Research Note

ZYMOMONAS MOBILIS *BATCH AND FED-BATCH FERMENTATION OF HIGH TEST MOLASSES*[1]

Bacterial ethanol fermentations with strains of *Zymomonas* have been found to offer many advantages over traditional yeast fermentations. These include a higher specific rate of sugar uptake and ethanol production, and improved yields.[2] Current literature describes experimentation with chemically defined media containing sucrose, glucose or fructose but little is known of *Zymomonas* performance in raw material of commercial interest such as molasses. Therefore, research was initiated at the Rum Pilot Plant[3] to ascertain the causes of the poor performance of *Z. mobilis* in HTM and BM, and to find ways to improve this performance.

Silman overcame the inhibition of growth and ethanol production by *Zymomonas mobilis* at concentrations of glucose greater than 8% by using a fed-batch process.[4] Our previous experience[3] pointed to the need of a more dilute molasses substrate. Our present investigation deals with the desirability of diluting our high test molasses mash through a similar fed-batch system. Growth curves of *Zymomonas mobilis* in high test molasses, sucrose, and glucose media are also presented.

Bacterial seed (1L) was prepared in each case by successive transfers of *Zymomonas* strain ATCC 29191 (B-2) from stock culture to 10 ml 7% glucose media, to 100 ml 7% glucose media, and to 900 ml high test molasses (HTM) diluted to 8° Brix supplemented with 0.5% each of ammonium sulphate and dehydrated yeast extract. The glucose-defined media also contained 1% yeast extract. These transfers were grown batchwise, static at 32° C, each for 16 to 24 hours. A second one liter seed was built up in the same way but with glucose media including the last transfer to the 900 ml amount.

Batch and fed-batch fermentations were conducted in 14-liter New Brunswick Magna Ferm Fermentors.[5] The seed (1L) was placed in the fermentor, 9 liters of 18° Brix mash was added, either batchwise or fed continuously, with a metering pump not exceeding a 12° Brix reading inside the fermentor to prevent inhibitory effects. Approximately 18% sugar was fed for a goal of 8 to 9% alcoholic yield. Agitation was set at

[1]Manuscript submitted to Editorial Board 21 October 1987.

[2]Rogers, P. L., K. J. Lee, M. L. Skotnicki and D. E. Tribe, 1981. Ethanol production by highly productive strains of *Zymomonas mobilis*. Advances in Biotechnol., Pergamon Press; Eds, M. Moo-Young and C. W. Robinson, Vol. 2 pp. 189–94.

[3]Murphy, N. F. de, 1987. Batch fermentation patterns for different strains of *Zymomonas* in high test molasses and blackstrap molasses. *J. Agric. Univ. P. R.* 72 (3): 00000.

[4]Silman, R. W., 1984. Ethanol production by *Zymomonas mobilis* in fed-batch fermentations. *Biotechnol. and Bioeng.* XXVI, pp. 247–51.

[5]Trade names are used solely for the purpose of providing specific information. Mention of trade names does not constitute a guarantee or warranty by the Agricultural Experiment Station of the University of Puerto Rico or an endorsement over other preparations not mentioned.

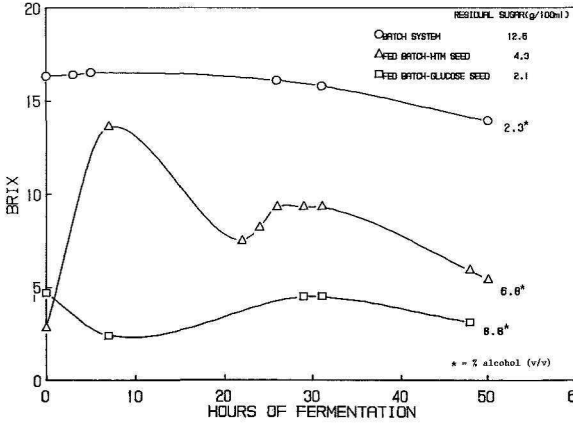

FIG. 1.—Batch and fed-batch fermentations of high test molasses by *Z. mobilis*.

150 r/m at 30° C. Periodic °Brix readings were recorded. Residual sugars and alcohol percentage were determined according to the methods of the Rum Pilot Plant.[6]

Figure 1 presents the results. Faster fermentation rates and higher alcohol yields were attained with the fed-batch system than with batch fermentation run at approximately 18% sugars. The fed-batch fermentations using solely glucose synthetic media for bacterial seed preparation gave the best results at conditions studied: 8.8% alcohol yields in 48 hours vs 2.3% in the batch system. Sugar was not fermented to completion in the 48-hour fermentation period; thus there was still capacity for improving the alcohol yield.

Preparing the seed in HTM reduced the fermentation rate. At the 48-hour fermentation period the sugar available was still too high, 4.3% with a 6.8% alcohol yield. Evidently, even at 8° Bx dilution there are inhibitors in molasses which affect *Zymomonas* growth and consequently the activity of the seed.

A comparative study on growth of *Zymomonas* in three different media provided information on optimal growth time for its use as seed and presented awareness of difficulties due to substrate composition.

All media were prepared for an initial sugar content of approximately 8%:

8% glucose 1% yeast extract	Medium 1
8% sucrose 1% yeast extract	Medium 2
HTM diluted to 8° Bx 0.5% yeast extract	Medium 3

Microorganisms were inoculated in duplicate tubes containing 6 ml of the different media under study, grown for 12 to 16 hours, and inoculated again in duplicate

[6]Manual de Métodos Analíticos de la P.P.R., Esta. Exp. Agric., Recinto de Mayagüez, Univ. P. R., Río Piedras, P. R.

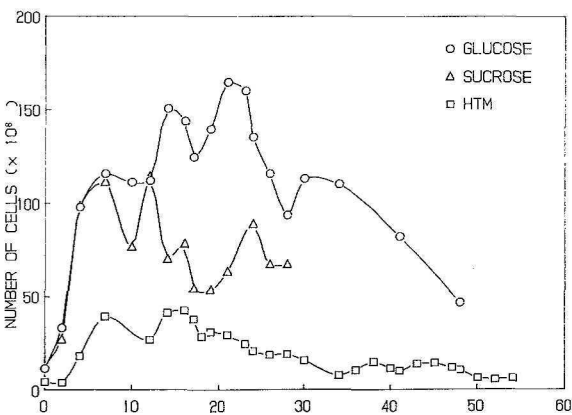

FIG. 2.—Comparative growth of *Zymomonas mobilis* in different sugar sources.

flasks containing 35 ml of the corresponding media. After 12 to 16 hours growth period, flasks containing 300 ml of the same broth were inoculated. All samples were incubated at 32° C. Bacteria were counted at the time of inoculation and every 2 hours for 48 hours. Counts were conducted on a Lumac Biocounter[6] where rapid measurement was attained by assay of ATP, which is present in all viable bacteria. The technique is based on a light producing reaction with a luciferase enzyme derived from fireflies.[7] Initial and final sugar and alcohol yield were analyzed by the methods in the Manual de Métodos Analíticas de la Planta Pilot de Ron.[8] 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.

Rapid growth and sustained viability was obtained in glucose broth (fig. 2). Cells were at their optimum at 14 to 22 hours, after which time cells decreased. Fermentation of glucose proceeded quantitatively with 97% efficiency (table 1). Growth in sucrose broth yielded lower counts than in an equivalent concentration of glucose; fermentation efficiency was also lower, 84%. The literature reported that some strains of *Zymomonas* produce levan from sucrose.[8] Since sucrose is one of the main sugars present in high test molasses, this sucrose content might be the cause for the lower alcohol productivity. Cell yield was also lower in HTM than in the defined media with equivalent concentrations of glucose and sucrose. Inhibited growth in the molasses was ob-

[7]Stanley, Philip, Rapid measurements of bacteria by ATP assay. Laboratory Equipment Digest, February 1982.

[8]Dawes, E. A., D. W. Ribbons, 1966. Sucrose utilization by *Zymomonas mobilis*: Formation of levan. *Biochem. J.* 98: 804–12.

TABLE 1.—*Sugar conversion to alcohol by* Zymomonas mobilis *in different sugar sources*

Fermentation parameters	Glucose	Sucrose	HTM molasses
Initial Sugar g/100 ml	8.54	8.73	7.41
Residual Sugar g/100 ml	0.64	1.42	0.75
% Alcohol v/v	5.1	4.34	3.59
% Efficiency	97.5	84	79

served from the beginning, when cell yields at 4 and 7 hours were approximately 1/4 of those grown in glucose media. These cell yields never attained values obtained in the other two defined media. Apparently cells were affected by other molasses components that interfered with their performance. This evident inhibition of *Zymomonas mobilis*, strain B-2, in HTM accounts for the slow fermentation rate observed.

A fed-batch system could decrease inhibition problems in fermentation of HTM by *Zymomonas mobilis*, but together with this technique other alternatives such as molasses desalting will be necessary to reduce the inhibitors and increase rate and productivity. Strains improved by mutation and adaptation may also help to attain a more efficient fermentation procedure.

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