ACTA CHEMICA SCANDINAVICA 20 (1966) 1016–1025

Yeast Growth and Glycerol Formation

KURT NORDSTRÖM

Division of Applied Microbiology, Royal Institute of Technology, Stockholm 70, Sweden

A theoretical discussion of the formation of a yeast cell from glucose, ammonium ions, sulphate and phosphate is presented. It is possible to trace the carbon skeletons of the cell constituents to a C_3-body and carbon dioxide, a procedure which facilitates a comparison with alcoholic fermentation. The latter involves no net oxidation or reduction. The biosynthesis of a yeast cell, on the other hand, was calculated to be an oxidation. Under anaerobic conditions, the only way of counteracting this oxidation is to reduce dihydroxyacetone phosphate to glycerol. The result of the calculation was that 5.0 or 7.5 mmoles of glycerol are formed per g of yeast (dry matter) synthesized dependent upon the reaction pathways considered. A few experimental results are discussed, which show that the type of theoretical approach presented is plausible.

A considerable quantity of glycerol is formed as a by-product of alcoholic fermentation. Genevois [1,2] and co-workers have shown that this formation of glycerol is caused by the redox balance of the cell. They also deduced quantitative correlations between the formation of glycerol and of other substances. However, information on one important product is lacking in their reports, namely cell substance. Even Genevois [2] has noted this omission. The purpose of this paper is to try to deduce a quantitative correlation between yeast growth and the formation of glycerol.

REDOX BALANCE IN ALCOHOLIC FERMENTATION

The reactions involved in alcoholic fermentation are shown in a simplified scheme in Fig. 1. Carbohydrate is transformed to fructose diphosphate, which is split into two molecules of triose phosphate (dihydroxyacetone phosphate and 3-phosphoglyceraldehyde). 3-Phosphoglyceraldehyde is then oxidized and transformed *via* several intermediates to pyruvate. The latter compound is decarboxylated and reduced to ethanol. There is one oxidation and one reduction per triose phosphate molecule, which makes alcoholic fermentation an inert pathway from the redox aspect.

If, however, acetaldehyde is not reduced but withdrawn in some way, the reduction step in Fig. 1 is impossible and alcoholic fermentation will be limited by NAD* deficiency (NAD is totally transformed to $NADH_2$). However,

* for abbreviations, see Ref. 3.

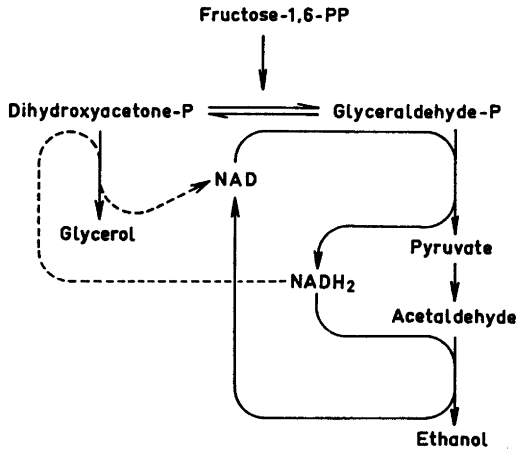

Fig. 1. Simplified scheme of glycolysis in yeast.

$NADH_2$ may be oxidized to NAD by reducing dihydroxyacetone phosphate to glycerol.[4] Hence, there is a quantitative correlation between the amount of acetaldehyde left in the medium and the formation glycerol.[1,2]

This relationship between acetaldehyde and glycerol was used on a commercial scale during the World War I in Germany.[5] By adding HSO_3^-, acetaldehyde formed an addition compound and considerable amounts of glycerol were formed. There are also other ways of disturbing the reduction of acetaldehyde to ethanol, giving rise to the formation of large quantities of glycerol.[5]

In analogous ways, correlations between other by-products and glycerol have been deduced by Genevois and co-workers.[1,2] Some of their deductions have been criticized recently [6] since they are based on the assumption that all by-products are completely formed from acetaldehyde, whereas a considerable part of the carbon atoms are derived from carbon dioxide. When this latter fact is taken into consideration, some of Genevois' coefficients must be changed.[6]

BASIC PRINCIPLES AND ASSUMPTIONS

A close study of the biosynthesis of various components of the cell shows that it is both possible and important to distinguish two sources of carbon: a C_2-body and CO_2. The C_2-body is acetate (acetyl $\sim$ SCoA) [7] or acetaldehyde (hydroxyethyl-thiamine-PP).[8,9] In some cases, this division is purely hypothetical, *e.g.* when hexoses or compounds utilizing pyruvate without decarboxylation are discussed. However, a separation of a C_2-body and CO_2 facilitates a comparison with alcoholic fermentation, since the latter yields a C_2-skeleton (ethanol) and CO_2 (Fig. 1).

Furthermore, it is convenient to assume that the C_2-body is ethanol since, in such a case, a comparison can be made with a neutral redox reaction.

In some cases a one-carbon fragment other than CO_2 is introduced. It is assumed in this paper that the fragment C_1 is formed according to eqn. 1.

$$\text{Pyruvate} \longrightarrow \text{serine} \longrightarrow \text{glycine} + \text{"formate"} \qquad (1)$$

Ehrensvärd [10] has indicated that "formate" originates from the methyl group of pyruvate, which makes $C_1 = 1/2\ C_2$.

In the following text, the biosynthesis of a yeast cell from ethanol and CO_2 is described. The calculations are performed schematically and it must be stressed that some of the biosynthetic pathways in yeast are uncertain and that there are alternative pathways for the formation of some compounds (glycine, for example, is formed in three ways and threonine in two ways, Fig. 2).

The citric acid cycle is assumed to be acyclic under anaerobic conditions, as has been shown by Vavra and Johnsson [11] for *Saccharomyces cerevisiae*. Succinate dehydrogenase, which utilizes FAD,[12] does not operate anaerobically. Succinate can be formed anaerobically *via* the citric acid cycle, but cannot be oxidized to fumarate. Hydrogen acceptors having a sufficiently high oxidation-reduction potential are not present [11] in the absence of O_2.

The cell is considered to be synthesized from carbohydrate, ammonium ion, phosphate and sulphate.

The yeast cell is assumed to contain 48 % of protein, [13,14] 35 % of carbohydrate,[14] 5 % of lipids,[15] 3 % of nucleic acids,[16] and 9 % of mineral constituents.[17] These figures are plausible for baker's and brewer's yeast, but it must be stressed that the composition may vary. Variations, however, do not alter the present discussion.

BIOSYNTHESIS OF VARIOUS COMPONENTS OF THE CELL

Proteins. The amino acid composition of the yeast cell was taken from Mojonnier *et al.*[13] (Table 1). Half the amount of aspartate and glutamate is assumed to be in the amide form.

The biochemical pathways giving rise to various amino acids are presented schematically in Figs. 2—4; for greater detail, reference should be made to Harris.[18] Fourteen amino acids are derived from pyruvate and nine of them are formed *via* oxaloacetate (Fig. 2).

Glycine may be formed in three different ways (Fig. 2); these are summarized in Table 2. Among these three pathways, the one from serine has been chosen as predominant, since the other two pathways give more extreme values of the relative contribution of the C_2-body and of CO_2 to the carbon skeleton of glycine. Vavra and Johnson [11] studied the incorporation of radioactivity into various amino acids when *Saccharomyces cerevisiae* was grown anaerobically on glucose in the presence of methyl-^{14}C-labeled acetate. The pathway from isocitrate to glycine seems to be excluded by their results which showed that very little radioactivity was incorporated into glycine from the acetate labeled with ^{14}C in the methyl group.

Threonine (and hence isoleucine and methionine) may be synthesized in two ways: *via* homoserine or *via* pyruvate. The latter pathway is analogous with the formation of leucine, lysine, and glutamate by the addition of acetyl $\sim$ SCoA to α-keto acids. Guymon *et al.*[19] have reported that two of three studied threonine deficient mutants of *Saccharomyces cerevisiae* were able to form propanol, which is a by-product of α-ketobutyrate. The latter substance can then be converted into threonine and isoleucine. In the experiment mentioned above, Vavra and Johnson [11] found that aspartate, glycine, and alanine

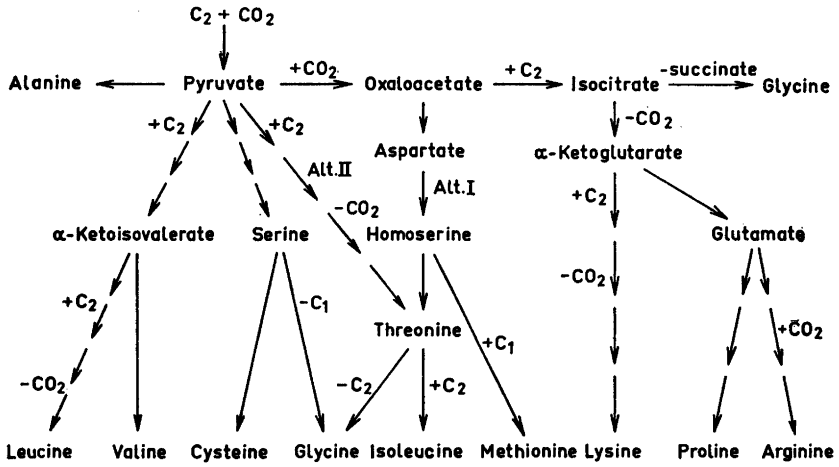

Fig. 2. Simplified scheme showing the biosynthetic pathways involved in the formation of some amino acids in yeast.

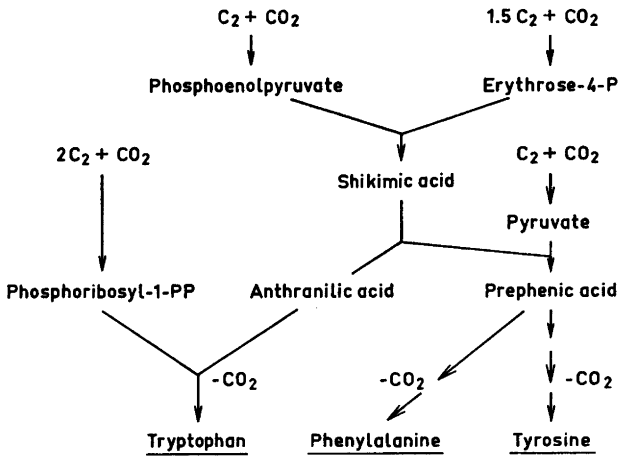

Fig. 3. Simplified scheme showing the biosynthesis of aromatic amino acids in yeast.

Fig. 4. Simplified scheme showing the biosynthesis of histidine in yeast.

were non-labeled, while glutamate had one labeled carbon atom per molecule. The specific activity of the sum of leucine and isoleucine was 0.859 labeled carbon atoms per molecule. If both these amino acids were formed by the incorporation of acetyl ~ SCoA, the label should be 1 and if only leucine formation involved acetyl ~ SCoA, the label should be about 2/3 (Table 1). The figure reported by Vavra and Johnson [11] is an intermediate of these two

Table 1. Biosynthesis of amino acids. Alternative I and II means that threonine is formed exclusively from homoserine and pyruvate, respectively.

Amino acid	Mole % in whole cells of yeast	Moles of C_2 and CO_2 in biosynthesis of 1 mole of each amino acid			
		C_2		CO_2	
		I	II	I	II
Alanine	8.3	1	1	1	1
Arginine	4.4	2	2	2	2
Aspartate	14.2	1	1	2	2
Cystein	0.2	1	1	1	1
Glutamate	12.0	2	2	1	1
Glycine	9.8	0.5	0.5	1	1
Histidine	1.8	2.5	2.5	1	1
Isoleucine	4.4	2	3	2	0
Leucine	7.8	3	3	0	0
Lysine	8.6	3	3	0	0
Methionine	1.2	1.5	1.5	2	2
Phenylalanine	3.5	3.5	3.5	2	2
Prolin	5.0	2	2	1	1
Serine	3.5	1	1	1	1
Threonine	4.9	1	2	2	0
Tryptophan	0.8	4.5	4.5	2	2
Tyrosine	2.8	3.5	3.5	2	2
Valine	6.8	2	2	1	1

Table 2. Formation of glycine. (Alt. I and II, see Table 1).

Reaction	Moles of C_2 and CO_2 in biosynthesis of 1 mole of glycine	
	C_2	CO_2
Serine → glycine + HCHO	1/2	1
Threonine → glycine + CH_3CHO, alt. I		2
» » » alt. II	1	
Isocitrate → glycine + succinate	1	

values, which may indicate that both pathways are used to synthesize isoleucine. In the following discussion the homoserine-threonine pathway is given as alternative I and the pyruvate-threonine pathway as alternative II.

It is easy to calculate from Figs. 2—4 how the carbon skeletons of the amino acids are derived from C_2 and CO_2 (Table 1). The result of such a calculation is summarized in Table 3.

As examples of the redox calculations performed, the synthesis of serine (eqn. 2) and glutamic acid (eqn. 3) are presented.

$$C_2H_5OH + CO_2 + NH_3 + NAD \longrightarrow CH_2OHCH(NH_2)COOH + NADH_2 \quad (2)$$

$$2C_2H_5OH + CO_2 + NH_3 + 3NAD \longrightarrow COOHCH_2CH_2CH(NH_2)COOH + 3NADH_2 \quad (3)$$

Thus, the biosynthesis of 1 mole of serine and glutamate gives rise to the formation of 1 and 3 moles of glycerol, respectively.

Table 3. Biosynthesis of protein, carbohydrate, lipids, and nucleic acids in yeast (alt. I and II, see Table 1).

Compounds	Occurrence in yeast (% of dry weight)	C-content (%)	C (% of dry weight) from	
			C_2	CO_2
Protein, alt. I	48	54	19.7	6.2
» alt. II	48	54	20.6	5.3
Carbohydrate	35	40	9.3	4.7
Lipids	5	77	3.8	0.1
Nucleic acids	3	33	0.7	0.3
The whole yeast cell, alt. I		45	33.5	11.3
» » » » alt. II		45	34.4	10.4

Table 4. Number of oxidation steps in biosynthesis of amino acids from ethanol and carbon dioxide (alt. I and II, see Table 1).

No. of oxidation steps	Amino acid	
	Alt. I	Alt. II
−7	Met	
−4	Cys	Cys
−3	Ileu	
−2	Thre	
−1		Met
0	Ala, Asp, Gly, Val	Ala, Asp, Gly, Val
1	Arg, Phe, Pro, Ser	Arg, Phe, Pro, Ser
2	Tyr	Tyr
3	Glu, Leu	Glu, Leu, Ileu
4	Lys, Try	Lys, Try, Thre
5	His	His

Biosynthesis of amino acids from carbohydrate and ammonium ions gives the redox results presented in Table 4. Four (two in alternative II) amino acids require reduction, ten (twelve) oxidation, while four are inert from a redox viewpoint. One of the amino acids that requires reduction, cysteine, is present in very low quantities in yeast. Thus, there is a considerable excess of oxidations; *cf.* Tables 5 and 6. Even if the amino acid composition of yeast were appreciably different from the one given in Table 1, oxidations would predominate over reductions.

Carbohydrates. The carbohydrates have been assumed to consist entirely of hexoses (Table 3). The cell wall of *Saccharomyces cerevisiae* contains 29 % glucan and 31 % mannan.[20] The pentoses that form part of the nucleic acids are accounted for within the last group of substances (see below).

The biosynthesis of carbohydrates has no influence on the redox balance of the cell (Tables 5 and 6).

Table 5. Formation of glycerol in biosynthesis of some cell constituents from ethanol, CO_2, NH_3, PO_4, and SO_4 (alt. I and II, see Table 1).

Compounds	Formation of glycerol			
	mmole/g of each substance		mmole/g of yeast (dry weight)	
	I	II	I	II
Proteins	9.1	14.4	4.4	6.9
Carbohydrates	0.0	0.0	0.0	0.0
Lipids	4.5	4.5	0.2	0.2
Nucleic acids	12.4	12.4	0.4	0.4
The yeast cell			5.0	7.5

Table 6. Biosynthesis of proteins, carbohydrates, lipids, nucleic acids and yeast (alt. I and II for proteins and cell substance, see Table 1).

$$a\ EtOH + b\ CO_2 + c\ NH_3 + d\ H_2SO_4 + e\ H_3PO_4 + f\ NAD \rightarrow CH_gO_hN_cP_eS_d + k\ H_2O + f\ NADH_2$$

Formula	Substance	Value of the coefficients								
		a	b	c	d	e	f	g	h	k
1a	Proteins, alt. I	0.38	0.24	0.31	0.003		0.21	1.61	0.28	0.59
1b	» alt. II	0.40	0.20	0.31	0.003		0.33	1.61	0.28	0.53
2	Carbohydrates	0.33	0.33					1.67	0.83	0.17
3	Lipids	0.49	0.03				0.07	1.95	0.11	0.43
4	Nucleic acids	0.34	0.32	0.39		0.11	0.41	1.28	0.71	0.71
5a	Yeast, alt. I	0.37	0.25	0.18	0.002	0.002	0.13	1.65	0.46	0.43
5b	» alt. II	0.38	0.23	0.18	0.002	0.002	0.19	1.65	0.46	0.40

Lipids. The lipid fraction of yeast consists of many groups of compounds: triglycerides, glycerophosphatides, cerebrins, sterols, and carotenoids.[17] The major part of these compounds are formed from C_2. Triglycerides and glycerophosphatides are quantitatively dominating.[21] These two groups of substances differ only with respect to one of the acids bound to glycerol, which makes the biosynthesis of them quite similar. Hence, the lipids have been represented by triglycerides in this paper (Table 3). The biosynthesis of fats implies an oxidation compared to alcoholic fermentation (Tables 5 and 6).

Nucleic acids. It was assumed that the pentose monophosphate pathway is acyclic under anaerobic conditions. The synthesis of purines and pyrimidines [18] is shown schematically in Fig. 5. The base composition was assumed to be 25 % adenine, 28 % guanine, 20 % cytosine, and 27 % uracil. This composition was reported by Crestfield *et al.*[22] for yeast RNA. RNA is the dominating nucleic acid in yeast; Enebo *et al.*[16] have reported that the yeast cell contains about 15 times more RNA than DNA.

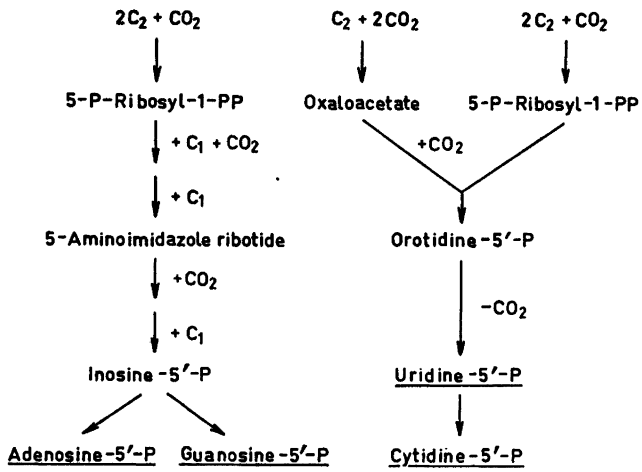

Fig. 5. Simplified scheme showing the biosynthesis of nucleotides in yeast.

The distribution between C_2 and CO_2 as carbon sources for nucleic acids is given in Table 3 and the redox conditions in Tables 5 and 6. Nucleic acid synthesis, thus, involves a considerable oxidation, compared to alcoholic fermentation.

BIOSYNTHESIS OF THE CELL

From the composition of the cell (Table 3) and the formulae 1—4 given in Table 6, formula 5 of Table 6 was calculated. This is a summarized formula for the biosynthesis of the cell. Growth involves a relatively strong oxidation compared to alcoholic fermentation; the synthesis of 1 g of yeast (dry matter) gives an excess of 5.0 and 7.5 mmoles of $NADH_2$, respectively, in the two alternatives considered. Note that formulae 1—4 account for 91 % of the dry weight of the cell. The only way to get rid of the excess of $NADH_2$ under anaerobic conditions seems to be reduction of dihydroxyacetone phosphate to glycerol (Fig. 1). Thus, yeast growth should give rise to the formation of glycerol. Battley [23] has shown experimentally that this prediction is correct. However, he has reported that much more $NADH_2$ (glycerol) is formed than according to formula 5 of Fig. 5. He has also reported that the incorporation of CO_2 is significantly lower than in formula 5, which suggests that the amino acids are almost completely derived from C_2 and none at all from CO_2; this is not plausible. Battley did not find any products other than ethanol, carbon dioxide, yeast and glycerol, which is surprising. His glycerol figures are 5 to 6 times higher than those given in Table 5.

As an illustration of the theory presented, some experimental results of the author [24,25] are given in Table 7. A synthetic medium (80 g/l of glucose, 36 mM of citrate, 5 g/l of ammonium sulphate, salts, trace elements, and vitamins) was fermented anaerobically at 20°C until the glucose was completely exhausted.[24] More than half of the amounts of glycerol formed could be accounted for by acetate and cell substance (Table 7); one mole of acetate

Table 7. Formation of glycerol in fermentation of some media.[24,25] The formation of acetate and yeast has been transformed to glycerol formation; 1 mole of acetate = 2 moles of glycerol (*cf.* eqn. 4) 1 g of yeast (dry matter) = 5.0 and 7.5 mmoles of glycerol in alternative I and II, respectively (Table 5).

Alt.	Substance	Formation of glycerol (mM)					
		Pantothenate (μM)		Biotin (μg/l)		2,4-Dinitrophenol (mM)	
		0.2	1.7	0.05	4	0.0	0.2
	Acetate	32.6	11.2	45.0	7.0	10.0	6.0
	Glycerol = G	58	48	71	51	57	30
I	Yeast	8.9	15.4	5.2	20.0	20.0	6.6
	Acetate + yeast = S	42	27	50	27	30	13
	S in % of G	72	56	70	53	53	43
II	Yeast	13.3	23.1	7.8	30.0	30.0	9.9
	Acetate + yeast = S	46	34	53	37	40	16
	S in % of G	79	71	75	72	70	53

corresponds to two moles of glycerol (eqn. 4) and the growth of the yeast can be related to glycerol formation by Table 5. It is also apparent that Battley's glycerol values are too high.

$$C_2H_5OH + 2NAD + H_2O \longrightarrow CH_3COOH + 2NADH_2 \quad (4)$$

It must be stressed, however, that although the results of Table 7 do not prove that the calculations reported in this paper are an exact representation of what occurs in practice, they do show that the calculations are plausible. To prove formula 5 of Table 6, a complete carbon and redox balance has to be drawn up.

Influence of the nitrogen source. Biosynthesis of protein contributes more to the formation of glycerol than does the formation of other constituents of the cell (Table 5). Hence, it is reasonable to assume that the formation of glycerol should be lower in a medium rich in amino acids (such as beer wort) than in a medium where ammonium ions are the sole nitrogen source. An indication that this assumption is correct is the report by Enebo [26] that the glycerol content of a Swedish beer was 21.0 mM, *i.e.* significantly less than the amounts found in a synthetic medium with the same concentration of fermentable carbohydrates (Table 7).

Acknowledgements. Financial support from *The Swedish Technical Research Council* and *Pripp-Bryggerierna AB*, Stockholm, is gratefully acknowledged.

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Received December 14, 1965.