GLYCEROL PRODUCTION OF VARIOUS STRAINS OF *SACCHAROMYCES*

F. Radler and H. Schütz

Institut für Mikrobiologie und Weinforschung der Johannes Gutenberg-Universität Mainz, Ernst-Ludwig-Strasse 10, D-6500 Mainz, Germany

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ABSTRACT

The quantity of glycerol as principal by-product of the alcoholic fermentation depends to a large extent on the yeast strain. Different strains of *Saccharomyces cerevisiae* were found to form amounts of glycerol varying between 4.2 to 10.4 g/L. The formation of glycerol is regarded as a result of the competition between alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase that compete for the reduced coenzyme $NADH_2$. High and low glycerol forming yeast strains showed large differences in the activity of glycerol-3-phosphate dehydrogenase and only small variations in the activity of alcohol dehydrogenase. The total amount of glycerol formed was also influenced by amino acids. In thiamine deficient media a decrease in glycerol formation was observed. Experiments indicate a correlation between the formation of acetaldehyde and glycerol and the production of cell mass that may be of practical interest.

Glycerol is by far the most important secondary product of fermentation. The glycerol content of wine may be of two different origins. A certain amount of glycerol is always formed by yeasts. Occasionally, glycerol is already present in the grape must, as has been observed by Mühlberger and Grohmann (7). This glycerol is formed by *Boyrytis cinerea,* a fungus which frequently attacks grapes when they are produced in humid climates. The amount of glycerol formed by yeasts is generally assumed to be in the range of 1/10 or 1/15 of the alcohol formed (11). The formation of glycerol is not constant but depends on various factors. Early observations date back to the last century. Because of the sweet taste that is similar to glucose (11), a high content of glycerol may have a favorable effect on the taste of wines.

Besides the yeast strain, such factors as oxygen, fermentation temperatures, and pH have been reported to influence the formation of glycerol. Within the "normal" range of conditions these factors are obviously not very important, particularly when the range of the pH is kept between 2.8 to 5.0.

There is no doubt that the formation of glycerol does not only depend on the yeast strain, but also to a large extent on the composition of the fermentation medium. It is the purpose of this paper to show how environmental factors and characters of yeast strains influence the formation of glycerol during fermentation.

MATERIALS AND METHODS

Cultures: The strains of the species *Saccharomyces cerevisiae* were all from the collection of this institute.

Culture media: a) *B-Medium* (6) modified as follows was used for most experiments: glucose 100 g, (or as indicated); inositol, 0.04 g; KH_2PO_4, 1 g; ammonium sulphate, 1.5 g; $MgSO_4 \times 7H_2O$, 1 g; $CaCl_2$, 0.5 g; potassium hydrogen tartrate, 4.5 g; L-alanine, 75 mg, L-arginine-HCl, 350 mg; L-histidine-HCl, 20 mg; L-methionine, 40 mg; L-serine, 50 mg; L-threonine, 200 mg; L-tryptophane 40 mg; L-aspartic acid, 50 mg; glutamic acid, 300 mg; 4-aminobenzoic acid, 0.2 mg; biotin, 0.02 mg; folic acid, 0.02 mg; nicotinic acid, 1 mg; Ca-D-pantothenic acid, 1 mg; pyridoxolium chloride, 1 mg; riboflavine, 0.5 mg; thiaminedichloride, 0.5 mg; boric acid, 2mg; $FeCl_3 \times 6H_2O$, 2 mg; $ZnSO_4 \times 7H_2O$, 2 mg; $MnSO_4 \times 1\ H_2O$, 2 mg; $AlCl_3$, 2 mg; KI, 1 mg; $CuSO_4 \times 5H_2O$, 1 mg; $Na_2MoO_4 \times 2H_2O$, 1 mg; $CoCl_2 \times 6H_2O$, 1 mg; $Li_2SO_4 \times 2H_2O$, 1 mg; H_2O is added to a total volume of 1000 mL, pH 3.2.

b) *YEP-Medium:* yeast extract, 2 g; pepton, 20 g; KH_2PO_4, 1 g; glucose, 200 g; H_2O added to a total volume of 1000 mL, pH 3.2.

c) *grape must (cultivar Müller-Thurgau):* glucose, 66 g/l; fructose, 63 g/L; total acid, 13.7 g/L; malic acid, 8.3 g/L; glycerol, 0.7 g/L; pH 3.2).

Fermentation experiments: Yeast cells were cultivated in 500 mL Erlenmeyer flasks with fermentation closures containing 200 mL medium at 25°C on a circular shaker (150 rpm) until the CO_2 production ceased. For enzymatic experiments 5 L Erlenmeyer flasks with fermentation closures containing 4 L medium were used. The medium was sparged with O_2-free nitrogen gas for 15 min before and after inoculation. As inoculum, 5% of cells grown anaerobically for 48 hr were used, unless indicated otherwise.

Enzyme preparation: The yeast cells were collected by centrifugation at the end of the exponential growth phase. They were washed twice in triethanolamine-HCl buffer, pH 7.6, and suspended in the same buffer. The suspension was shaken in a refrigerated ball mill Braun MSK with glass beads (0.45 – 0.5 mm diameter). Twenty gram cells (fresh weight), 20 mL buffer and 75 g glass beads were homogenized for 90 s. After decanting, the beads were washed with 20 mL buffer. The liquids (homogenate) were pooled.

Analytical determinations: Protein was determined by the biuret method. The assay of glycerol was performed by the enzymatic method of Eggstein and Kuhlmann (5) with glycerokinase. Glucose and fructose were determined enzymatically with hexokinase, glucose-6-phosphate-dehydrogenase, and phospho-glucose-isomerase (4). Ethanol was determined with alcohol dehydrogenase (2). Pyruvate was assayed by the enzymatic method of Czok and Lamprecht (3). Acetaldehyde was determined by a colorimetric method using 3-methyl-2-benzo-thiazolon-hydrazon as reagent (12).

Enzyme determinations: Specific activities are expressed as μMol substrate converted per mg protein and minute. Alcohol dehydrogenase (E.C. 1.1.1.1.) was determined according to Bergmeyer et al. (1). For the determination of glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8.), the method of Nader et al. (8) was modified. The reaction was carried out in 20 mM imidazole/HCl buffer, pH 7.0 containing 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM NADH, 3 mM fructose-1.6-diphosphate, 0.9 U aldolase and 100 U triosephosphate isomerase.

Chemicals: All enzymes, co-enzymes and pyruvate were purchased from Boehringer-Mannheim. All the other chemicals were purchased from Merck-Darmstadt.

RESULTS AND DISCUSSION

It is well established and documented by the work of Rankine (9) that differences exist in the amount of glycerol formed by various yeast strains during fermentation. In our own experiments with 23 different yeast strains, B-medium and comparable conditions of fermentation, even within the species *Saccharomyces cerevisiae*, a considerable strain variation was observed. The average amount was 5.9 g glycerol per L, the extreme values were 4.2 and 10.4 g glycerol per liter.

When the glycerol formation of seven yeast strains was compared in three different media, the highest amounts of glycerol were produced in grape must (Table 1). The glycerol formation in YEP-medium and B-medium were similar. In spite of the variation of the total amounts of glycerol, the yeast strains seemed to show a similar behavior. At least the highest and lowest amounts of glycerol were formed by the same strains in all three media.

It was assumed that the amount of yeast cells used as inoculum might influence the glycerol production of yeasts. In one experiment that was carried out with three different yeast strains the inoculum was varied from .25% to 5% (about $5 \cdot 10^5$ to 10^7 cells per mL of the final volume). No significant differences in glycerol formation were observed.

Table 1. The production of glycerol by several strains of *Saccharomyces cerevisiae* during the fermentation of different media. (Culture conditions: 200 mL medium in a 500 mL Erlenmeyer flask with fermentation closures. Incubation at 25°C on a circular shaker. YEP-medium and B-medium contained 20% glucose, grape must 14% reducing sugar. Incubation time 4 to 14 days. For all cultures 10 mL of a 48 hr culture in the same medium were used as inoculum.)

Yeast strain	Amount of glycerol formed (g/L)		
	YEP-medium	B-medium	Grape must
S. cerevisiae 35	10.4	11.6	6.3
S. cerevisiae 33	8.2	9.1	5.8
S. cerevisiae 101	6.4	7.0	4.7
S. cerevisiae 29	5.9	7.8	5.3
S. cerevisiae Wal.	5.4	7.2	4.5
S. cerevisiae 7	5.3	8.0	4.9
S. cerevisiae 93	5.1	6.7	4.4

However, when the range of the inoculum was greatly varied (1% to 100%, corresponding to ca. $2 \cdot 10^6$ to $2 \cdot 10^8$ cells per mL) the highest glycerol formation was observed with the largest inoculum (Table 2). Of course, such an inoculum is not applied in wine making. The same experiment showed another interesting result. The fermentations were carried out in Erlenmeyer flasks closed with fermentation traps. One series of flasks was left standing on the shelf whereas the other series was continuously shaken on a circular shaker at 150 rpm. Shaking resulted in a considerable increase in cell mass and glycerol formation as indicated in Table 2. With the yeast strain No. 35, the glycerol production increased from 6g to 7 g/L to 11g to 13 g/L in the agitated culture. A similar increase was observed with the second yeast strain. This effect is difficult to explain. It is unlikely to be caused by an increased availability of oxygen in the agitated cultures, for all vessels were closed with fermentation traps. In order to avoid misleading results all experiments were carried out in flasks incubated on a circular shaker.

Table 2. The influence of the amount of inoculum and the method of incubation of the fermentation vessels (standing vs. incubation on rotary shaker) on the formation of glycerol by two strains of *Saccharomyces cerevisiae* (B-Medium, incubation at 25°C, 8-10 days).

Yeast strain No.	Amount of inoculum used (%)	Final cell mass (g/L wet weight)	Glycerol formed (g/L)
Fermentation vessels not shaken			
35	1	13.0	5.8
35	10	14.0	5.9
35	100	18.5	7.1[a]
16	1	12.0	4.8
16	10	14.0	4.6
16	100	26.0	5.3[a]
Fermentation vessels incubated on rotary shaker			
35	1	20.5	11.3
35	10	19.0	10.8
35	100	30.5	13.6[a]
16	1	18.5	7.8
16	10	18.5	9.0
16	100	27.5	9.0[a]

a) Figures corrected for the amount of glycerol (about 0.1g per liter) introduced with the inoculum. The cells used as inoculum were centrifuged and resuspended in the culture medium.

Generally, more ethanol and more glycerol are formed at high than at low sugar concentrations. However, the ratio, glycerol:ethanol (expressed as g glycerol formed per 100 g ethanol) varies with the concentration of glucose. Fig. 1 shows that a minimum of this ratio is observed at 150 g glucose per L. At lower and at higher sugar concentrations comparatively more glycerol is formed. No reasonable explanation has been found for this observation.

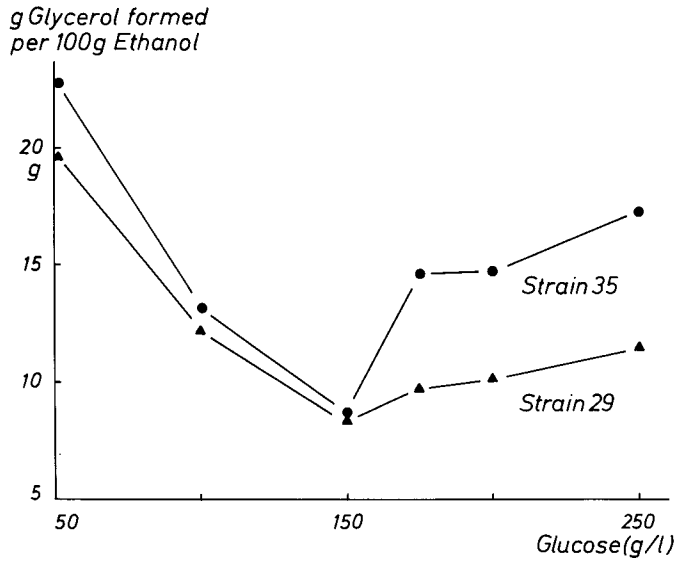

Fig. 1. The influence of glucose concentration on the amount of glycerol (g) formed per 100 g of ethanol during fermentation of B-medium by two strains of *Saccharomyces cerevisiae* (Culture conditions as indicated in Table 1).

Yeasts show considerable differences in their requirements for growth factors. However, if no particular care was taken to free even a synthetic medium from contaminating compounds, it was observed that only an omission of thiamine lowered the formation of glycerol in this experiment. This is shown in Table 3. The cell yields were similar except for pantothenic acid. When this factor was omitted, only half of the amount of cells was produced. Probably this particular yeast strain has a requirement for pantothenic acid. When the medium contained no thiamine, only about two thirds of the amount of glycerol was formed, whereas the cell yield remained in the normal range.

The quantity of the nitrogen available for yeast and the quality of the nitrogen source have a pronounced effect on yeast growth and fermentation. Therefore, some experiments were performed to investigate the influence of the nitrogen metabolism on glycerol formation by yeast. If ammonium sulphate served as a sole nitrogen source, it did not influence the glycerol formation unless the amount of nitrogen was insufficient for yeast growth, that is below about 200 mg N/L in a medium containing 200 g glucose per liter (Fig. 2). Similar observations were made if a mixture of amino acids was the nitrogen source.

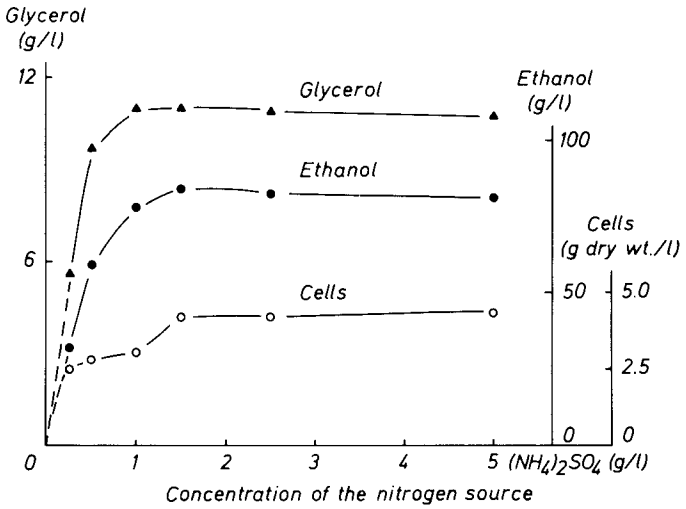

Fig. 2. The influence of the amount of nitrogen in the synthetic modified B-medium on growth and glycerol formation of *Saccharomyces cerevisiae* 35 (Culture conditions as indicated in Table 3).

If single amino acids were used as the nitrogen source for the yeasts, some gave results identical with the control which consisted of a mixture of amino acids. Alanine, asparagine, serine and valine lowered the glycerol formation (see Table 4). In another experiment it was found that if the mixture of the nine amino acids of the B-medium (alanine, arginine, histidine, methionine, serine, threonine, tryptophane, aspartic acid, glutamic acid) was the source of nitrogen, it was of little influence if one of the acids was omitted. An exception was methionine; if this compound was missing the amount of glycerol formed by the yeast decreased.

Table 3: The influence of growth factors on the formation of glycerol by *Saccharomyces cerevisiae* 35 in the modified synthetic B-medium. (Culture conditions as indicated in Table 1, except that the cells of 1 mL of a 48 hr culture were used as inoculum after being washed twice in sterile distilled water and resuspended in 0.1 M KCl/HCl-buffer, pH 3.2)

Factor omitted	Cell yield (dry weight g/L)	Glycerol formed (g/L)
None (complete medium)	7.0	13.0
4-Amino-benzoic acid	6.6	13.3
Biotin	6.5	13.8
Folic acid	6.6	12.3
Nicotinic acid	6.5	14.0
Pantothenic acid	3.3	12.5
Pyridoxine	6.9	11.2
Riboflavine	6.6	13.3
Thiamine	6.5	7.6

Table 4: Formation of glycerol by *Saccharomyces cerevisiae* 35 during fermentation in the presence of different single amino acids as nitrogen source (500 mg N/L). (Culture conditions as indicated in Table 3).

Nitrogen source	Glycerol formed (g/L)
Control (Complete B-medium)	10.4
Arginine, aspartic acid, glutamic acid, methionine or threonine	9.3 - 11.1
Alanine	7.9
Asparagine	7.8
Serine	7.1
Valine	5.9
(Incomplete fermentation with cysteine, hystidine and tryptophane)	

The relations between the metabolism of nitrogen compounds and the formation of glycerol by fermenting yeast have already been investigated by Ribéreau-Gayon et al. (10). Obviously, intricate regulatory mechanisms are involved. As stated above, alanine decreases the glycerol formation. Therefore, it was assumed that pyruvate, being a precursor or metabolite of alanine, may influence glycerol formation. However, an addition of 1 g pyruvate to 1 L medium did not affect glycerol fermentation significantly.

A detailed investigation of the course of glycerol production during growth and fermentation was made with two yeast strains. A high yielding yeast strain (No. 35) formed glycerol during the growth phase and after cessation of growth when the fermentation of glucose continued (Fig. 3). There is no indication that glycerol is primarily formed at the beginning of fermentation although the amounts of glycerol formed during the early stages of fermentation are slightly higher than during the later stages. Another yeast strain (No. 29) that formed average amounts of glycerol only, produced more cell mass and showed slower growth than the previous strain. Glycerol formation continued as long as growth occurred and sugar was fermented.

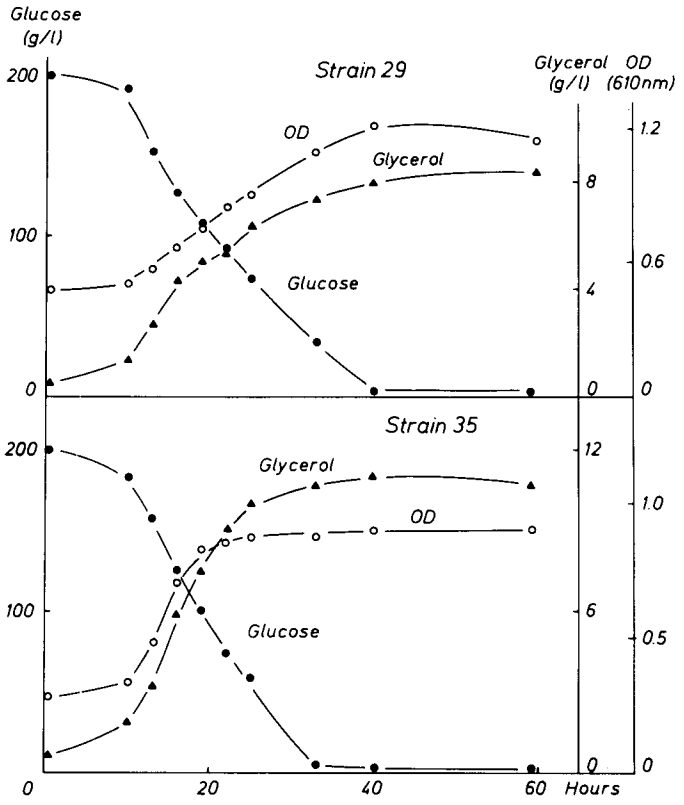

Fig. 3. Glycerol formation, sugar consumption and cell growth (OD - optical density) by two strains of *Saccharomyces cerevisiae* (strains 29 and 35) (Culture conditions as indicated in Table 1).

It is interesting to note that different amounts of pyruvate and acetaldehyde were formed by the two yeast strains (Fig. 4). The yeast strain forming small amounts of glycerol only, produced very small amounts of acetaldehyde and pyruvate during fermentation. Much more of these two metabolites were formed during fermentation by the yeast strain that produced comparatively large amounts of glycerol. However, at the end of fermentation, both strains showed similar concentrations of acetaldehyde and pyruvate. A high amount of acetaldehyde during fermentation could mean that more hydrogen (bound to the coenzyme NAD) is converted to glycerol in this strain, whereas a low concentration of acetaldehyde in the other strain could mean that this compound is reduced more rapidly to ethanol than in the other strain.

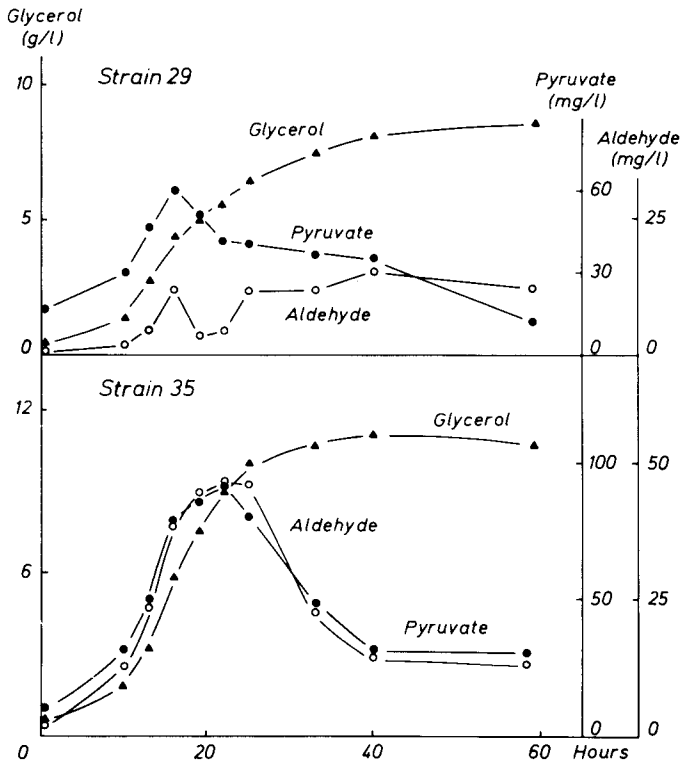

Fig. 4 Formation of acetaldehyde and pyruvate during fermentation by two strains of *Saccharomyces cerevisiae* (strains 29 and 35) Culture conditions as indicated in Table 1).

The enzymes leading directly to ethanol and glycerol-3-phosphate are alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase respectively. The latter enzyme has recently been investigated in *Saccharomyces carlsbergensis* by Nader et al. (8). This enzyme is strongly inhibited by anions, particularly phosphate. In a preliminary investigation, we observed that the content of alcohol dehydrogenase did not vary greatly in various yeast strains. The specific activity was found to be high and in the range of 2 to 8 U/mg protein. Much greater variations were found in our experiments with glycerol-3-phosphate dehydrogenase as shown in Table 5. The enzyme activities recorded in this table are mean values of two determinations. A high activity of glycerol-3-phosphate dehydrogenase was found in strain 35 that produced the largest amounts of glycerol. Hardly detectable amounts of this enzyme were observed in the strains 42 and 13 that formed very little glycerol. Not included

Table 5: A comparison of the content of glycerol-3-phosphate dehydrogenase and alcohol dehydrogenase in yeast strains differing in glycerol formation. (Culture conditions as indicated in Table 1. The yeast cells were harvested after 48 hours in the early stationary phase).

Yeast strain No.	Glycerol formed (g/L)	Glycerol-3-phosphate dehydrogenase (specific activity mu/mg)	Alcohol dehydrogenase (specific activity mu/mg)
35	12.4	245	3200
104	9.7	52	2800
29	8.4	155	5300
79	8.2	140	4400
149	8.0	46	2500
146	7.6	26	8700
13	2.8	3	1500
42	2.5	6	4000

in this table are the results of a previous study with yeast strain No. 44 that produces small amounts of glycerol, but showed an activity of glycerol-3-phosphate dehydrogenase that was higher than expected.

Too few experiments have been made to prove the hypothesis outlined in Fig. 5 that the formation of glycerol can be regarded as the result of the competition between the two enzymes alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase that compete for the reduced coenzyme $NADH_2$. Although this postulated competition of two enzymes is probably not the only factor involved in glycerol formation, it is likely that the amount of glycerol-3-phosphate dehydrogenase present in a yeast may have some importance for its capacity to form glycerol during fermentation. Of course, not only the amount of the enzyme, but also its regulation by ions or metabolites, may be significant.

If further investigations should prove that a general correlation exists between high glycerol formation, a high activity of glycerol-3-phosphate dehydrogenase and a high formation of acetaldehyde, such yeast strains may be of little value for wine fermentation where acetaldehyde is not wanted. On the other hand, yeast strains that show rapid fermentation with the development of small amounts of cell mass would be of interest. Certainly, it appears premature to draw conclusions based on the few observations presented. Perhaps it may be wishful thinking, but for wine fermentation, a yeast strain should be developed that produces a large quantity of glycerol and ferments rapidly to dryness by producing well settling yeast cells and small amounts of acetaldehyde. On the other hand, for distillery purposes a yeast strain forming abundant glycerol would be of little value if the yield of alcohol is lowered simultaneously. Genetic experiments should demonstrate the possibilities.

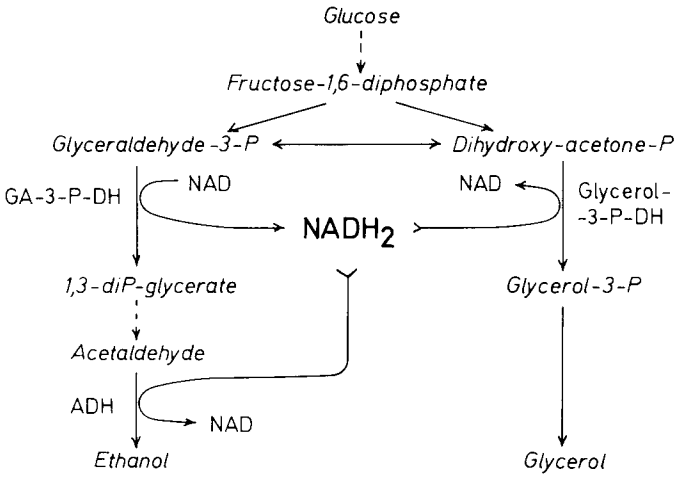

Fig. 5. Simplified scheme showing the competition of glycerol-3-P-dehydrogenase and alcohol dehydrogenase (ADH) for $NADH_2$ during the alcoholic fermentation of yeast.

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