SHORT COMMUNICATION

Theoretical Analysis of Media Used in the Growth of Yeasts on Methanol

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A theoretical analysis has been made of five different media which have been used for growth of the methylotrophic yeasts *Hansenula polymorpha* and *Candida boidinii*. The media compositions were found to differ significantly in their content of trace elements. Chemostat studies revealed that low growth yields, particularly at high dilution rates, were probably due to unexpected limitations in trace elements.

INTRODUCTION

Amongst methylotrophic yeasts the two species *Hansenula polymorpha* and *Candida boidinii* are the best investigated organisms with respect to biochemistry and growth characteristics. Although the biochemistry of the dissimilatory and assimilatory pathways was reported to be identical in both yeasts (van Dijken, 1976; Sahm, 1977; van Dijken *et al.*, 1978; Egli *et al.*, 1980), the growth yields during growth with methanol as carbon source vary from 0.15 g dry wt (g methanol)⁻¹ (Ogata *et al.*, 1969) to 0.66 (Malashenko *et al.*, 1976).

Since it is well known that the composition of the medium can affect the growth yield with respect to the limiting substrate (Pirt, 1975), several of the synthetic media previously used for the cultivation of *H. polymorpha* and *C. boidinii* have been compared. The basis of the comparison was the extent to which individual elements were in theoretical excess over carbon. This calculation was based on the average growth yields with respect to different elements as reported in the literature.

METHODS

Media. The following media reported to support growth of strains of either *Candida boidinii* or *Hansenula polymorpha*, with methanol as sole source of carbon and energy, were chosen for the theoretical analysis.

Medium A (Ogata *et al.*, 1969): CH₃OH, 20.0 g; NH₄Cl, 4.0 g; KH₂PO₄, 1.0 g; K₂HPO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; thiamin, 1.0 mg; riboflavin, 1.0 mg; pyridoxine, 1.0 mg; pantothenic acid, 1.0 mg; nicotinic acid, 1.0 mg; *p*-aminobenzoic acid, 0.2 mg; folic acid, 10 µg; biotin, 10 µg; water to 1.0 l. It is not stated in this reference whether distilled water or tap water was used; in a later paper the same authors reported that tap water was used and the concentrations of all vitamins were 100 times lower (Tani *et al.*, 1972). The medium was reported to support growth of *Kloeckera* sp. 2201 (now classified as *Candida boidinii*; Fukui *et al.*, 1975) on methanol.

Medium B (Sahm & Wagner, 1972): CH₃OH, 31.2 g; KH₂PO₄, 1.0 g; K₂HPO₄, 2.0 g; (NH₄)₂SO₄, 2.0 g; NH₄NO₃, 2.0 g; Na₂HPO₄·2H₂O, 1.0 g; KCl, 0.20 g; MgSO₄·7H₂O, 0.20 g; H₃BO₃, 0.50 mg; CuSO₄·5H₂O, 0.040 mg; KI, 0.10 mg; FeCl₃·6H₂O, 0.20 mg; MnSO₄·H₂O, 0.40 mg; ZnSO₄·7H₂O, 0.40 mg; (NH₄)₆Mo₇O₂₄·4H₂O, 0.20 mg; biotin, 10.0 µg; distilled water to 1.0 l. This medium was reported to support growth of *Candida boidinii* on methanol.

Medium C (van Dijken, 1976): CH₃OH, 5.0 g; (NH₄)₂SO₄, 1.50 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.20 g; ZnSO₄·7H₂O, 4.4 mg; CaCl₂, 1.1 mg; MnCl₂·4H₂O, 1.01 mg; FeSO₄·7H₂O, 1.0 mg; (NH₄)₆Mo₇O₂₄·4H₂O,

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0.22 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.31 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.32 mg; biotin, 5.0 μg ; thiamin, 300 μg ; EDTA, 10.0 mg; distilled water to 1.0 l. This medium was reported to support growth of *Hansenula polymorpha* on methanol.

Medium D (Dziengel *et al.*, 1977): CH_3OH , 30.0 g; $(\text{NH}_4)_2\text{SO}_4$, 6.0 g; KH_2PO_4 , 0.71 g; K_2HPO_4 , 0.94 g; NaCl , 0.08 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.06 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.012 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.82 g; H_3BO_3 , 4.0 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 mg; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.0 mg; KI, 0.6 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.20 mg; biotin, 0.11 mg; thiamin.HCl, 1.40 mg; distilled water to 1.0 l. This medium was reported to support growth of *Candida boidinii* variant 60 (a strain derived from *Kloeckera* sp. 2201; Schlender *et al.*, 1978).

Medium E (constructed as a consequence of the present study): carbon up to 4.55 g; NH_4Cl , 7.63 g; KH_2PO_4 , 2.81 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.59 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 55.0 mg; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 37.5 mg; $\text{MnSO}_4 \cdot 3\text{H}_2\text{O}$, 17.0 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 22.0 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 4.0 mg; $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 2.80 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2.60 mg; H_3BO_3 , 4.0 mg; KI, 0.60 mg; biotin, 50 μg ; thiamin.HCl, 5.0 mg; meso-inositol, 47.0 mg; pantothenic acid, 23.0 mg; pyridoxine, 1.20 mg; EDTA, 0.45 g; antifoam (polypropylene glycol), 40.0 mg; distilled water to 1.0 l. Besides biotin and thiamin, which were reported to be especially important for yeasts during growth on methanol (van Dijken & Harder, 1974; Sahm & Wagner, 1972), the vitamins meso-inositol, pantothenic acid and pyridoxine were included in this medium in order to enable control experiments to be performed with the non-methylotrophic yeast *Saccharomyces cerevisiae* H1022 without changing the medium composition. These additional vitamins did not affect the growth yields of *H. polymorpha* or *Kloeckera* sp. 2201 with respect to glucose or methanol (T. Egli, unpublished).

Calculation of theoretical excess factors for elements. Theoretical growth yield factors for elements or vitamins ($Y_{X/E}$ = g dry wt cells per g element or vitamin, as appropriate) were calculated from literature data on the elementary composition and vitamin content of dried yeast cells (Silbereisen, 1960; Butschek & Kautzmann, 1962; Harrison, 1967; Burrows, 1970; Peppler, 1970; Aiba *et al.*, 1973). They were found to vary within the following limits: $1.02 < Y_{X/C} < 1.15$; $8.3 < Y_{X/N} < 20$; $38.2 < Y_{X/S} < 3.3 \times 10^4$; $22.7 < Y_{X/P} < 285$; $25 < Y_{X/K} < 111$; $200 < Y_{X/Mg} < 1670$; $111 < Y_{X/Ca} < 2.8 \times 10^4$; $200 < Y_{X/Fe} < 5 \times 10^4$; $1.4 \times 10^4 < Y_{X/Mn} < 2.5 \times 10^5$; $5 \times 10^3 < Y_{X/Zn} < 2.6 \times 10^4$; $5 \times 10^3 < Y_{X/Cu} < 10^5$; $2.5 \times 10^4 < Y_{X/Co} < 2 \times 10^5$; $10^5 < Y_{X/Mo} < 10^6$; $2 \times 10^5 < Y_{X/I} < 2 \times 10^6$; $2.8 \times 10^4 < Y_{X/Biotin} < 2 \times 10^6$; $6 \times 10^3 < Y_{X/Thiamin} < 2 \times 10^5$. No data on the boron content of dried yeast cells were reported in the above cited references. The theoretical growth yield factors for *Klebsiella aerogenes* with respect to elements (Pirt, 1975) and for either *Streptococcus* or mouse LS cells with respect to vitamins (Pirt, 1975) were found to be in the same ranges as those calculated here for yeast cells. Consequently, theoretical growth yield factors appear to be, in general, more dependent upon growth conditions than on cell type or species. Bearing this fact in mind and in the absence of a single, well-documented series of theoretical growth yield factors for a given yeast, most of the factors used in the calculations presented in this paper were taken from the detailed figures available for a bacterium, *K. aerogenes* (Pirt, 1975): $Y_{X/C}$, 1.1; $Y_{X/N}$, 8.75; $Y_{X/S}$, 333; $Y_{X/P}$, 39.1; $Y_{X/K}$, 59.5; $Y_{X/Mg}$, 430; $Y_{X/Ca}$, 3.3×10^3 ; $Y_{X/Fe}$, 6.7×10^3 ; $Y_{X/Mn}$, 2×10^4 ; $Y_{X/Zn}$, 2×10^4 ; $Y_{X/Cu}$, 10^5 ; $Y_{X/Co}$, 10^5 ; $Y_{X/Mo}$, 10^5 ; $Y_{X/I}$, 2×10^5 ; $Y_{X/Biotin}$, 10^6 ; $Y_{X/Thiamin}$, 10^4 .

All media were reported to be carbon-limited. Therefore, the theoretical excess factors of elements and vitamins (F_C) were computed with respect to the element carbon using the equation:

$$F_C = (Y_{X/E} \times C_E) / (Y_{X/C} \times C_C)$$

where $Y_{X/E}$ is the average growth yield [g dry wt (g element)⁻¹], $Y_{X/C}$ is the average growth yield [g dry wt (g carbon)⁻¹], C_E and C_C are the concentrations of element E and carbon (g l⁻¹), respectively.

Micro-organisms and cultivation. *Hansenula polymorpha* de Morais et Maia CBS 4732 and *Kloeckera* sp. 2201 were used for the growth yield studies. Both yeasts were cultivated under the chemostat conditions described by Egli *et al.* (1980). For dry weight measurements the cells were centrifuged for 5 min at 5000 g, washed once with distilled water and dried to constant weight at 105 °C. Parallel samples showed less than 1% deviation. Residual concentrations of glucose and methanol were measured as described previously (Egli *et al.*, 1980).

RESULTS AND DISCUSSION

The theoretical excess factors for elements and vitamins with respect to carbon (F_C) calculated for media used by Ogata *et al.* (1969), Sahm & Wagner (1972), van Dijken (1976) and Dziengel *et al.* (1977) are listed in Table 1. All values of F_C less than 1 indicate a theoretical limitation, whereas factors greater than 1 mark a theoretical excess of an element or vitamin. Differences in the compositions of the four media are obvious. F_C values for trace elements, in particular, differ over a wide range up to 90-fold. A correlation between theoretically limited medium composition and low growth yield can be seen (media B and D). In medium B iron might be a limiting factor because all methylotrophic yeasts contain high amounts of the iron-containing enzyme catalase when grown with methanol as the carbon

Table 1. Theoretical excess factors for elements and vitamins with respect to carbon (F_c) and maximal growth yield reported for different carbon-limited media used for growth of yeasts with methanol as sole source of carbon

Element/ Vitamin	Medium†...	Theoretical excess factors, F_c				
		A	B	C	D	E
C		1.00	1.00	1.00	1.00	1.00
N		1.11	0.76	1.35	0.90	3.50
S		2.63	13.3	63.1	42.1	5.12
P		1.92	2.33	4.32	1.04	5.00
K		5.31	5.96	8.30	3.01	9.60
Mg		2.57	0.66	4.12	2.81	5.00
Ca		*	—	0.48	4.36	9.90
Fe		*	0.02	0.83	1.34	10.3
Mn		*	0.20	2.71	0.64	19.5
Zn		*	0.14	9.72	0.73	20.0
Cu		*	0.08	3.83	0.41	20.4
Co		*	—	3.85	—	19.9
Mo		*	0.87	5.98	3.20	20.6
I		*	1.19	—	7.41	18.3
Biotin		1.21	0.78	2.43	8.88	10.0
Thiamin		1.21	—	1.46	1.13	10.0
Maximum growth yield reported‡ [g dry wt (g methanol) ⁻¹]		0.22 ^a	0.27 ^c	0.38 ^d	0.32 ^b	{ 0.38 ^d 0.40 ^a

—, Not added.

* Tap water was used instead of trace element solution.

† A, Ogata *et al.* (1969); B, Sahm & Wagner (1972); C, van Dijken (1976); D, Dziengel *et al.* (1977); E, this study, and Egli (1980).

‡ a, *Kloeckera* sp. 2201; b, *Candida boidinii* variant 60; c, *Candida boidinii*; d, *Hansenula polymorpha* CBS 4732.

source (Sahm, 1977). For medium A no calculation of F_c for trace elements was possible, but the use of tap water as a source of trace elements seemed to lower the growth yield for methanol of *Kloeckera* sp. 2201 to 0.22 g dry wt (g methanol)⁻¹, because on growth of this yeast with medium D an enhanced growth yield of 0.32 was reported.

As a consequence of this study, medium E was constructed and was shown to be carbon-limited for growth of *H. polymorpha* and *Kloeckera* sp. 2201 with methanol and glucose as a carbon source (see Egli, 1980). With this medium an enhanced growth yield for *Kloeckera* sp. 2201 over medium A was observed in methanol-limited chemostat cultures (Table 1).

The growth yields listed in Table 1 were estimated either in batch cultures (media A, B) or in chemostat cultures (media C, D, E). To eliminate any possible effect of the different conditions, *H. polymorpha* and *Kloeckera* sp. 2201 were grown in chemostat cultures under identical conditions with either medium B (which is theoretically limited in trace element composition) or medium E. Methanol or glucose was used as carbon source. During methanol-limited growth at a dilution rate of 0.05 h⁻¹ only slightly lowered growth yields for both yeasts were found with medium B as compared with medium E (Table 2). However, at a dilution rate of 0.09 h⁻¹ a significant drop in growth yield and detectable residual methanol was found in cultures of *H. polymorpha* and *Kloeckera* sp. 2201 growing in medium B as compared with medium E. Similar results were obtained with glucose-limited cultures.

The results presented suggest that the reason for the variation of growth yields of 'methanol-limited' cultures of methylotrophic yeasts reported in the literature may, in fact, be mainly due to the media being limited in both carbon and a trace element (and in some cases,

Table 2. Growth yields for methanol and residual methanol concentrations as a function of medium and dilution rate during growth of *H. polymorpha* and *Kloeckera* sp. 2201 in chemostat cultures with methanol as sole source of carbon and energy

Organism	Medium*	Dilution rate (h ⁻¹)	Methanol concn† (g l ⁻¹)	Growth yield [g dry wt (g methanol) ⁻¹]
<i>H. polymorpha</i>	B	0.05	ND	0.34
	E	0.05	ND	0.36
	B	0.09	2.81	0.27
	E	0.09	ND	0.38
<i>Kloeckera</i> sp. 2201	B	0.05	ND	0.33
	E	0.05	ND	0.36
	B	0.09	3.63	0.28
	E	0.09	ND	0.36

* B, Sahn & Wagner (1972); E, this study, and Egli (1980).

† Methanol concentration in the inflowing medium was 6.80 g l⁻¹. ND, Not detectable.

nitrogen as well). This would be in accordance with the report by Pirt (1975) that a reduction of growth yields can occur during trace element-limited growth at high dilution rates.

During the preparation of this manuscript, Tsuchiya *et al.* (1980) reported on a medium optimization for the production of biomass and vitamin B₁₂ from *Pseudomonas* AM1 using the same theoretical approach as described above. Although the authors did not take into account the contribution of potassium, their results for the methylotrophic bacterium *Pseudomonas* AM1 confirmed the data presented here for methylotrophic yeasts.

This emphasizes the importance of ensuring that in cultivating micro-organisms account is taken, not only of the more common limitations in C, N, P or S, but also possible limitations in trace elements.

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