Research Note

BATCH FERMENTATION PATTERNS FOR DIFFERENT STRAINS OF ZYMOMONAS IN HIGH TEST MOLASSES AND BLACKSTRAP MOLASSES[1]

Almost all alcoholic beverages in the western world depend on yeast fermentation. The most common yeast strain is *Saccharomyces cerevisiae*. In many tropical areas of America, Africa and Asia, other types of alcoholic beverages, such as pulque and palm wines, are very popular. These beverages consist of plant saps that undergo a mixed natural fermentation that depends mostly on bacteria of the genus *Zymomonas*.

These *Zymomonas* fermentations date back to ancient times, but only recently extensive studies have acknowledged their potential. Many investigators[2, 3, 4] have found in *Zymomonas mobilis* some attributes which make it an attractive alternative, with advantages over the traditional yeasts for producing ethanol. These attributes include higher specific rate of sugar uptake, ethanol production and improved yields.

Current literature describes experimentation on sucrose, glucose and fructose defined media. However, for a rum fermentation process that employs this bacterium, the microorganisms must have an affinity for sugarcane molasses substrate. The Rum Pilot Plant, updating our investigation, initiated research in this field.

Our initial investigation focused on performance of different strains of *Zymomonas* in cane molasses to provide the necessary information for future efforts on process optimization. Until more is learned about *Zymomonas* in molasses, the fermentation procedures used, although not necessarily the most convenient, will be those traditionally known for yeasts: batchwise at approximately 18% initial sugar concentration (total as invert). Experimentation was done in high test molasses (HTM) and blackstrap molasses (BM) with three different strains of *Zymomonas* obtained from the American Type Culture Collection: 10988 *Z. mobilis* (B-1), 29191 *Z. mobilis* (B-2), 29501 *Z. anaerobia* (B-4). They were maintained by periodic transfers to fresh media containing 1% of each yeast extract and peptone, 2% glucose and 4% agar. To prepare an active bacterial seed, I transferred the three bacterial strains from stock culture slants to the same glucose broth and incubated them for 18 to 24 h at 30° C in a CO_2 incubator. To insure actively growing cells, I conducted a second transfer to fresh glucose broth and incubated the three strains again in the same manner.

Fermentation mashes were prepared by dilution with tap water to the desired approximately 18% sugar concentration. This concentration was obtained at 23.2° Bx with BM and at 17.6° Bx with HTM. Three hundred (300) ml amounts were distributed in 500 ml Erlenmeyer flasks and sterilized at 121° C for 15 min. They were analyzed initially for pH, total acidity and total sugar concentration and then inoculated with 5% and 10% (v/v) actively growing bacterial seed of each of the three strains of *Zymomonas*. Inoculations were conducted in duplicate to perform at 32° C in a CO_2

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[2]Swings, J. and J. Deley, 1977. The biology of *Zymomonas*, Bacteriol. Rev. 41, pp 1–46.

[3]Rogers, P. L., D. Phil, K. J. Lee and D. E. Tribe, 1980. High productivity ethanol fermentations with *Zymomonas mobilis*. *Process Biochem.*, Aug.-Sept. 7–11.

[4]Lyness, E. and H. W. Doelle, 1981. Fermentation pattern of sucrose to ethanol conversions by *Zymomonas mobilis*. *Biotechnol. Bioeng.* 23: 1449–460.

TABLE 1.—*Comparative blackstrap molasses fermentations with different strains of* Z. mobilis *2-week fermentation period*

Strain		B-1				B-2				B-4			
Inoculum v/v		5%		10%		5%		10%		5%		10%	
Incubation conditions		RT[1]	30°C[2]	RT	30°C	RT	30°C	RT	30°C	RT	30°C	RT	30°C
pH	Initial	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9
	Final	3.9	3.6	4.6	3.6	4.5	3.6	4.4	3.7	3.9	3.6	4.5	3.6
Acidity g/L	Initial	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
	Final	11.8	13.6	4.8	13.4	5.5	13.6	5.7	12.7	10.5	12.8	4.8	13.6
	Produced	8.1	9.9	1.1	9.7	1.8	9.9	1.9	9.0	6.8	9.1	1.1	1.9
°Brix	Initial	23.2	23.2	23.2	23.2	23.2	23.2	23.2	23.2	23.2	23.2	23.2	23.2
	Final	19.2	9.7	14.6	9.2	14.0	10.0	11.1	11.5	22.9	9.7	19.6	9.5
	Change in Brix	4.0	13.5	8.6	14.0	9.2	13.2	12.1	11.7	0.3	13.5	3.6	13.7
Total sugars g/100 ml	Initial	18.4	18.4	18.4	18.4	18.4	18.4	18.4	18.4	18.4	18.4	18.4	18.4
	Final	9.0	1.4	3.8	1.4	6.9	1.6	4.0	3.2	13.4	1.3	12.5	1.3
	Utilized	9.4	17.0	14.6	17.0	11.5	16.8	14.4	15.2	5.0	17.0	5.9	17.1
% Sugar consumed		51	92	79	92	62	91	78	83	27	92	32	93
% Alcohol v/v		1.5	6.1	6.2	5.8	4.6	5.8	5.8	4.5	0	6.2	0.6	5.6
% Efficiency		13	54	55	51	41	51	51	40	0	55	6	50

[1]RT = Room temperature.
[2]30°C in a CO_2 incubator.

incubator and at room temperature (approximately 26° C).

Set 1: Erlenmeyer flasks fermenters plugged with cotton were incubated at 32° C in a CO_2 incubator. Flasks were periodically taken out, transferred to a cylinder for °Brix readings and returned to the flasks and incubator.

Set 2: Erlenmeyer flasks were rubber stoppered and an outlet glass tube was provided for CO_2 escape to a water vessel. To establish anaerobic conditions, initially the flasks were incubated in the CO_2 incubator for 30 minutes. Fermentations were then conducted at room temperature, manual agitation provided periodically, and initial and final °Brix were recorded.

After a 2-week fermentation, mashes were analyzed for total acidity, pH, total sugars and percentage alcohol. Methods for analyses, described in Manual de Métodos Analíticos de la Planta Piloto de Ron, included Lane Eynon for sugars and immersion refraction for alcohol determinations. Fermentation efficiency was calculated as follows:

$$\frac{1.633\ (\%\ \text{alcohol/volume})(100)}{\text{Initial total sugars as invert}}$$

where, 1.633 is a constant derived from Pasteur theoretical efficiency of 100/61.23. Tables 1 and 2 and figures 1 through 4 present the data collected.

Discussion of preliminary results

Zymomonas can grow in and ferment sugarcane molasses. This finding is evidenced by the almost total consumption of sugars and ethanol production in nearly all cases studied here. The slow fermentation rate and low alcoholic yield experienced were expected possibilities because proper environmental conditions for efficient molasses *Zymomonas* fermentations are not yet known.

It is well known that many substrate components at certain concentration may exert inhibitory effects on microorganisms. The prolonged lag that the fermentation of both BM and HTM mashes experienced indicates such inhibition occurred at the initial substrate sugar level studied (± 18%). The characteristic high mineral content of sugarcane molasses may account for this inhibition. If mineral content would exert a certain degree of inhibition on *Zymomonas*, then the effect would be stronger in BS molasses than in HTM molasses since the percentage of inorganic salts in HTM is approximately 8 times lower than in BM. Besides, HTM has a higher total sugar content, which is mostly reducing sugars with a low sucrose content. BM has a lower total sugar content, which is mostly sucrose, as shown below:

	HTM	BM
Total sugars	74 to 79%	55 to 60%
Sucrose	15 to 26%	25 to 40%
Reducing sugars	50 to 65%	12 to 25%
Sulfated ash	2 to 3%	7 to 15%

These differences in the concentration of the major constituents possibly explain the observed differences in fermentation behavior, which was better for HTM. The fermentation of HTM reached its logarithmic phase faster than that of BM: 24 to 50 h in HTM vs. 75 to 210 h in BM. Moreover, a tendency to higher efficiencies was observed in HTM.

Although a relatively high percentage of sugar was consumed in most cases, alcoholic fermentation efficiencies were low at conditions studied. This finding suggests excessive sugar deviation to by-product formation. This is wasteful in terms of alcohol production. Considerable amounts of acids were produced, higher in BM. A study underway on secondary products includes their identification and process manipulation to discourage their formation.

Smaller inoculum (5%) usually behaved more slowly initially. This behavior was more evident in BM and was minimized later during this long term fermentation.

Microorganisms differ from one another, even members of the same kind. Fermentation curves of B-1 in both kinds of molasses had a rapid start, then a stationary phase, and then accelerated again. B-1 and B-4 usually presented shorter lag periods than B-2, but B-2 although with a slow start, completed fermentation faster in most

TABLE 2.—*Comparative high test molasses fermentations with different strains of* Z. mobilis *2-week fermentation period*

Strain		B-1				B-2				B-4			
Inoculum v/v		5%		10%		5%		10%		5%		10%	
Incubation conditions		RT[1]	30°C[2]	RT	30°C	RT	30°C	RT	30°C	RT	30°C	RT	30°C
pH	Initial	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
	Final	3.8	3.4	3.8	3.8	4.1	3.7	4.1	3.5	4.3	3.5	4.3	3.5
Acidity g/L	Initial	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
	Final	3.8	8.2	4.1	7.8	3.2	6.4	3.1	8.0	2.8	7.1	2.8	7.6
	Produced	1.9	6.3	2.2	5.9	1.3	4.5	1.2	6.1	0.9	5.2	0.9	5.6
°Brix	Initial	17.6	17.6	17.6	17.6	17.6	17.6	17.6	17.6	17.6	17.6	17.6	17.6
	Final	9.5	4.1	7.5	3.6	4.2	4.7	4.3	4.9	5.8	4.7	4.3	4.7
	Change in Brix	8.1	13.5	10.1	14.0	13.4	12.9	13.3	12.7	11.8	12.9	13.3	12.9
Total sugars g/100 ml	Initial	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3
	Final	4.8	1.0	5.2	0.8	2.1	1.3	2.0	1.2	2.9	1.4	2.0	1.5
	Utilized	12.5	16.3	12.1	16.5	15.2	16.0	15.3	16.1	14.4	15.9	15.3	15.8
% Sugar consumed		72	94	70	95	88	92	88	93	83	92	88	91
% Alcohol v/v		5.2	5.0	4.4	5.8	6.8	5.5	6.4	5.0	6.1	5.5	6.1	4.9
% Efficiency		49	47	42	55	64	52	60	47	58	52	58	46

[1] RT = Room temperature.
[2] 30°C in a CO_2 incubator.

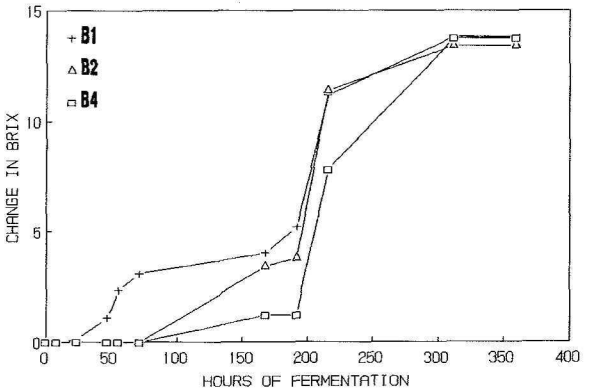

FIG. 1.—Fermentation patterns of different strains of *Z. mobilis* in blackstrap molasses 5% inoculum (v/v)

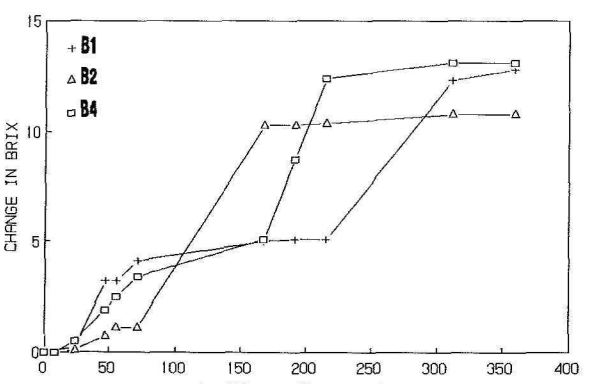

FIG. 2.—Fermentation patterns of different strains of *Z. mobilis* in blackstrap molasses 10% inoculum (v/v)

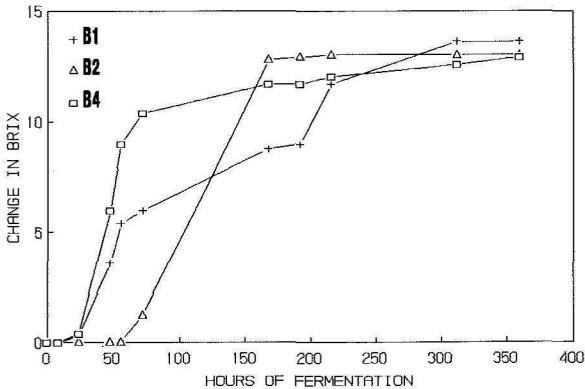

FIG. 3.—Fermentation patterns of different strains of *Z. mobilis* in high test molasses 5% inoculum (v/v)

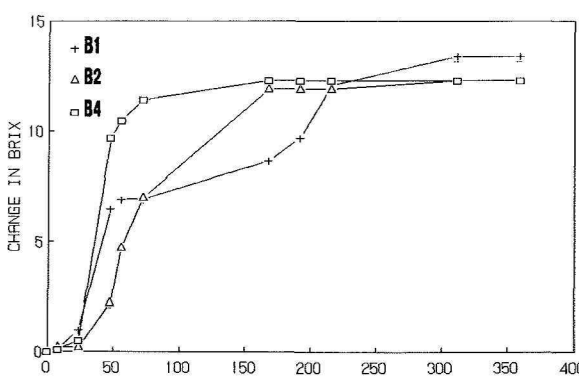

FIG. 4.—Fermentation patterns of different strains of *Z. mobilis* in high test molasses 10% inoculum (v/v)

cases. B-4 was the fastest in HTM, but problems are visualized with this strain in BM, where, with a 5% inoculum at room temperature, it did not even start. From the literature[5] it is known that this strain does not attack sucrose, which is the main constituent in BM. Sucrose utilization accomplished in BM with this strain was probably as result of the changes that occurred in the medium during this relatively long fermentation time.

The highest alcohol yields in HTM were obtained with strain B-2. In BM, strain B-1 produced higher yields but this strain showed inconsistency in BM. At room temperature and 5% inoculum B-1 showed a very poor fermentation behavior. B-2 is then our choice to continue our investigation; it showed a slight but consistently better performance in cane molasses.

In this present investigation three strains of *Z. mobilis* were tested for their ability to ferment HTM and BM. Since there is remarkably little published material available for the performance of *Zymomonas* in molasses, these initial experiments introduced our investigation to the field and offered valuable information for a better understanding of these organisms in our molasses substrate.

The data collected permitted us to design the experimental approach to be followed in our efforts to optimize the process. It is hoped that two major limitations will be overcome: substrate inhibition and by-product formation.

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[5]Breed, R. S., E. G. D. Murray and N. R. Smith, Bergey's Manual of Determinative Bacteriology. The Wilkins & Wilkins Co.