transferred to plates of a nutrient synthetic medium (11) and incubated at 32°C. After approximately a 6-hour incubation period, cultural behavior of the yeasts subjected to the several conditions was observed microscopically.

It also was desirable to check the fermentative capabilities of the preserved yeasts to determine if adverse variations on the behavioral pattern occurred as a result of the calcium carbonate. Batch fermentation experiments were conducted on a laboratory scale with *Saccharomyces* yeast strains PPR-3, 184, 191, 193, and 194, each from three different sources: (a) Stock pure cultures of a nutrient synthetic medium (11) free from $CaCO_3$ stored at 5° to 10° C. (employed as controls); (b) yeast cultures stored at 5° to 10° C. for 18 months in their growth liquid molasses treated with $CaCO_3$; and (c), same as (b) but recovered and purified (11) on plates of nutrient synthetic media after an 18-month storage period.

The diluted mash necessary for this test was prepared by diluting the blackstrap molasses to a 22° Brix, followed by the addition of 1.5 g./liter of ammonium sulfate. Portions of 35 ml., 250 ml., and 2 liters of the mash were sterilized and larger quantities were bulk-pasteurized.

Before being used, yeast starters were activated and increased in volume by growing them in a suitable pure culture system. The yeast strains were transferred to 125-ml. Erlenmeyer flasks containing 35 ml. of molasses mash and allowed to grow under constant mechanical agitation for approximately 24 hours. They were again subcultured to 500-ml. flasks containing 250 ml. of the mash, and shaken for another 24 hours. Finally, transfers of the fully active yeasts were made to 4-liter flasks containing 2 liters of the molasses mash and kept overnight at room temperature without shak-

TABLE 2.—*Effect of calcium carbonate on yeast preservation (added initially to growth medium)*

Yeast number	Treatment	Percent viability (monthly readings)									
		1	2	3	4	5	6	7	8	9	10
3	Control	80	58	20	12	4	0	0	0	0	0
3	NaOH	95	67	40	16	10	2	0	0	0	0
3	$CaCO_3$	97	78	47	48	30	22	22	17	11	5
3	Charcoal	85	58	49	19	8	7	3	0	0	0
184	Control	74	54	53	18	12	5	0	0	0	0
184	NaOH	89	68	59	37	27	6	0	0	0	0
184	$CaCO_3$	91	58	58	44	42	22	14	14	10	7
184	Charcoal	73	69	50	33	19	16	4	2	1	0
191	Control	89	64	26	13	7	3	0	0	0	
191	NaOH	98	84	59	32	15	11	5	0	0	
191	$CaCO_3$	96	92	48	48	27	23	18	11	8	
191	Charcoal	95	82	52	30	16	11	2	1	1	
193	Control	88	63	30	15	10	4	4	0	0	
193	NaOH	92	83	60	38	24	11	4	1	0	
193	$CaCO_3$	89	80	57	41	31	23	15	10	7	
193	Charcoal	89	80	59	33	19	8	4	2	2	
194	Control	94	57	33	11	11	4	0	0	0	
194	NaOH	97	80	58	25	16	5	0	0	0	
194	$CaCO_3$	95	84	73	46	35	27	15	15	12	
194	Charcoal	95	72	52	27	17	10	6	3	2	
239	Control	38	2	0	0	0	0	0	0	0	
239	NaOH	53	5	0	0	0	0	0	0	0	
239	$CaCO_3$	54	17	13	5	5	0	0	0	0	
239	Charcoal	36	9	0	0	0	0	0	0	0	
263	Control	41	12	1	0	0	0	0	0	0	
263	NaOH	50	12	1	0	0	0	0	0	0	
263	$CaCO_3$	60	18	15	11	6	6	4	2	0	
263	Charcoal	47	16	8	3	0	0	0	0	0	

ing. These yeast samples were used as starters for the fermentation experiments.

Molasses mash was pasteurized and cooled, and 14-liter portions were introduced into 20-liter bottles to conduct all the fermentation experiments. Each bottle was inoculated with 2 liters of the yeast starter and allowed to ferment for 26 hours. Finally the fermented mashes were analyzed for percent alcohol by volume according to the method previously described (10). Fermentation efficiencies were calculated using the outlined formula (11):

$$\text{Percent efficiency} = \frac{1.633\ (\text{percent alcohol by volume})(100)}{\text{Total sugar (as invert in dilute molasses)}}$$

TABLE 3.—*Effect of calcium carbonate on yeast preservation (added after yeast growth terminates)*

Yeast number	Treatment	Percent viability (monthly readings)									
		1	2	3	4	5	6	7	8	9	10
3	Control	80	58	20	12	4	0	0	0	0	0
3	NaOH	92	66	50	25	14	7	5	0	0	0
3	$CaCO_3$	95	75	64	38	22	15	9	6	2	1
3	Charcoal	81	64	43	18	10	5	1	1	0	0
184	Control	74	54	53	18	12	5	0	0	0	0
184	NaOH	83	74	58	47	32	16	9	3	2	1
184	$CaCO_3$	79	77	41	39	32	28	22	18	14	12
184	Charcoal	87	76	29	17	14	9	3	0	0	0
191	Control	89	64	26	13	7	3	0	0	0	
191	NaOH	90	75	52	29	19	9	6	2	1	
191	$CaCO_3$	87	70	31	33	16	22	16	9	9	
191	Charcoal	88	62	37	15	10	10	3	0	0	
193	Control	88	63	30	15	10	4	4	0	0	
193	NaOH	89	72	50	21	20	12	6	2	1	
193	$CaCO_3$	95	92	66	38	32	27	13	11	9	
193	Charcoal	94	80	60	36	26	16	5	3	4	
194	Control	94	57	33	11	11	4	0	0	0	
194	NaOH	84	63	49	32	22	10	6	5	2	
194	$CaCO_3$	97	66	68	45	34	28	20	16	12	
194	Charcoal	87	86	66	27	17	11	10	4	2	
239	Control	38	2	0	0	0	0	0	0	0	
239	NaOH	50	17	9	0	0	0	0	0	0	
239	$CaCO_3$	64	28	25	9	4	0	0	0	0	
239	Charcoal	46	19	15	0	0	0	0	0	0	
263	Control	41	12	1	0	0	0	0	0	0	
263	NaOH	57	12	2	0	0	0	0	0	0	
263	$CaCO_3$	56	8	4	0	0	0	0	0	0	
263	Charcoal	49	8	5	0	0	0	0	0	0	

RESULTS

The results of these investigations are given in tables 1, 2, and 3 and figure 1. Figure 1 shows the behavior of yeast strain 193 during a 9-month storage period. The other yeast strains behaved similarly. Findings indicate the usefulness of calcium carbonate as an additive to molasses media

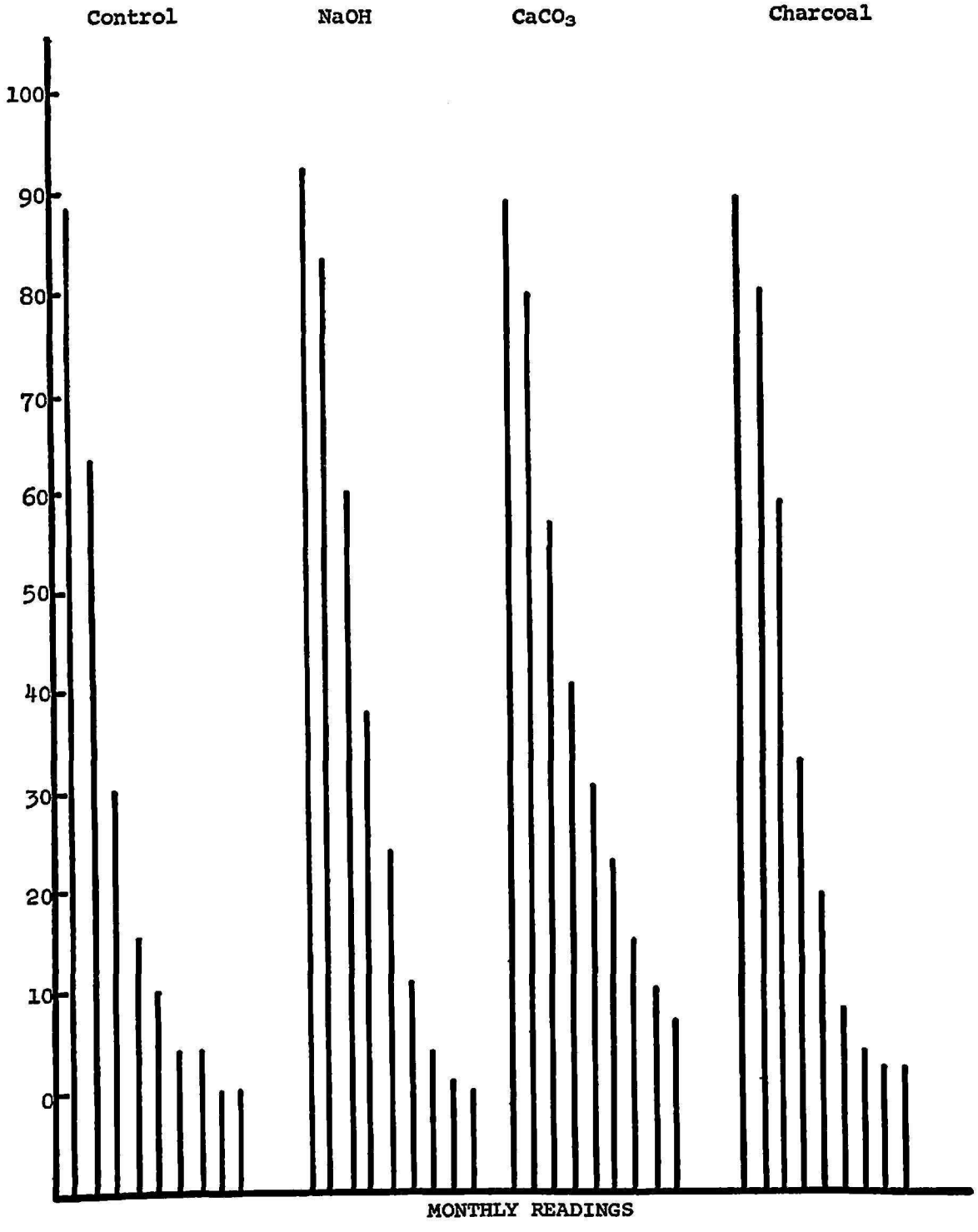

FIG. 1.—Percent viability of yeast strain 193 showing its behavior during a 9-month storage period.

for long-term survival of the cells of yeast in storage. All yeast strains tested were well preserved in the presence of calcium carbonate. Neither charcoal nor NaOH were as effective in lengthening the life of yeast cells as $CaCO_3$. After a 9-month period, the cells survived practically only where calcium carbonate was present.

As shown in tables 2 and 3, no significant differences in its preservation capacity were found when calcium carbonate was added before starting yeast growth or after cessation of growth. Therefore, its effectiveness as a yeast preservative is not affected by the method of growing the yeast.

Direct microscopic observations of the yeast cells corroborate these

TABLE 4.—*Fermentation tests using yeast preserved with $CaCO_3$*

Fermentation number	Yeast number	Storage conditions	Alcohol volume	Fermentation efficiency
			Percent	*Percent*
1	3	Cultures not exposed to $CaCO_3$	7.1	88
2	184		6.9	86
3	191		6.9	86
4	193		7.0	86
5	194		7.0	86
6	3	Exposed to $CaCO_3$	7.1	88
7	184		7.1	88
8	191		7.0	86
9	193		6.8	84
10	194		7.0	86
11	3	Exposed to $CaCO_3$ and purified after	7.2	89
12	184	18 months	7.0	86
13	191		6.8	84
14	193		6.9	86
15	194		7.0	86

findings. Live cells in calcium carbonate, when stimulated to grow on a solid synthetic medium, appeared to be healthier, bigger, more uniform in size, and very actively budding, as compared with live cells in controls that showed shrunken cells with much smaller and irregular buds.

Table 4 summarizes the results of laboratory-scale fermentation tests conducted with the different strains of yeast exposed to calcium carbonate during 18 months of storage.

Results of the fermentation tests showed that the yeasts exposed to calcium carbonate behaved similarly to the controls or unexposed yeasts. Yeasts purified after exposure to calcium carbonate for 18 months behaved similarly. Percent alcohol produced by volume and fermentation efficiencies were approximately the same in all cases. Consequently, it is concluded

that yeasts exposed to $CaCO_3$ retain their good fermenting capacity over an 18-month storage period.

SUMMARY

A simple and efficient method is described for the preservation of yeasts in liquid molasses media. The technique involves the addition of calcium carbonate to the medium either prior to yeast inoculation or after cessation of growth. After undergoing different periods of storage, the properties of the yeasts were checked both by microscopic observation and by fermentation tests. The results showed that yeasts stored under the conditions described remain viable and suitable for use in fermentation for at least 18 months. Improved storage life of distillery yeasts has been achieved without loss of fermentative power.

RESUMEN

Se describe un método sencillo y eficiente para preservar levaduras en mieles finales (melaza). El método consiste simplemente en añadir carbonato de calcio a la miel antes de su inoculación con la levadura o después de terminado el período de crecimiento. Durante el almacenamiento, se estudiaron las características de las levaduras periódicamente, mediante pruebas de viabilidad en placas, observaciones microscópicas y pruebas fermentativas. Los resultados demuestran que el carbonato de calcio favorece la conservación de levaduras en baticiones de miel, manteniéndolas vivas y aptas para la fermentación por un período de por lo menos 18 meses. Esto se logró sin que las levaduras perdieran sus propiedades fermentativas.

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