Biochemical Limits to Microbial Growth Yields: An Analysis of Mixed Substrate Utilization

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A theoretical analysis has been made of carbon conversion efficiency during heterotrophic microbial growth. The expectation was that the maximal growth yield occurs when all the substrate is assimilated and the net flow of carbon through dissimilation is zero. This, however, is not identical to a 100% carbon conversion, since assimilatory pathways lead to a net production of CO_2. It can be shown that the amount of CO_2 produced by way of assimilatory processes is dependent upon the nature of the carbon source, but independent of its degree of reduction and varies between 12 and 29% of the substrate carbon. An analysis of published yield data reveals that nearly complete assimilation can occur during growth on substrates with a high energy content. This holds for substrates with a heat of combustion of ca. 550 kJ/mol C, or a degree of reduction higher than 5 (e.g. ethane, ethanol, and methanol). Complete assimilation can also be achieved on substrates with a lower energy content, provided that an auxiliary energy source is present that cannot be used as a carbon source. This is evident from the cell yields reported for *Candida utilis* grown on glucose plus formate and for *Thiobacillus versutus* grown on acetate plus thiosulfate. This evaluation of the carbon conversion efficiency during assimilation also made it possible to compare the energy content of the auxiliary energy substrate added with the quantity of the carbon source it had replaced. It will be shown that utilization of the auxiliary energy source may lead to extreme changes in the efficiency of dissimilatory processes.

INTRODUCTION

During the last two decades the energetics of growth of microorganims has been subject of many studies.[1-4] One of the central issues that has been raised is the theoretical maximum yield of biomass from a single carbon source. In parallel studies, it has been pointed out that during the assimilation of a carbon source to biomass it is inevitable that some of the substrate used is converted to CO_2.[5-7] This is caused by the metabolic constraints posed upon a growing organism during the assimilation of its substrate. In the total of CO_2-consuming and CO_2-producing assimilatory reactions in the cell, the balance shows a net CO_2 production. Surprisingly, this fact has not generally been taken into account in the analysis of microbial growth yields.[2-4,8] Although it was concluded that a 100% substrate to biomass conversion is apparently impossible, an adequate explanation for this phenomenon has not been advanced.

The aim of this article is to present experimental and theoretical evidence demonstrating that though a carbon conversion of 100% is intrinsically impossible, in certain cases substrates can nevertheless be used completely for assimilatory purposes. Maximal carbon conversion (100% assimilation) is reached when the cell yield is not limited by the availability of energy. Such a situation is defined in this study as carbon-limited growth and may be encountered in two cases. First, during growth on energy rich substrates the assimilation may liberate enough energy to sustain the overall process of biomass formation. Secondly, addition of another energy source during growth on low energy substrates may also result in carbon-limited growth. A prerequisite for an evaluation of the energetics of these situations is the proper calculation of the net assimilatory and dissimilatory flows from yield data. The calculations, presented in this article clearly establish the existence of biochemical limits to growth yields as a result of CO_2 production during assimilatory processes. Moreover, especially in the case of mixed substrate utilization, these calculations also provide a useful tool for the analysis of the efficiency of respiratory processes.

THEORY AND CALCULATIONS

Calculation of Assimilatory CO_2 Production

The metabolic pathways leading from a growth substrate molecule to the various cell components include many carboxylation and decarboxylation steps. In (nonautotrophic) biomass synthesis, the balance shows a net production of CO_2 for most heterotrophic growth substrates. A notable exception is the growth of certain bacteria on methanol via the serine pathway. Only in this case generation of C_3

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Table I. Net CO_2 production and substrate requirement during synthesis of 1 g cell polymer from glucose, acetate, or malate. Numbers were calculated as published in ref. 6 using the overall stoichiometries of generally accepted biochemical pathways. All quantities are given in mmoles.

Substrate polymer	Glucose required	CO_2 produced	Acetate required	CO_2 produced	Malate required	CO_2 produced
Protein	8.49	5.95	30.29	15.58	15.15	15.58
Nucleic acids	4.14	−3.14	14.59	1.17	7.29	1.17
Lipids	14.12	29.72	28.15	1.30	28.93	60.70
Polysaccharides	6.33	0	24.67	12.33	12.33	12.33

Table II. Comparison of CO_2 production during the assimilation of glucose, acetate, and malate to bacterial or yeast cells. The CO_2 production per gram of polymer is taken from Table I. The cell compositions represent (from ref. 2) average bacterial cells (52.4% protein, 18.9% nucleic acids, 9.4% lipids, and 16.6% polysaccharides) and (from ref. 6) average yeast cells (40% protein, 7% nucleic acids, 7% lipids, and 42% polysaccharides).

Substrate cell type	Glucose required	CO_2 produced	Acetate required	CO_2 produced	Malate required	CO_2 produced
Average bacterial cell (%C ⟶ CO_2)	7.65	5.32 (11.6)	25.38	10.55 (20.8)	14.09	16.14 (28.6)
Average yeast cell (%C ⟶ CO_2)	7.34	4.24 (9.6)	25.47	11.58 (22.7)	13.78	15.74 (28.6)

compounds from the growth substrate requires a net input of CO_2 via PEP carboxylase.[9] Consequently, during utilization of methanol via the serine pathway up to 50% of the cellular carbon is exchangeable with CO_2.[10] This exceptional case is not considered in this article.

The net CO_2 production during synthesis of the different cell polymers from either glucose, acetate, or malate was calculated via summation of all biochemical reactions leading to the various monomers used for polymer synthesis. The assumptions underlying these calculations and the monomer composition of the cell polymers were as described by Bruinenberg and co-workers.[6] The results presented in Table I show that, except for the formation of nucleic acids and polysaccharide from glucose, synthesis of cell polymers is necessarily associated with the production of CO_2.

With the data from Table I, the CO_2 produced during the conversion of a substrate to biomass of a known polymer composition can be calculated (Table II). The influence of the cell composition is rather small but the difference between yeast and bacterial biomass is not negligible. The amounts of CO_2 produced during the formation of bacterial biomass from a number of substrates have been summarized in Table III. The data clearly show that the degree of carbon conversion during formation of biomass is not linked to the energy content of the growth substrate.

Calculation of Assimilatory and Dissimilatory Flows

The quantity of substrate needed to form a certain amount of biomass can theoretically be divided into two flows (Fig. 1). The assimilatory fraction is the amount of substrate converted to biomass via the assimilatory pathways. The dissimilatory fraction is defined here as the amount of substrate used for other purposes (energy generation, transport, maintenance, futile cycles, etc.). As stated in the introduction, carbon-limited growth is defined as the growth condition under which the substrate is utilized solely for assimilatory purposes. Energy-limited growth is defined as the growth conditions under which part of the substrate is dissimilated.

For growth on a single substrate, assimilatory and dissimilatory flows can easily be calculated from the cell yield on the substrate using the data presented in Table III. This, however, only holds when biomass and CO_2 are the sole products of metabolism. In the case that other prod-

Table III. Overall carbon losses, as CO_2, during the complete assimilation of several commonly used carbon and energy sources. Calculations were performed assuming that assimilation of the carbon sources proceeds via the following pathways: glucose via the Embden–Meyerhof pathway; gluconate via the Entner–Doudoroff pathway; methane, methanol, and formaldehyde via the ribulose–monophosphate pathway of formaldehyde fixation (ref. 9); acetate via the glyoxylate cycle and isocitrate-lyase; and oxalate via the glycerate pathway (ref. 32).

Percentage of substrate carbon converted to CO_2 during assimilation	Substrates
12	Glucose, gluconate, mannitol, glycerol, methane, methanol, formaldehyde, lactate, pyruvate
21	Acetate, ethanol, ethane, hexadecane
29	Malate, oxalate, succinate, fumarate

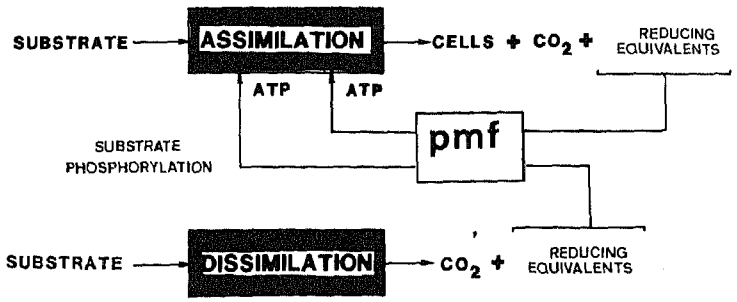

Figure 1. Simplified scheme of growth on a heterotrophic substrate illustrating the carbon and energy flows. It should be noted that during the assimilation of highly oxidized organic substrates the assimilation process may not generate $NADH_2$/reducing power at all and part of the substrate dissimilated is used to provide for the reduction equivalents necessary during the conversion of substrate to biomass.

ucts are formed, such as siderophores (i.e. *Paracoccus denitrificans*[11]), construction of carbon balances for assimilatory and dissimilatory processes is impossible and will therefore not be considered in this article. Furthermore, in the calculations presented below only yield data obtained with chemostat cultures grown under carbon and/or energy limitation are used. Only under these conditions may balanced growth with optimal efficiency be assumed.

A situation slightly more complicated than carbon and/or energy limited growth on a single substrate arises in the case of growth on two substrates of which one substrate contributes to the dissimilatory flow only and cannot be used as a carbon source. A schematic representation of this type of mixed substrate utilization and of growth on a single substrate is shown in Figure 2. The first substrate is considered to be the only carbon source under all growth

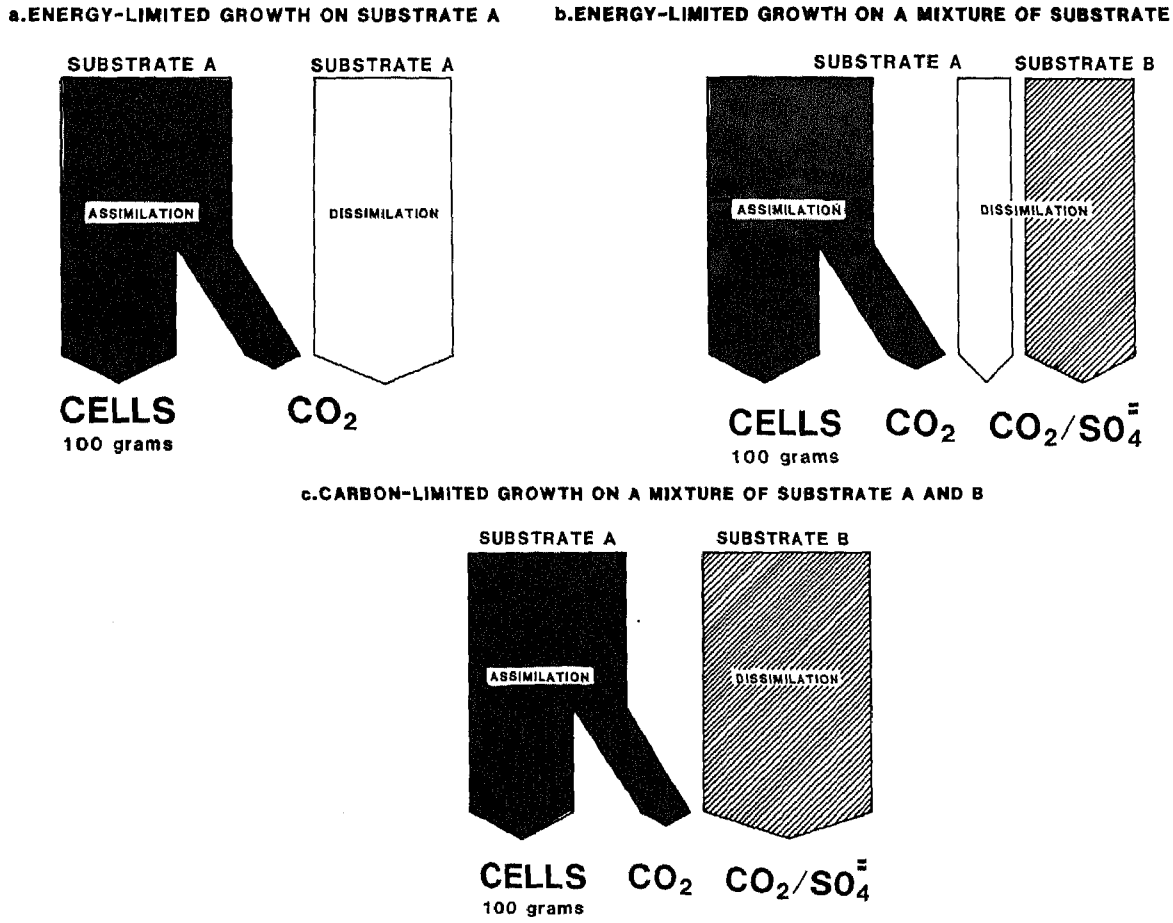

Figure 2. Schematic representation of the influence of an auxiliary energy source (substrate B) on the assimilatory and dissimilatory flows of a sole carbon source (substrate A) under substrate limiting conditions. Both CO_2 and $SO_4^=$ are shown as possible products formed during the oxidation the auxiliary energy source (e.g. formate or thiosulphate).

conditions. This implies that the assimilatory CO_2 production is independent of the ancillary energy source, and thus can be calculated from Table III in the same way as for single substrates. Autotrophic CO_2 fixation (via the Calvin cycle) complicates the construction of carbon balances for the assimilatory and dissimilatory flows on the basis of yield data. Therefore, only those cases will be considered where information is available on the absence of autotrophic CO_2 fixation as measured by enzyme activities of the Calvin cycle via appropriate methods. To facilitate comparison of biomass yields during single and mixed substrate growth, it is necessary to express all data as moles of substrate needed to form 1 g of biomass (dry wt). This is equivalent to the inverse of the molar growth yield expressed as gram dry weight formed per mole of substrate. In this way, the quantity of growth substrate that has been replaced by the auxiliary energy source can be estimated.

For instance, in the case of *Pseudomonas oxalaticus* the experimental data[12] are (Table IV): 30m*M* acetate yields 648 mg dry wt/L and 30m*M* acetate + 10m*M* formate yields 717 mg dry wt/L. Thus during growth on acetate alone, 46.3 mmol acetate are needed to form 1 g dry wt cells, whereas in the presence of formate (acetate:formate ratio of 3:1) only 41.8 mmol are utilized for the formation of this amount of biomass (Table IV). With the data presented in Table III, it is possible to calculate the amount of the carbon substrate needed for assimilatory purposes (for the synthesis of 1 g biomass). In the case of *P. oxalaticus*, 25.4 mmol acetate are needed for these assimilatory purposes (Table IV). The amount of substrate A (acetate) dissimilated is therefore 46.3–25.4 = 20.9 mmol in the case

Table IV. The effect of an auxiliary energy source upon the assimilatory and dissimilatory flows of the carbon and energy substrate in aerobic chemostat experiments in which no autotrophic CO_2 fixation occurred. For an explanation of the different columns see the Theory and Calculations section. All amounts are expressed as mmoles. The experimental ratio is calculated from the amount of reduction equivalents added (substrate B) and the amount of substrate A spared. The expected ratios of the energetic value of the reduction equivalents of substrate A and B were 1 except in the case of acetate and thiosulphate when the ratio was assumed to be 2 (for further details see the Theory and Calculations section).

	Substrate (mmol) needed to synthesize g biomass									
	Experimental				Substrate A		Percentages		Ratio of the energetic value of the reduction equivalents generated from substrates A and B	
Organisms	Substrate A		Substrate B		Assimilated (theor)	Dissimilated	Assimilated	Dissimilated	(Experimental/expected)	Reference
Pseudomonas oxalaticus	ACET[a]	46.3	—		25.4	20.9	55	45	—	12
	ACET	41.8	FORM[b]	14.0	25.4	16.4	61	39	1.3	
	ACET	37.7	FORM	25.2	25.4	12.3	67	33	1.3	
	ACET	34.7	FORM	34.7	25.4	9.3	73	27	1.3	
	ACET	31.9	FORM	42.6	25.4	6.5	80	20	1.3	
Pseudomonas oxalaticus	OXAL[c]	250	—		28.2	221.8	11	89	—	18
	OXAL	227	FORM	23	28.2	198.8	12	88	1.0	
	OXAL	204	FORM	47	28.2	175.8	14	86	1.0	
	OXAL	177	FORM	71	28.2	148.8	16	84	1.0	
Thiobacillus versutus	ACET	48.5	—		25.4	23.1	52	48	—	27
	ACET	33.2	THIO[d]	22.1	25.4	6.8	77	23	1.4	
	ACET	27.1	THIO	36.1	25.4	1.7	94	6	1.2	
	ACET	23.5	THIO	47.0	25.4	−1.9	100	0	1.1	
Thiobacillus Q	ACET	62.9	—		25.4	37.5	40	60	—	28
	ACET	55.0	THIO	16.5	25.4	29.6	46	54	1.0	
	ACET	50.8	THIO	25.4	25.4	25.4	50	50	1.0	
	ACET	47.2	THIO	33.0	25.4	21.8	54	46	1.0	
Acinetobacter calcoaceticus	ACET	83.8	—		25.4	58.4	30	70	—	29
	ACET	75.3	XYLO[e]	12.1	25.4	49.9	34	66	2.9	
	ACET	65.1	XYLO	26.3	25.4	39.7	39	61	2.9	
	ACET	61.1	XYLO	30.7	25.4	35.7	42	58	2.9	
	ACET	53.6	XYLO	43.6	25.4	28.2	47	53	2.9	
Candida utilus	GLUC[f]	11.1	—		7.3	3.8	66	34	—	6
	GLUC	9.1	FORM	18.2	7.3	1.8	80	20	1.3	
	GLUC	7.9	FORM	27.8	7.3	0.6	92	8	1.4	

[a] Acetate.
[b] Formate.
[c] Oxapate.
[d] Thiosulfate.
[e] Xylose.
[f] Glucose.

of growth on acetate alone. In the cases of mixed substrate growth, part of this acetate is replaced by the energy source (formate) and therefore the fraction of the carbon source (acetate) dissimilated decreases with decreasing ratios of acetate:formate (Table IV and Fig. 2).

Energetics of Single and Mixed Substrate Utilization

When it is assumed that the energy requirements for the assimilation of a carbon source to a fixed amount of biomass is independent of the presence of an auxiliary energy source, the energetic value of this energy source can be evaluated. For this purpose, the fraction of the primary energy source replaced by the auxiliary energy source must be calculated. For example, in the case of *P. oxalaticus* growing on acetate and formate (molar ratio of 3:1), 14 mmol formate replace 20.9 − 16.4 = 4.5 mmol acetate which are dissimilated during growth on acetate alone (Table IV). If reducing equivalents are utilized with the same efficiency during single and mixed substrate utilization, the amount of reducing equivalents from acetate (4 × 4.5) should be equal to that of formate obtained from formate oxidation (14.0). However, reducing equivalents derived from formate are apparently 4 × 4.5/14.0 = 1.3 times more efficiently utilized than anticipated on the basis of this assumption.

In order to compare the energetic value of the reducing equivalents of the two substrates used in the cases presented in Table IV and Figure 4 the following assumptions were made: 1) The reducing equivalents from acetate, formate, oxalate, and glucose all enter the respiratory chain at the level of $NADH_2$ and are equivalent. 2) The oxidation of xylose to xylonic acid results in the production of one reducing equivalent as $PQQH_2$ (PQQ = pyrrolo-quinoline quinone) which enters the respiratory chain at the level of cytochrome b.[13] For simplicity, the energetic value of the reducing equivalents from $PQQH_2$ in Table IV were based upon equivalency of $PQQH_2$ and $NADH_2$. 3) The oxidation of thiosulphate to sulphate yields reducing equivalents that are fed into the respiratory chain at the level of cytochrome c.[14] The respiratory chain contains one proton loop between cytochrome c and O_2. For a comparison with the reducing equivalents derived from acetate ($NADH_2$) the number of proton translocating loops that exist between the level of $NADH_2$ and cytochrome c must be known. A conservative estimate, based on the relatively low yields of thiobacilli on acetate, would be one proton translocating loop although two loops can not be excluded. For the calculation of the energetic value of the reducing equivalents from thiosulphate and acetate, it was assumed that the electrons from acetate pass two loops and those from thiosulphate one.

RESULTS AND DISCUSSION

Single Substrate Utilization

The relationship between substrate consumption and formation of biomass was first noted by Monod in 1942.[15] Attempts to correlate observed yields with the quantity of electrons available from the substrate were first published by Payne and co-workers.[16] Linton and Stephenson[8] collected yield data from the literature and related the maximum observed growth yield to the energy content of the growth substrates. They suggested a classification of growth substrates into two distinct groups: energy-limited and carbon-limited substrates. For unknown reasons, maximal carbon–carbon conversion was found to be less than 100%. This unknown factor has now been explained in terms of obligatory CO_2 production during biosynthesis of biomass. This assimilatory CO_2 production is substrate dependent (Table III) and determined by the conversion of substrate into molecules needed for cell synthesis.[5–7] It is evident therefore that for an appropriate interpretation of the bioenergetic relationship between growth yields and the energy content of substrates (expressed as heat of combustion (kJ/mol C), or as the degree of reduction which is analogous) the amount of substrate assimilated rather than the carbon conversion must be considered.[17] As is evident from Figure 3 only the amount (or %) of substrate assimilated allows a separation of growth substrates into energy-limited and carbon-limited compounds. Above ca. 550 kJ/mol C, which corresponds to a degree of reduction of 5 per carbon mole, the amount of substrate assimilated approaches the theoretical maximum. One always must ask if the very high yields are due to measurement errors, but the experiments referred to in Figure 3[8,18–25] all involved carefully selected results from chemostat experiments. Below 550 kJ/mol C, the fraction of substrate assimilated decreases with decreasing energy content. It is therefore justified to classify these substrates as energy limited.[8] Indeed, as discussed below utilization of auxiliary energy sources can increase the cell yield on such substrates.

Our calculations (Fig. 3) thus confirm the suggested classification of substrates on the basis of their degree of reduction. Originally, this classification was based on the apparent relation between yields and energetic content of growth substrates. Growth yields, however, have no meaning as such in mechanistic terms. When our definition of carbon-limited growth is accepted (complete assimilation of the substrate) it follows that above a heat of combustion of ca. 550 kJ/mol C, substrates approach carbon-limitation: the limit of the cell yields on these compounds is set by the overall stoichiometry of the assimilatory processes. In this case, utilization of an auxiliary energy source is not expected to lead to an increase in cell yield. This remains to be verified experimentally.

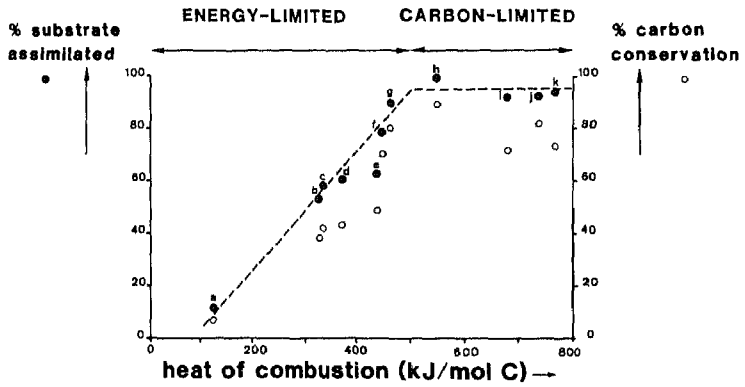

Figure 3. Amount of a single limiting carbon and energy source converted to biomass [expressed as percentage carbon conversion (open symbols) and as percentage of substrate assimilated (closed symbols)] as a function of the heat of combustion of the substrate. An increase in the heat of combustion corresponds to an increase in the degree of reduction of the substrates (550 kJ/mol C is equivalent to a degree of reduction of 5). Complete assimilation of the different substrates results in carbon conversions as presented in Table III. The literature data used in this figure represent the highest growth yields measured in chemostat studies as published for these substrates. The letters in the graph correspond with the substrates listed below. Their degree of reduction is given, followed by the appropriate reference: (a) oxalate, 1, ref. 18; (b) malate, 3, ref. 8; (c) fumarate, 3, ref. 21; (d) succinate, 3.5, ref. 8; (e) acetate, 4, ref. 24; (f) lactate, 4, ref. 24; (g) glucose, 4, ref. 19; (h) glycerol, 4.7, ref. 20; (i) ethanol, 6, ref. 22; (j) methanol, 6, ref. 23; and (k) ethane, 7, ref. 25.

Mixed Substrate Utilization

The strict discrimination between substrate dissimilation and assimilation as pointed out above is particularly necessary in the interpretation of microbial growth yields obtained under dual substrate limitation, when an organic carbon and energy source is consumed simultaneously with a substrate that can be used for energy generation only. Data from the literature and our own work (Table IV and Fig. 4) show that addition of a separate energy source indeed can result in an enhancement of the molar growth yield on the carbon and energy source.[6,12,26-29]

In two cases, *Thiobacillus versutus* and *Candida utilis* the stepwise increase of the energy source eventually resulted in assimilation of substrate A carbon of more than 90%. Actually, both cases can be said to exhibit 100% assimilation. For *Thiobacillus versutus* this is obvious. With respect to *Candida utilis* Bruinenberg and co-workers[6] showed that the last 8% of the glucose is used for the production of NADPH for biosynthetic purposes. This is due to the fact that oxidation of formate only yields NADH and cannot replace this amount of glucose, because yeasts lack transhydrogenase activity ($NADH_2 + NADP \rightarrow NAD + NADPH_2$). In *Candida utilis* utilization of excess formate (i.e. at molar ratios formate/glucose higher than 3.5) no longer increased the cell yield, although formate was utilized completely. Only above a molar ratio of 5.5 the formate concentration in the fermentor effluent became detectable.[26] In the case of *Thiobacillus versutus* growing on mixtures of acetate and thiosulphate the occurrence of 100% assimilation coincided with the appearance of autotrophic CO_2 fixation, thus confirming the carbon-limited status of the organism under these growth conditions.[27] Interestingly, Gottschal and Kuenen, neglecting assimilatory CO_2 production from acetate, concluded that only 75% of the acetate was assimilated by *T. versutus* at the onset of autotrophic CO_2 fixation. *Pseudomonas oxalaticus* does not reach 100% assimilation of acetate when grown on acetate with excess formate. The CO_2 fixing capability appeared at 82% assimilation of the acetate (Fig. 3).[12] When grown on oxalate + formate, the autotrophic CO_2 fixing ability appeared at even lower assimilation percentages (16%).[18] Apparently, unlike *T. versutus*, autotrophic CO_2 fixation in *P. oxalaticus* is not correlated with growth under conditions of carbon limitation.

The introduction of a theoretical assimilatory flow and the normalization of all yield data to mmoles of carbon source utilized per gram biomass allows a comparison of the energy content of the auxiliary energy substrate B with that of A. Surprisingly, in some cases this leads to the conclusion that dissimilatory processes, during growth in the presence of an auxiliary energy source, proceed with unexpected high efficiency. This is substantiated below.

Utilization of Formate by Glucose-Limited Cultures of Candida utilis

The data summarized in Table IV show that the energy value of the average reducing equivalent from glucose is only 0.75 of those from formate. The unexpected high energetic value of formate reducing equivalents in this case has been noted before.[26] This phenomenon becomes even more significant when it is taken into consideration that probably also NAD-independent oxidation of formate is

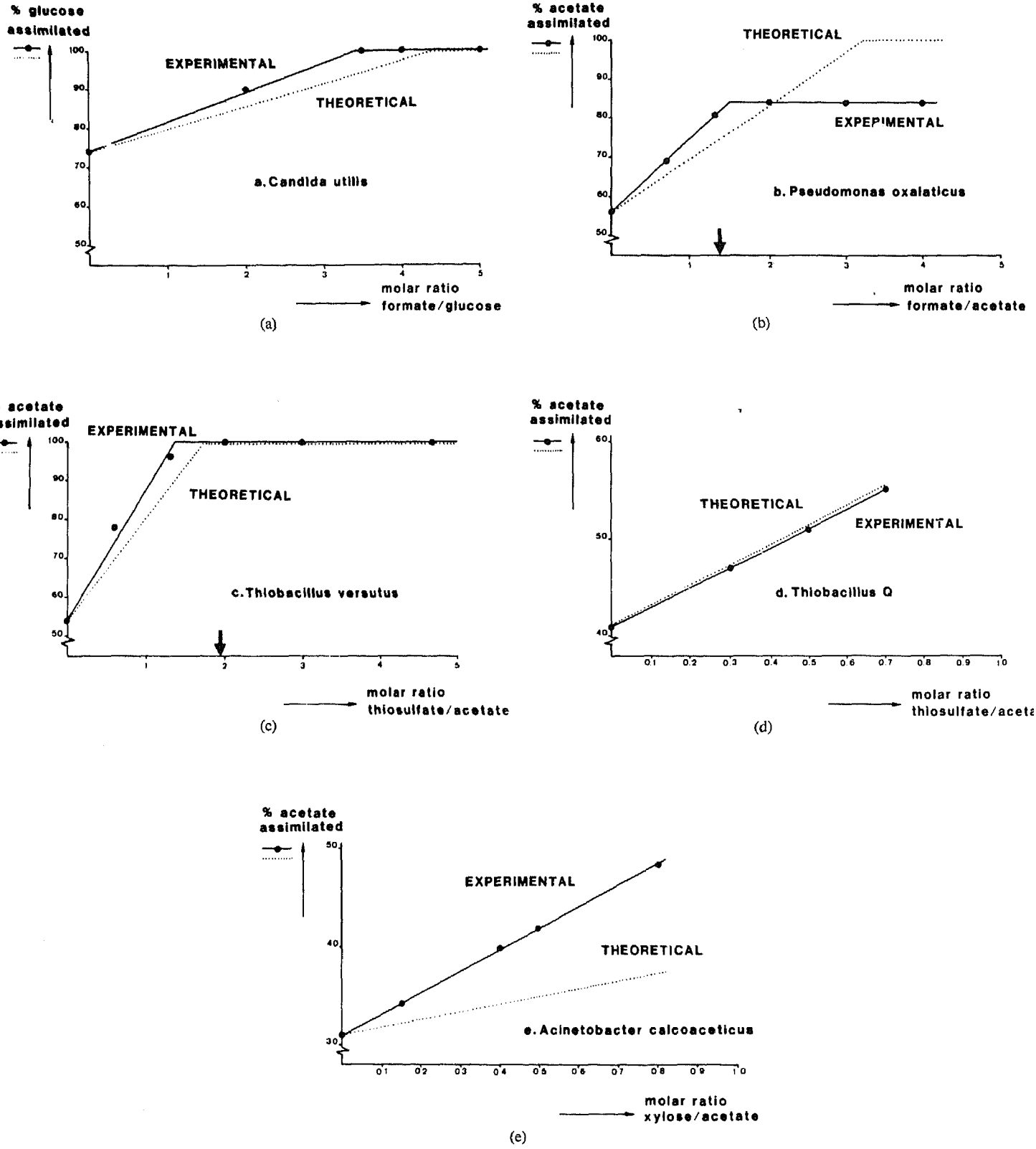

Figure 4. The percentage of a carbon source assimilated as a function of the ratio of the auxiliary energy source and the carbon source in the reservoir medium of chemostat cultures of five microorganisms. The dotted line represents the theoretical prediction of this parameter. It was calculated assuming an equivalency of the electrons of formate to those of acetate and glucose. In the case of thiosulfate the electrons were assumed to have half the energetic value of those derived from acetate. In the case of the oxidation of xylose to xylonic acid by *Acinetobacter calcoaceticus* reduction equivalents (i.e. $PQQH_2$) were taken to be equivalent to $NADH_2$. The critical ratios at which *in vivo* CO_2 fixation was found are indicated by an arrow: (a) *Candida utilis* growing on mixtures of glucose and formate (ref. 26); (b) *Pseudomonas oxalaticus* growing on mixtures of acetate and formate (ref. 12); (c) *Thiobacillus versutus* growing on mixtures of acetate and thiosulfate (ref. 27); (d) *Thiobacillus* Q growing on mixtures of acetate and thiosulfate (ref. 28); and (e) *Acinetobacter calcoaceticus* growing on mixtures of acetate and xylose (ref. 29).

carried out by this yeast.[26] Furthermore, in order to yield energy, formate must be transported into the cell and therefore the costs of transport per reducing equivalent generated are most likely higher for formate than for glucose.

Utilization of Formate by Acetate-Limited Cultures of Pseudomonas oxalaticus

As in the case of *Candida utilis* also utilization of formate by acetate-limited *Pseudomonas oxalaticus* is unexpectedly efficient. The same arguments as mentioned for *C. utilis* further strengthen this conclusion. Per reducing equivalent transport costs are higher for formate and also NAD-independent oxidation of formate may occur in this organism.[30]

Utilization of Thiosulphate by Acetate-Limited Cultures of Thiobacillus *spp*

As mentioned in the Theory and Calculations section, the energetic value of the average reducing equivalents from acetate oxidation can be estimated to be twice of that from thiosulphate. In the case of *Thiobacillus* Q the observed yield increase caused by the addition of thiosulphate to acetate-limited cultures is according to the prediction, but if reducing equivalents from $NADH_2$ would pass more than an average of two loops the yield increase would be unexpectedly high. In the case of *Thiobacillus versutus,* however, the energetic value of the reducing equivalents is higher than expected in any case. This leads to the conclusion that during the mixed substrate utilization an increase in energy efficiency is observed. It remains to be investigated whether this is due to more efficient use of reducing equivalents from either or both thiosulphate and acetate.

Utilization of Xylose as an Energy Source by Acetate-Limited Cultures of Acinetobacter calcoaceticus

Acinetobacter calcoaceticus constitutively synthesizes an aldose dehydrogenase which contains pyrollo-quinoline quinone (PQQ) as a prosthetic group.[31] The enzyme probably feeds its electrons into the electron transport chain at the level of cytochrome b and is located at the outer face of the cytoplasmic membrane.[13] *A. calcoaceticus* is unable to utilize xylose as a carbon source. Utilization of this aldose by acetate-limited chemostat cultures results in the stoichiometric conversion of xylose to xylonic acid.[29] The high energetic value of reducing equivalents obtained from xylose oxidation (as $PQQH_2$) being three times that of the reducing equivalents from acetate (largely $NADH_2$) is even more significant when it is taken into account that in view of the likely occurrence of proton translocation between $NADH_2$ and cytochrome b, $NADH_2$ should yield more energy than $PQQH_2$.

Utilization of Formate by Oxalate-Limited Cultures of Pseudomonas oxalaticus

Taking assimilatory CO_2 production into account, assimilatory and dissimilatory flows of carbon can be calculated both during growth on single and on mixed substrates. In the latter case, it is a prerequisite that the auxiliary energy source does not serve as a carbon source. In four or, depending on the assumptions made, even five of the cases considered, growth of *C. utilis* on glucose and formate, growth of *P. oxalaticus* on acetate and formate, *A. calcoaceticus* on acetate and xylose, and *Thiobacillus* Q and *Thiobacillus versutus,* both grown on acetate and thiosulphate, it was calculated that the reducing equivalents of the auxiliary energy source have a much higher energetic value than those of the carbon source. It must be stressed, however, that this is merely a conclusion expressed in mathematical terms. It is highly unlikely that the reducing equivalents from the auxiliary energy source can yield more ATP than those of the carbon source. For example, in the case of formate and acetate both compounds yield mainly $NADH_2$ and thus in biochemical terms the energetic value of reducing equivalents derived from formate should be equal to those from acetate or less (depending on the contribution of NAD-independent formate oxidation and the energy requirements of transport). In the case of xylose oxidation by *A. calcoaceticus,* the energetic value of the reducing equivalents is most probably not even equal but less than those from acetate since reducing equivalents are channeled into the electron transport chain at the level of cytochrome b. At its best these reducing equivalents ($PQQH_2$) can be equal to $NADH_2$ in the case of absence of proton translocation between $NADH_2$ and cytochrome b.

The apparent contradiction between the biochemical identity of the reducing equivalents and their calculated energetic value can only be explained when it is assumed in the above five cases that utilization of the auxiliary energy source leads to an increased proton translocation with both the reducing equivalents of the carbon source and the auxiliary energy source. This may be accomplished in two ways: either synthesis of additional components of the electron transport chain exhibiting proton translocation, or a readjustment of the electron flow over another branch of the chain that is more efficient (i.e. contains more proton translocating loops). The former explanation has been put forward by van Verseveld and co-workers for *Paracoccus denitrificans* to explain the high growth yields on mixtures of mannitol and methanol.[11] Unfortunately we cannot use their results in terms of carbon flows over assimilatory and dissimilatory pathways due to the reported production of (complex) siderophores. In any case, increased efficiency of respiration is not unique to methanol utilization by *Paracoccus denitrificans* since it was also observed for formate and xylose oxidation by other organisms (Table IV). It must be admitted, however, that one of the assumptions underlying our calculations (unchanged energy requirement of assimilatory

processes in the presence of an auxiliary energy source) may not hold and that a decreased energy requirement of assimilation rather than increased efficiency of dissimilation is the basis for the excessive increase in cell yields mentioned above. At present, no reasonable mechanism can be put forward to substantiate this possibility. It must be stressed that our calculations have comparative value only. They merely elucidate the relative values of the reducing equivalents from 2 substrates. Whether or not the increased efficiency during mixed substrate utilization in certain cases can be adequately explained by the introduction of one extra proton translocating loop (phosphorylation site) is beyond the scope of this article. Finally it should be reiterated that we have confirmed the concept of Linton and Stephenson[8] that organic substrates can be classified as energy and carbon limited. The condition for the classification is that the maximum carbon conversion be calculated from the biochemical pathways, taking into account assimilatory CO_2 production.

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References

1. J. P. van Dijken and W. Harder, *Biotechnol. Bioeng.*, **27**, 15 (1975).
2. A. H. Stouthamer, Int. Rev. Biochem. Microbiol. Biochem., **21**, 1 (1979).
3. J. A. Roels, *Biotechnol. Bioeng.*, **22**, 2457 (1980).
4. H. V. Westerhoff, J. S. Lollema, R. Otto, and K. J. Hellingwerf, *Biochim. Biophys. Acta*, **683**, 181 (1982).
5. E. Oura, "The effect of aeration on the growth energetics and biochemical composition of baker's yeast," Ph.D. thesis, University of Helsinki, 1972.
6. P. M. Bruinenberg, J. P. van Dijken, and W. A. Scheffers, *J. Gen. Microbiol.*, **129**, 953 (1983).
7. W. Babel and R. H. Muller, *Appl. Microbiol. Biotechnol.*, **22**, 201 (1985).
8. J. D. Linton and R. J. Stephenson, *FEMS Microbiol. Lett.*, **3**, 95 (1978).
9. J. R. Quale and T. Ferenci, *Microbiol. Rev.*, **42**, 251 (1978).
10. P. J. Large, D. Peel, and J. R. Quayle, *Biochem. J.*, **81**, 470–480 (1961).
11. H. W. van Verseveld, J. P. Boon, and A. H. Stouthamer, *Arch. Microbiol.*, **121**, 213 (1979).
12. L. Dijkhuizen and W. Harder, *Arch. Microbiol.*, **123**, 47 (1979).
13. B. J. van Schie, K. J. Hellingwerf, J. P. van Dijken, M. G. L. Elferink, J. M. van Dijl, J. G. Kuenen, and W. N. Konings, *J. Bacteriol.*, **163**, 493 (1985).
14. D. P. Kelly, *Phil. Trans. R. Soc. Lond. B*, **298**, 499 (1982).
15. J. Monod, *Recherches sur la croissance des cultures bacteriennes* (Herman en Cie, Paris, 1942), p. 210.
16. W. J. Payne, *Ann. Rev. Microbiol.*, **24**, 17 (1970).
17. L. E. Erickson, *Biotechnol. Bioeng.*, **22**, 791 (1981).
18. L. Dijkhuizen, W. Harder, *Arch. Microbiol.*, **123**, 55 (1979).
19. S. A. Palombo and L. D. Witter, *Appl. Microbiol.*, **18**, 137 (1969).
20. C. W. Jones, J. M. Brice, and C. Edwards, *Arch. Microbiol.*, **115**, 85 (1977).
21. H. W. van Verseveld and A. H. Stouthamer, *Arch. Microbiol.*, **107**, 241 (1976).
22. T. A. Chistyakova, I. G. Minkevich, V. K. Eroshin, *Eur. J. Appl. Microbiol. Biotechnol.*, **18**, 225 (1983).
23. W. Hazeu, J. C. de Bruyn, and J. P. van Dijken, *Arch. Microbiol.*, **135**, 205 (1983).
24. C. Edwards, J. A. Spode, and C. W. Jones, *FEMS Lett.*, **1**, 67 (1977).
25. W. P. Hempfling and S. E. Mainzer, *J. Bacteriol.*, **123**, 1076 (1975).
26. P. M. Bruinenberg, R. Jonker, J. P. van Dijken, and W. A. Scheffers, Arch. Microbiol., **142**, 302 (1985).
27. J. C. Gottschal, J. G. Kuenen, *Arch. Microbiol.*, **126**, 33 (1980).
28. P. J. F. Gommers and J. G. Kuenen, unpublished results.
29. B. J. van Schie, J. T. Pronk, R. J. Rouwenhorst, J. P. van Dijken, and J. G. Kuenen, "Energy transduction *in vivo* and *in vitro* by a bacterial membrane-bound glucose dehydrogenase," 13th International Congress Biochemistry, Amsterdam, 1985.
30. L. Dijkhuizen, J. W. C. Timmerman, and W. Harder, *FEMS Microbiol. Lett.*, **6**, 53 (1979).
31. J. A. Duine, J. Frank Jzn and J. K. van Zeeland, *FEBS Lett.*, **108**, 443 (1979).
32. L. Dijkhuizen, "Regulation of autotrophic and heterotrophic metabolism in *Pseudomonas oxalaticus* OX1," Ph.D. thesis, University of Groningen, The Netherlands, 1979.