Molar Growth Yields of Yeasts in Anaerobic Batch Cultures

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SUMMARY

Molar growth yields have been determined for *Saccharomyces cerevisiae*, two laboratory strains of *Saccharomyces carlsbergensis*, *Schizosaccharomyces pombe*, *Candida utilis* and *Metschnikowia pulcherrima* under different anaerobic growth conditions. Yeast dry weight and analyses of end products were used for calculation of Y_{ATP} . The reported data correspond to the theoretical Y_{ATP} values calculated from material-balance equations. In complex media, Y_{ATP} values averaging 11.2 and ranging from 9.2 to 14.0 were obtained shortly before glucose depletion. Lower values were observed in minimal media. Y_{ATP} were highly dependent on growth time, and seemed to decrease linearly. The data presented indicate that a change in Y_{ATP} from the accepted 10.5 to 15 to 18 may occur because of variation in time between inoculation and sampling. Previously reported Y_{ATP} values are discussed.

INTRODUCTION

The concept of Y_{ATP} (defined as gram dry weight of micro-organisms produced per mole of ATP formed) was introduced by Bauchop & Elsdon (1960), whose experiments with three different micro-organisms indicated that Y_{ATP} averaged 10.5 anaerobically in complete media with the energy source as the limiting factor. This work has been extended to other organisms (Senez, 1962; Stouthamer, 1962; Twarog & Wolfe, 1963; Chen, 1964; Hadji-petrou, Gerrits, Teulings & Stouthamer, 1964; Oxenburgh & Snoswell, 1965; Belaich & Senez, 1965; Buchanan & Pine, 1967; Forrest, 1967; Hernandez & Johnson, 1967; Korman-čikowa, Kovac & Vidrova, 1969). These observations are consistent with the assumption that the relation between growth and energy production is a biological constant for micro-organisms (Senez, 1962). Reports have appeared, however, indicating that certain bacteria do not follow this pattern (Hungate, 1963; Hobson, 1965; Hobson & Summers, 1967; Moustafa & Collins, 1968, 1969; De Vries, Kapteijn, Van der Beek & Stouthamer, 1970), and that Y_{ATP} values in the range of 15 to 23 may be obtainable.

In an earlier investigation (Lie, 1965) we noted that the observed Y_{ATP} in a malt-extract fermentation tended to decrease with time. The purpose of the present study was to examine the effect of medium composition and time of growth on the relation between growth and energy production. Yeast organisms were selected because their anaerobic energy metabolism is well known. In the reported experiments the amount of energy produced is calculated from analysis of end products only.

METHODS

Organisms. The following cultures were used: two strains of brewer's yeast, *Saccharomyces carlsbergensis* laboratory strain no. 10 and 20; *Saccharomyces cerevisiae* NCYC 240; *Schizosaccharomyces pombe*, Lindner CBS 311; *Candida utilis* ATCC 8205; *Metschnikowia pulcherrima*, Pitt & Miller (1968) laboratory strain DH-131. All were maintained on agar

slants at 5°. *Saccharomyces cerevisiae* and both strains of *S. carlsbergensis* showed an absolute requirement for biotin (Haukelí & Lie, 1971). *Schizosaccharomyces pombe* had a complex requirement, and was grown on a medium containing yeast extract. *Metschnikowia pulcherrima* and *C. utilis* required no vitamins. *Candida utilis*, however, needed an addition of 0.1 mmole O₂/h. when grown in a minimal medium. In a complete vitamin medium addition of trace amounts of oxygen were not necessary.

Media, fermenter, inoculum and measurements of dry weight. These have been described (Haukelí & Lie, 1971).

Analytical methods. Ethanol was determined quantitatively by a simple gas-chromatographic technique (Lie, Haukelí & Gether, 1970). The directly injected samples were separated on a 'Porapac Q' column (1.5 m. × 3.2 mm.) at 135° with acetone as internal standard. Total time of one analysis was 3 min., and the observed variation coefficient was less than 1%. Cellular nitrogen was determined by a semi-micro Kjeldahl method. Glycerol was determined by thin layer chromatography (Erdal, 1964), and glucose by the method of Somogyi (1937).

Calculations. Material-balance equations were calculated according to Maxon & Johnson (1953) and Chen (1964), and were based on the elementary composition of a standard yeast as reported by Sperber (1946). This composition is in good agreement with the average nitrogen content of the yeasts used in our experiments. The total material-balance equation is divided into three separate equations, I, II and III. In equation III it is assumed that one mole of glucose yields two moles of ethanol and two moles of ATP:

RESULTS

The observed molar growth yields from completed fermentation experiments are given in Table 1. All media contained 1.8% (w/v) glucose as energy and carbon source. The Y_{ATP} values listed were observed when more than 97% of the glucose had been consumed, but before the weight of yeast had been reduced owing to possible changes in the carbohydrate reserves and autolysis. Y_{ATP} values observed shortly before glucose exhaustion in complex media average 11.2, ranging from 9.2 to 14.0. The obtained average yield of ethanol was 96.5%. Glycerol was the only by-product present in amounts large enough to affect the results. The ATP-production from the synthesis of glycerol gives a maximum error in the obtained molar growth yields of 1.5%. The Y_{ATP} values in Table 1 and in the Figures are not corrected for this error.

Formation of cell mass and energy production were presumed to follow the material-balance equations outlined under Methods. Observed cell yield accorded strictly with the calculated values from the equations.

Each of the growth experiments listed in Table 1 was followed by four to eight observations of yeast production, ethanol formed, glucose consumed and nitrogen content of the yeasts. In all the experiments performed, the observed Y_{ATP} decreased with increasing time of growth. This was true of the experiments in which glucose seemed to be the sole limiting factor, and of those in which a changing generation time indicated that the cells were limited in growth by nutrients other than glucose. This was clearly demonstrated for

Saccharomyces carlsbergensis, *Candida utilis* and *Metschnikowia pulcherrima* grown in defined media (Fig. 1). In mineral medium supplemented with biotin only, the observed Y_{ATP} of *S. carlsbergensis* decreased from 9 to 5, whereas the Y_{ATP} values of *M. pulcherrima* in a minimal medium without any growth factors decreased from 5.4 to 3.5.

Table 1. Yields of yeasts grown anaerobically at 25°, observed immediately before glucose depletion^a

Organism addition to minimal medium ^c	Growth yield (mg./ml.)	% N in cells	ATP estimated from ethanol formed (mmole/ml.)	Y_{ATP}^d	Gen. time (h.)
<i>Saccharomyces carlsbergensis</i> , strain no. 10					
Biotin	0.72	11.5	0.153	4.7	30–65
Biotin, pyridoxine	0.96	8.5	0.162	5.9	25–50
Biotin, pyridoxine, thiamine	1.07	7.5	0.164	6.5	15–25
Nine vitamins	1.26 ^b	6.5	0.164	7.7	6
Nine vitamins, histidine, methionine, tryptophane	1.24 ^b	—	0.163	7.6	8
Yeast extract	1.80 ^b	7.3	0.168	10.7	3½
<i>S. carlsbergensis</i> , strain no. 20					
Biotin	1.25	7.5	0.159	7.9	15–30
<i>S. cerevisiae</i> , NCYC 240					
Biotin	1.07 ^b	6.5	0.165	6.5	50
Nine vitamins	1.74 ^b	7.5	0.157	11.1	6
Nine vitamins, Casamino acids	2.19 ^b	6.5	0.169	13.0	5
Yeast extract	2.31 ^b	8.0	0.165	14.0	4½
<i>Metschnikowia pulcherrima</i> , DH-131					
Minimal medium	0.51	6.6	0.153	3.3	12–140
Biotin	0.74	8.4	0.159	4.7	10–70
Biotin, Casamino acids	0.79	—	0.161	4.9	10–70
Nine vitamins	1.42	8.5	0.175	8.1	9
Nine vitamins, Casamino acids	—	7.2	—	8.2	8–21
Yeast extract	1.98 ^b	8.8	0.172	11.5	3
<i>Candida utilis</i> , ATCC 8205					
Nine vitamins	0.86	11.0	0.158	5.4	30–60
Yeast extract	1.62 ^b	9.0	0.176	9.2	6½
<i>Schizosaccharomyces pombe</i> , Lindner CBS 311					
Yeast extract	1.70 ^b	7.9	0.158	10.8	7

^a All media contained ergosterol (7 µg./l.).

^b Glucose the limiting factor.

^c 1.8% (w/v) glucose and mineral salts (Haukeli & Lie, 1971).

^d g. dry weight of cells produced per mole of ATP estimated from ethanol formed.

When complex media were used, the highest observed values for Y_{ATP} were 17.2 for *Saccharomyces cerevisiae* at 15° and after 44 h. growth, 15.2 for *Metschnikowia pulcherrima* (25°, 20 h. growth), and 15.0 for *S. cerevisiae* (25°, 30 h growth).

To indicate what Y_{ATP} values could have been expected if the cells from the fermentation experiments in complex media had been harvested very early, the Y_{ATP} observations were plotted in Fig. 2 as a function of number of generations. The number of generations was here calculated as time of growth from inoculation until the samples were harvested, divided by the constant generation time. The observed Y_{ATP} values for each of these three experiments show linear correlation when they are plotted against time of growth or number

Fig. 1. Y_{ATP} observed during growth of *Saccharomyces carlsbergensis* strain no. 10 (●) in a minimal medium containing mineral salts, glucose and biotin only; *Candida utilis* (△) in a complete vitamin medium; and *Metschnikowia pulcherrima* (▲) in a minimal medium with only mineral salts and glucose. To all media were added ergosterol ($7 \mu\text{g./l.}$). Fermentation temperature was 25° .

Fig. 2

Fig. 3

Fig. 2. The relation between growth and energy production, Y_{ATP} at 25° , plotted against number of generations. □, *Saccharomyces cerevisiae* grown in a complete vitamin medium plus 0.1% casamino acids; ●, *Schizosaccharomyces pombe*; △, *Candida utilis*, both grown in 0.3% yeast extract medium.

Fig. 3. Y_{ATP} observed during fermentation, plotted against % cell yield at 25° . Broken line represents the theoretical relation between Y_{ATP} and % cell yield according to the material-balance equations. ●, *Saccharomyces cerevisiae* in a complete vitamin medium; △, *Saccharomyces carlsbergensis* in biotin medium; ▲, *Schizosaccharomyces pombe* in yeast extract medium; ○, *Metschnikowia pulcherrima* in vitamin medium plus casamino acids; ■, *M. pulcherrima* in minimal medium.

of generations. The experimental technique used made it difficult to obtain accurate observations of yeast yield and ethanol formation after very short incubation time. From the results in Fig. 2 Y_{ATP} as high as 17 to 18 might be expected under our experimental conditions for *Saccharomyces cerevisiae* if the cells had been harvested at an early time.

DISCUSSION

In the present investigation Y_{ATP} values have been observed for several yeasts (Table 1). All Y_{ATP} values showed a clear tendency to decrease with increasing time between inoculation and sampling. The results obtained at the point of glucose exhaustion (48 to 72 h.) were in accord with the findings of Bauchop & Elsdon (1960). Their substrate concentration was 6 to 16 $\mu\text{mole/ml.}$ compared to 100 $\mu\text{mole/ml.}$ used in the present study. Although our findings indicate that Y_{ATP} is not constant during fermentation this does not diminish the importance of the Y_{ATP} concept, but merely emphasizes the need for accurately defined experimental conditions; in particular it seems necessary to take the length of time between inoculation and sampling into account. For *Streptococcus diacetilactis* and *Streptococcus faecalis* Y_{ATP} values ranging from 15.3 to 17.4 have been reported in batch cultures with glucose as energy source (Moustafa & Collins, 1968). From their growth data it can be calculated that Y_{ATP} were observed after 4.5 to 5 generations. If our observations with *Saccharomyces cerevisiae* in partially defined medium (Fig. 2) are extrapolated to 4.5 generations, a Y_{ATP} value of 15.0 would be expected.

The possibility that the efficiency of ATP-production may vary with changing substrate concentrations led Forrest & Walker (1965) to suggest that 3 moles ATP might be formed per mole of glucose at low substrate levels (6 $\mu\text{mole/ml.}$). However, Beck & Shugart (1966) found that this additional yield could occur at concentrations of sugar up to seven times greater than 6 $\mu\text{mole/ml.}$ At higher concentrations of energy source the normally accepted formation of 2 moles ATP seemed to occur, if one assumes an average Y_{ATP} of 10.5. Moustafa & Collins (1968), having eliminated the hypothesis that *Streptococcus diacetilactis* could gain 3 moles of ATP per mole of glucose metabolized, suggested that 17.0 was more nearly correct than the generally accepted value of 10.5. If we suppose an invariant Y_{ATP} (Hemphfling, Mainzer & Van Demark, 1969) of 17.0 or 10.5 based on variable ATP-production, the molar production of ATP of *Saccharomyces cerevisiae* per mole of glucose must decrease from 2.0 to 1.6 or from 3.0 to 2.5 respectively (Fig. 2). Our estimates, however, are based on an invariant ATP-production equal to one mole ATP per mole of ethanol formed, in accordance with the material-balance equation used.

At the beginning of this investigation we suspected that the high yields obtained after a short period of growth might partly be due to accumulation in the cells of glycogen or other compounds requiring less energy for polymerization than the normal cell material (Lie, 1965; Brown, 1968). The cells formed early in the fermentation, however, had normal nitrogen content, indicating normal amount of carbohydrate reserves, and did actually follow the material-balance equation (Fig. 3). This supports the view that the energy yield per mole ethanol was constant during the reported fermentations.

Lysis of bacteria has been observed after exhaustion of glucose (Hadjipetrou & Stout-hamer, 1963), and it has also been stressed that some lysis may occur during the active growth phase of bacteria and influence growth yields (Moustafa & Collins, 1968). In our experiments the difficulties with auto-fermentation (Joslyn, 1955) and lysis after cessation of growth were eliminated, because Y_{ATP} was determined repeatedly during growth, and the final sample taken just before glucose depletion. Lysis of yeast cells during the logarithmic growth phase is generally assumed to be minimal. It is well known that yeast may release

nitrogen compounds into the surrounding medium during growth and fermentation (Hoppe, 1956; Lewis & Phaff, 1964). Delisle & Phaff (1961) showed that such a release of nitrogenous substances does not come from autolysis, but is a physiological phenomenon in healthy yeast cells. It is our opinion that the high Y_{ATP} values observed in the recorded experiments represent normal growth. When Y_{ATP} equal to 10.5 is used for evaluation of ATP-production, the molar growth yield should be calculated at different time intervals or for a range of substrate concentrations, to ensure that the high values that evidently may occur for some organisms in early stages of fermentation are avoided.

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REFERENCES

- BAUCHOP, T. & ELSDEN, S. R. (1960). The growth of micro-organisms in relation to their energy supply. *Journal of General Microbiology* **23**, 457-469.
- BECK, R. W. & SHUGART, L. R. (1966). Molar growth yields in *Streptococcus faecalis* var. *liquefaciens*. *Journal of Bacteriology* **92**, 802-803.
- BELAICH, J. P. & SENEZ, J. C. (1965). Influence of aeration and of panthothenate on growth yields of *Zymomonas mobilis*. *Journal of Bacteriology* **89**, 1195-1200.
- BROWN, J. P. (1968). Anomalous anaerobic growth yields of *Lactobacillus casei* strain 103 in complex medium. *Applied Microbiology* **16**, 805-807.
- BUCHANAN, B. B. & PINE, L. (1967). Path of glucose breakdown and cell yields of a facultative anaerobe, *Actinomyces naeslundii*. *Journal of General Microbiology* **46**, 225-236.
- CHEN, S. L. (1964). Energy requirement for microbial growth. *Nature, London* **202**, 1135-1136.
- DELISLE, A. L. & PHAFF, H. J. (1961). The release of nitrogenous substances by brewers' yeast. *American Society of Brewing Chemists Proceedings*, pp. 107-118.
- DE VRIES, W., KAPTEIJN, W. M. C., VAN DER BEEK, E. G. & STOUTHAMER, A. H. (1970). Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and in continuous cultures. *Journal of General Microbiology* **63**, 333-345.
- ERDAL, K. (1964). Tyndlagschromatografisk kulhydratbestemmelse. *Brygmesteren* **12**, 281-286.
- FORREST, W. W. (1967). Energies of activation and uncoupled growth in *Streptococcus faecalis* and *Zymomonas mobilis*. *Journal of Bacteriology* **94**, 1459-1463.
- FORREST, W. W. & WALKER, D. J. (1965). Synthesis of reserve materials for endogenous metabolism in *Streptococcus faecalis*. *Journal of Bacteriology* **89**, 1448-1452.
- HADJIPETROU, L. P., GERRITS, J. P., TEULINGS, F. A. G. & STOUTHAMER, A. H. (1964). Relation between energy production and growth of *Aerobacter aerogenes*. *Journal of General Microbiology* **36**, 139-150.
- HADJIPETROU, L. P. & STOUTHAMER, A. H. (1963). Autolysis of *Bacillus subtilis* by glucose depletion. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* **29**, 256-260.
- HAUKELI, A. D. & LIE, S. (1971). Experimental conditions affecting yeast growth. *Journal of the Institute of Brewing* **77**, 253-258.
- HEMPFLING, W. P., MAINZER, S. E. & VAN DEMARK, P. J. (1969). Invariance of Y (adenosine triphosphate) of *Streptococcus faecalis* 10Cl during anaerobic continuous culture. *Bacteriological Proceedings* **69**, 143-144.
- HERNANDEZ, E. & JOHNSON, M. J. (1967). Anaerobic growth yields of *Aerobacter cloacae* and *Escherichia coli*. *Journal of Bacteriology* **94**, 991-995.
- HOBSON, P. N. (1965). Continuous culture of some anaerobic and facultatively anaerobic rumen bacteria. *Journal of General Microbiology* **38**, 167-180.
- HOBSON, P. N. & SUMMERS, R. (1967). The continuous culture of anaerobic bacteria. *Journal of General Microbiology* **47**, 53-65.

- HOPPE, W. (1956). Zur Kenntnis des Stickstoff-stoffwechsels der Hefe. Ein Beitrag zur Stickstoffdissimilation. *Brauwissenschaft* **9**, 34–41.
- HUNGATE, R. E. (1963). Polysaccharide storage and growth efficiency in *Ruminococcus albus*. *Journal of Bacteriology* **86**, 848–854.
- JOSLYN, M. A. (1955). Yeast autolysis. I. Chemical and cytological changes involved in autolysis. *Wallerstein Laboratories Communications* **18** **61**, 107–122.
- KORMANČIKOVA, V., KOVAC, L. & VIDROVA, M. (1969). Oxidative phosphorylation in yeast. V. Phosphorylation efficiencies in growing cells determined from molar growth yields. *Biochimica et biophysica acta* **180**, 9–17.
- LEWIS, M. J. & PHAFF, H. J. (1964). Release of nitrogenous substances by brewer's yeast. III. Shock excretion of amino acids. *Journal of Bacteriology* **87**, 1389–1396.
- LIE, S. (1965). *Energy efficiency of yeast growth in fermentations*. European Brewery Convention, Proceeding of the 10th Congress, Stockholm 1965, pp. 292–304. Amsterdam: Elsevier.
- LIE, S., HAUKELI, A. D. & GETHER, J. J. (1970). Gas chromatographic determination of ethanol. *Brygmesteren* **11**, 281–291.
- MAXON, W. D. & JOHNSON, M. J. (1953). Aeration studies on propagation of baker's yeast. *Industrial and Engineering Chemistry* **45**, 2554–2560.
- MOUSTAFA, H. H. & COLLINS, E. B. (1968). Molar growth yields of certain lactic acid bacteria as influenced by autolysis. *Journal of Bacteriology* **96**, 117–125.
- MOUSTAFA, H. H. & COLLINS, E. B. (1969). Molar growth yield of *Streptococcus faecalis* on pyruvate. *Journal of Bacteriology* **97**, 1496–1497.
- OXENBURGH, M. S. & SNOSWELL, A. M. (1965). Use of molar growth yields for the evaluation of energy-producing pathways in *Lactobacillus plantarum*. *Journal of Bacteriology* **89**, 913–914.
- PITT, J. I. & MILLER, M. W. (1968). Sporulation in *Candida pulcherrima*, *Candida reukaufii* and *Chlamydozyma* species: their relationships with *Metschnikowia*. *Mycologia* **60**, 663–685.
- SENEZ, J. C. (1962). Some considerations on the energetics of bacterial growth. *Bacteriological Reviews* **26**, 95–107.
- SOMOGYI, M. (1937). A reagent for copper-iodometric determination of very small amounts of sugar. *Journal of Biological Chemistry* **117**, 771.
- SPERBER, E. (1946). Studies in the metabolism of growing *Torulopsis utilis* under anaerobic conditions. *Arkiv för Kemi, Mineralogi och Geologi* **21 A**, 1.
- STOUTHAMER, A. H. (1962). Energy production in *Gluconobacter liquefaciens*. *Biochimica et biophysica acta* **56**, 19–32.
- TWAROG, R. & WOLFE, R. S. (1963). Role of butyryl phosphate in the energy metabolism of *Clostridium tetanomorphum*. *Journal of Bacteriology* **86**, 112–117.