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FERMENTATION YIELDS

Factors Influencing the Production of Polyhydric Alcohols by Osmophilic Yeasts

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Certain osmophilic yeasts produce considerable quantities of glycerol, erythritol, and D-arabitol during normal growth. A culture producing good yields of glycerol and D-arabitol was grown successfully in 5-liter stainless steel fermentors. Satisfactory yields of glycerol and D-arabitol were obtained using a glucose–yeast extract–urea medium, but corn steep liquor could be substituted for yeast extract if higher concentrations of urea were used. Increased rates of aeration decreased the rate of glucose utilization and the yield of ethyl alcohol and increased the glycerol yield, while the yield of D-arabitol was not affected by changes in aeration. Increasing the fermentation temperature, to 37° C., increased the yield of glycerol and the rate of glucose utilization. The initial glucose concentration could be raised to 30% without decreasing the amount of glucose converted to polyhydric alcohols. Ratios of glycerol and D-arabitol produced to glucose metabolized of 0.29 and 0.31 gram per gram, respectively, giving a combined yield of 0.60 gram of polyols per gram of glucose, have been obtained.

Fermentative glycerol production of yeast "steered" with sulfite or alkali has been known for some time. However, the use of these salts increases the cost of the product, both by increasing the cost of materials and by increasing the difficulty of recovery of glycerol, so there is still interest in unsteered fermentative processes for glycerol production.

Several organisms have been found to produce glycerol during normal fermentation (*4, 6, 8*). According to these reports, *Zygosaccharomyces acidifaciens*, a yeast isolated by Nickerson (*7*) from sour wine, possessed the most desirable characteristic—i.e., production of good yields of glycerol, in the absence

of sulfites or alkalies, from glucose in moderately concentrated solutions. Preliminary experiments in these laboratories by Neish, Blackwood, and coworkers showed that some similar but more strongly osmophilic yeasts, classified by Lodder and Kreger-van Rij (*3*) as *Saccharomyces rouxii* and *Saccharomyces mellis*, also produced polyhydric alcohols. These organisms grew more rapidly in concentrated media and produced higher yields of polyols than did *Z. acidifaciens* (*10*), and they were selected for further investigation.

Studies of one of these strains showed that apparent yields of glycerol, as determined by periodate oxidation, of nearly 50% of the glucose fermented could be obtained. Quantitative recovery of the glycerol showed that these estimates were actually far too high, and further investigations showed that high yields of D-arabitol were produced as well. A survey of several osmophilic yeasts showed that erythritol and mannitol were also produced by some strains, and a preliminary study of polyhydric alcohol production was made by Spencer and Sallans (*10*).

These polyhydric alcohols have not previously been reported as products of yeast fermentations, and since erythritol and D-arabitol are at present relatively scarce compounds, a study was begun of factors influencing their production in stirred fermentors. The results obtained with a strain producing glycerol and D-arabitol, and results of early studies of erythritol production are reported.

Materials and Methods

Fermentations. The yeast strain used was isolated from pollen from a sample of brood comb. This strain was the same as was used in earlier studies (*10*) of polyhydric alcohol formation.

The fermentors were the 5-liter stainless steel fermentors used in the study of the *Ustilago zeae* fermentation (*9*). A volume of 3 liters of medium per fermentor was used at all times. Glucose was used as the substrate, and nitrogenous materials were added to the glucose medium as specified in the different experiments. Unless otherwise stated, the initial glucose concentration was 20%.

Inoculum for the small fermentors was prepared by inoculating 150 ml. of 20% glucose plus 1% of yeast extract medium in a 250-ml. Erlenmeyer flask, either from a previous culture flask or from a tube of liquid culture and incubating the flasks for 2 to 3 days on a rotary shaker, eccentricity 0.5 inch, at 160 r.p.m. A 5% inoculum was used. For three of the later experiments, 500-ml. Erlenmeyer flasks, and a shaker having an eccentricity of 1 inch, operating at 230 r.p.m., were used. A more vigorous inoculum was obtained, in 2 days or less.

Table I. Effect of Yeast Extract and Calcium Carbonate on Polyhydric Alcohol Production[a]

$CaCO_3$, %	Yeast Extract, %	Fermentation Time, Days	Total Polyols as Glycerol, Mg./Ml.	Final pH
0.75	0.5	17	37	6.6
0.75	0.75	7	53	6.1
nil	0.75	7	56	4.2
nil	1.0	7	77	4.1

[a] Basal medium 20% glucose plus 0.114% urea. Sulfite oxidation rate 26 mmoles O_2/liter/hour, temperature 30° C.

Analytical Methods. The analytical methods used were those described by Neish (*5*), except where otherwise specified.

Reducing sugars were determined colorimetrically with alkaline copper reagent and arsenomolybdate reagent.

Polyhydric alcohols were determined by oxidation with periodic acid and colorimetric determination of the formaldehyde thus formed with chromotropic acid reagent. When glycerol and D-arabitol were not separated and determined individually, the yield was calculated as glycerol.

Ethyl alcohol was determined by oxidation with acid potassium dichromate in a Conway microdiffusion cell, and excess dichromate was determined with potassium iodide and sodium thiosulfate.

Glycerol and D-arabitol were separated by partition chromatography on water-Celite columns using a 25% benzene–75% butyl alcohol mixture, saturated with water, as a developing solvent.

Paper chromatograms were used to check the progress of the fermentations qualitatively. A butyl alcohol–acetic acid–water mixture (5 to 1 to 2 by volume) as described by Hough (*1*) was used as a developing solvent, and sugars and polyhydric alcohols were detected with alkaline silver nitrate by the procedure of Trevelyan, Procter, and Harrison (*11*).

Recovery of Polyhydric Alcohols. The broth was clarified with charcoal, water was removed by distillation, and ethyl alcohol was added in a 5 to 1 ratio by volume. This precipitated most of the polysaccharide gums. The preferred recovery method was then to allow D-arabitol or erythritol and mannitol, according to the products obtained, to crystallize from ethyl alcohol solution. The amount of water and glycerol present affected the recovery of other polyhydric alcohols considerably. Details of the recovery methods will be published later.

Table II. Effects of Yeast Extract, Corn Steep Liquor, and Aeration Rate on Polyhydric Alcohol Production[a]

Yeast Extract or Corn Steep Liquor, %		Sulfite Oxidation Rate, Mmoles O_2/Liter/Hour	Rate of Glucose Utilization, G./Liter/Day	Total Polyols as Glycerol, Mg./Ml.	Final pH
Yeast extract	1.0	26	40	48	4.2
		37	35	80	4.1
		57	37	79	4.2
Corn steep liquor	1.5	26	39	52	3.3
		37	38	55	3.4
		57	35	53	3.3
	2.0	26	57	24	3.9
		37	54	25	3.6
		57	46	31	3.5
	1.4	68	28	48	5.7[b]

[a] Basal medium 20% glucose plus 0.114% urea, temperature 30° C.
[b] Calcium carbonate added in excess.

Table III. Effect of Corn Steep Liquor and Urea on Polyhydric Alcohol Production[a]

Corn Steep Liquor, %	Urea, %	Fermentation Time, Days	Total Polyols as Glycerol, Mg./Ml.
0.8	0.20	8	85
0.8	0.26	8	87
1.0	0.20	7	78
1.0	0.26	6	82

[a] Basal medium 20% glucose. Sulfite oxidation rate 72 mmoles of O_2/liter/hour, temperature 30° C.

Results and Discussion

The data in Tables I and II are from individual fermentors. Those in Tables III to VI are averages of three replicates.

Effects of Medium Composition. Data in Table I show the effect of increasing the concentration of yeast extract. The time of fermentation was greatly reduced when the concentration of yeast extract was increased to 0.75%, but the highest yield of polyhydric alcohols was obtained when the yeast extract concentration was 1%.

Yeast extract, however, is impractical as a source of nitrogen and growth factors for large scale fermentations, so it was desirable to replace it with corn steep liquor. When high concentrations of corn steep liquor were used (Table II), the rate of glucose utilization was satisfactory, but the yield of polyols was very low. Reduction of the concentration of corn steep liquor resulted in slow fermentations and a low increase in yield. Reducing the concentration of corn steep liquor and increasing the urea concentration produced the desired results (Tables III and IV). A corn steep liquor concentration of 0.8% and urea concentration of 0.26 to 0.32% gave satisfactory yields and fairly rapid fermentations.

In glycerol production by *Saccharomyces cerevisiae* fermentation, the yield of glycerol increases as the pH of the fermentation is raised. As the pH of media containing relatively high concentrations of corn steep liquor was low, calcium carbonate was added to a fermentation containing 1.4% corn steep liquor. The final pH of the fermentation was raised from about 3.5 (Table II) to 5.7, but there was no increase in the yield of polyols. Similarly, when calcium carbonate was added to a medium containing yeast extract (Table I) the final pH was raised from 4.2 to 6.1 without affecting the polyol yield. Under the conditions of these experiments, then, the pH of the medium probably had little effect on the yield of polyhydric alcohols.

Effect of Aeration. It had been noted (*10*) that the osmophilic yeasts showed a marked Pasteur effect. Quantitative studies of the relationship between glycerol production and rate of aeration, as measured by the sulfite oxidation method, showed (Table V) that the glycerol yield increased with the aeration rate, up to a sulfite oxidation rate of 38 mmoles of oxygen per liter per hour. Further increases in aeration rate had no effect on the polyol yield, though the yield of ethyl alcohol and the rate of glucose utilization continued to decrease. The yield of D-arabitol was unaffected by changes in the rate of aeration.

Effects of Temperature and Initial Glucose Concentration. The effects of these two factors are interrelated. Lochhead and Heron (*2*) found that their cultures of osmophilic yeasts failed to grow at 37° C. in media of low sugar concentration. When higher concentrations of growth factors and nitrogen sources were used, as in these experiments (Table VI), the maximum temperature for growth in low glucose concentrations was shifted upward, the rate of glucose utilization increasing as the temperature was raised to 37° C. At a temperature of 40° C. the phenomenon observed by Lochhead and Heron was again encountered. No growth took place in fermentations at 40° C. or higher when the initial glucose concentration was 20%, though living yeast cells were recovered after 4 days from these fermentations. At initial glucose concentrations of 30 and 35%, growth took place, although the rate of glucose utilization was slower and decreased to zero when the glucose concentration fell to 12 to 15%. The higher absolute concentration of growth factors and nitrogen sources may also have influenced the behaviour of the organisms.

The changes in the yields of polyols were obscured somewhat by the presence of substances reacting as glycerol, but not yet identified. These substances, which have higher R_f values than glycerol (*11*), are reported in Table VI as "Fraction 1." In previous experiments and in Experiments I and II, Table VI, the concentrations of these unknowns were low and were not reported, but in Experiments III to V a more vigorous culture, resulting from a change in the method of preparing the inoculum, was used, and a larger yield of compounds other than glycerol and D-arabitol was obtained. In addition, paper chromatograms showed that a trace of glycerol appeared in Fraction 1 when the contaminating unknowns were present in larger quantities, so that the figures reported in that column include a little of the glycerol as well.

In Experiments I and II, Table VI, the final concentration of D-arabitol remained almost constant, while the increases in total polyol yields with increasing temperature and glucose concentration were almost entirely due to increased glycerol production. In Experiments III to V the effects of temperature changes were not so well defined. Increases in total yield were apparently due to increases in concentrations of D-arabitol. Increases in yield with increasing glucose concentration were due to increases in glycerol yield and yield of the unknown Fraction 1.

The highest yields of glycerol and D-arabitol as a percentage of the glucose utilized were obtained with the less vigorous culture, where a polyol-glucose ratio of 0.60 was obtained. Lower percentage yields were obtained in other experiments. As the initial glucose concentration was increased, the percentage yield of glycerol and D-arabitol

Table IV. Effect of Urea Concentration on Polyhydric Alcohol Production[a]

Sulfite Oxidation Rate, Mmoles O_2/Liter/Hour	Urea, %	Fermentation Time, Days	Total Polyols as Glycerol, Mg./Ml.
72	0.26	6	81
72	0.32	6	88
74	0.26	6	84
74	0.32	6	88

[a] Basal medium 20% glucose plus 0.8% corn steep liquor, temperature 30° C.

Table V. Effect of Aeration on Polyhydric Alcohol Production[a]

Sulfite Oxidation Rate, Mmoles O_2/Liter/Hour	Fermentation Time, Days	Ethyl Alcohol (at 5 Days), Mg./Ml.	Total Polyols as Glycerol, Mg./Ml.	Final Glycerol, Mg./Ml.	Final D-Arabitol, Mg./Ml
5	5	58	31	10	40
12	5	28	61	30	50
38	6	2	87	62	46
74	7	nil	86	..	..

[a] Medium 20% glucose plus 0.8% corn steep liquor plus 0.35% urea, temperature 30° C.

decreased. If the unknown contaminating product was included, however, the yield was somewhat higher, the ratio of total polyols to glucose utilized being about 0.5 rather than 0.36.

Production and Recovery of Erythritol. As the strains of osmophilic yeasts which produce erythritol were found to differ in some of their cultural characteristics from those producing high yields of D-arabitol, studies of factors influencing erythritol production were begun. Preliminary results showed that all the glucose in a 20% solution was utilized in about 7 days, using concentrations of yeast extract and urea of 1.3% and 0.3%, respectively, a sulfite oxidation rate of 58 mmoles of oxygen per liter per hour, and a fermentation temperature of 30° C. The rate of aeration was similar to that used for strains producing D-arabitol, but the concentrations of yeast extract and urea required were considerably higher. Good yeast growth was obtained if the temperature was raised to 37° C., but the yield of erythritol was reduced to a very low figure.

The fermentation liquor analyzed 4.4% erythritol, most of which could be recovered by procedures similar to those used for D-arabitol. A small quantity of mannitol was recovered during the process. Very little glycerol was produced by this strain. More detailed studies of the factors influencing the production of erythritol are in progress, and the results of these investigations will be published later.

Table VI. Effects of Temperature and Initial Glucose Concentration on Polyhydric Alcohol Production[a]

Initial Glucose Concn., Mg./Ml.	Temperature, ° C.	Rate of Glucose Utilization G./Liter/Day	Total Polyols as Glycerol, Mg./Ml.	Fraction 1 as Glycerol, Mg./Ml.[b]	Glycerol, Mg./Ml.	D-Arabitol, Mg./Ml.	Polyol/Glucose Ratio
			Experiment I				
200	25	11[c]	29	..	...	..	...
200	30	24	76	..	39	61	0.45
200	33.5	29	96	..	57	65	0.55
200	37	42	102	..	64	70	0.60
			Experiment II				
200	30	20	91	..	...	..	...
230	30	23	97	..	73	55	0.50
260	30	26	117	..	85	55	0.48
290	30	29	127	..	103	51	0.48
			Experiment III				
200	30	29	80	17	40	56	0.48
200	37	40	92	13	36	63	0.50
200	40	0	...	..	...	..	...
200	43	0	...	..	...	..	...
			Experiment IV				
300	30	47	119	30	63	49	0.37
300	37	50	130	21	60	85	0.48
300	40	28[c]	70[d]	..	...	..	...
			Experiment V				
350	30	49	128	42	60	64	0.36
350	37	64	144	47	59	65	0.36
350	40	30[c]	80[d]	40	24	34	0.29

[a] Ratio of glucose to corn steep liquor and urea 2000:75:35. Sulfite oxidation rate, 62 mmoles O_2/liter/hour.
[b] First fraction from Celite-water column used in separating glycerol and D-arabitol. Composition unknown.
[c] Glucose utilization stopped after glucose concentration fell to 120 to 150 g./liter.
[d] Determined after active glucose utilization ceased.

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