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A METHOD FOR THE CONTROL OF QUANTITATIVE FIT OF ELEMENTAL AND MACROMOLECULAR COMPOSITIONS OF MICROBIAL BIOMASS

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Introduction

The wide use of microbial cells in biotechnology has given rise to problems connected with the quantitative control of the physiological state of the culture. At the macroscopic level the growth can be characterized quantitatively by the stoichiometric equation where the biomass elemental composition is included (see Herbert, 1976; Madron, 1979). At the level of intracellular metabolism models have been worked out enabling to evaluate the energetic efficiency of the cells (Stouthamer, 1973; Ingraham et al., 1983) and the pattern of metabolic fluxes (Скурида et al., 1984; Вилу et al., 1990) which are based on the quantitative data of biomass monomer composition. Stoichiometric equation of growth and flux model of metabolism form the basis for comprehensive quantitative description of processes of microbiological synthesis and also for cell design (Vilu, 1990; Vilu and Paalme, 1990). The link connecting the macroscopic characteristics of growth with the flux model is the quantitativly fitting data of biomass element and macromolecular composition. So far the analysis of microbial cells with approaches described above have all been based on average biomass composition data compiled from different experiments. At the same time it is well known that the biomass composition changes significantly depending on the growth conditions (Herbert, 1976). The experimental determination of biomass complete composition, however, needs laborious analytic work which gives nevertheless not very reliable results (see Dekkers et al., 1981, table 1). Apparently because of this no data could be found in literature where all the macromolecular components were analyzed in the same experiment. In this article we describe a new approach which enables to obtain quantitatively fitting data on the contents of the complete macromolecular and element composition of biomass. The method is based on the material balance equations between the biomass macromolecular and element compositions. The equations allow to choose the optimal set of components to be experimentally assayed and those which are calculated indirectly. In this work we carried out an experimental study of biomass composition and checked the consistency of the data obtained from one and the same experiment with Escherichia coli. It was shown that in practice only the use of balance equations coupled with the determinations of elemental and macromolecular composition enables to quantify correctly all the elemental and macromolecular components and the residual water in dried biomass samples. The method can be used for the study of any kind of microbial biomass, provided the element composition of the macromolecular components is known.

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Escherichia coli K 12 W 3350 was cultivated in chemostat regime with aeration at 37 °C and pH 6.8 in the medium of following composition (in g/l): NH₄Cl 0.4, KH₂PO₄ 1.5, Na₂HPO₄×12H₂O 8.8, MgSO₄ 0.2. Glucose was autoclaved separately and added in final concentration of 5 g/l. The aim of high glucose/NH4Cl ratio was to obtain the conditions of nitrogen limitation. Dry weight was estimated by straight weighing of washed and dried biomass samples. The biomass was first kept overnight at 70 °C and then at room temperature for three days. Atfer weighing and making elemental analysis, the samples were ground and the powder was dried at 70 °C and kept in the desiccator under vacuo at room temperature until the second run of elemental analyses. From the washed biomass protein was determined by the Folin method (Lowry et al., 1951) with bovine serum albumin as a standard. For the extraction of nucleic acid the material was incubated 25 min at 70 °C in 0.5 N perchloric acid. After centrifuging, RNA was measured from the supernatant by the orcinol method (Ceriotty, 1955) and DNA by the Burton diphenylamine method (Manual of methods . . ., 1981). Ribose was used as a calibration standard for RNA and salmon sperm DNA for the DNA. Polysaccharides were determined with anthrone reagent (Herbert et al., 1971), using glucose as a standard. Purity of the standards was checked, using elemental analysis. The C, H, and N contents were measured by using a Perkin-Elmer model 240 B elemental analyzer. Standard errors were calculated on the 95% confidence level.

Results and discussion

Biomass material balance analysis based on literature data. The most abundant elements in biomass are C, H, O, and N. The bacterial biomass consists of nucleic acids, proteins, lipids and polysaccharides which altogether make over 95% of the cellular dry matter (Stouthamer, 1973). Table 1 shows the average chemical compositions of macromolecular fractions calculated for E. coli. The chemical composition of DNA was calculated for the mean E. coli DNA base composition (Davidson, 1972). The chemical composition of RNA was calculated, assuming that it consists of 80% rRNA, 15% tRNA and 5% mRNA (ibid.). Each fraction was characterized by its mean base composition. The elemental compositions of DNA and RNA vary only a little on changing the base compositions of the fractions in the ranges observed for different species. According to Cedergren and Holme (Neidhardt, 1963) most of E. coli's polysaccharides are in the form of polyglucose. Other sugars are mainly hexoses forming about 1-2% of dry weight (Ingraham et al., 1983). Therefore, the elemental composition of polysaccharide may also be considered constant, and the same as that of glycogen. The elemental composition of protein fraction was calculated as a mean of the amino acid composition data for E. coli: total protein (Morowitz, 1968; Lehninger, 1972; Ingraham et al., 1983),

Table 1

Elemental composition (%; w/w) of the major cell constituents of Escherichia coli

Macromolecular component	С	Н	0	N	Р
Protein	53.38	7,07	21.39	17.12	
Polysaccharides	44.53	6.18	49.29		
Lipid	63.52	8.65	22.24	1.42	4.17
RŇA	35.44	3.71	34.47	16.77	9.60
DNA	38.83	4.15	29.25	17.44	10.36

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total cytoplasmic protein and mean of 30 different proteins (Alroy, Tannenbaum, 1977). The lipid fraction of *E. coli* was considered to consist of 78% of phosphatidylethanolamine, 18% of phosphatidyl-glycerol and 4% of cardiolipin with average fatty acid composition of 35% palmitoleic and 30% *cis*-vaccenic acid (Raetz, 1978; Carty, Ingram, 1981). For yeast the lipids fraction elemental composition is 76.4% C, 12.0% H and 11.5% O (Babel, Müller, 1985), which shows that this fraction is rather variable in different species. Let us designate the contents of RNA, DNA, protein (prot.), lipid (lip.), polyglucose (p.gluc.) and residual water in the biomass samples as n_1, \ldots, n_6 , respectively. Equations of carbon, hydrogen, oxygen and nitrogen balance (Eq. (1)—(4)) show that the elemental and macromolecular compositions of the biomass are related if the macromolecules' chemical compositions are fixed:

biomass	RNA	DNA	prot.	lip.	p.gluc.	water
$\begin{array}{l} C & a & = \\ H & b & = \\ O & c & = \\ N & d & = \end{array}$	$a_1n_1 + b_1n_1 + c_1n_1 + d_1n_1 + d_1n_1$	$\begin{array}{rrrr} a_2n_2 &+\\ b_2n_2 &+\\ c_2n_2 &+\\ d_2n_2 &+ \end{array}$	$a_3n_3 + b_3n_3 + c_3n_3 + d_3n_3$	$\begin{array}{r}a_4n_4 + \\b_4n_4 + \\c_4n_4 + \end{array}$	$a_5n_5 + b_5n_5 + c_5n_5 + d_5n_5$	$ \begin{array}{c} (1) \\ -1/9n_6 \\ -8/9n_6 \\ (4) \end{array} $

a, b, c and d are the respective contents of C, H, O, and N in the total biomass and a_i , b_i , c_i , d_i the corresponding contents of the elements in the six fractions. All the contents are given in weight/weight units. Negligible error is made while taking the chemical composition of RNA for the chemical composition of the whole n.a. fraction (cf Table 1). The described approach was applied to data from the literature. Mean elemental compositions of the biomass of different microbes (Roels, 1980; Ishikawa, Shoda, 1983) were used, and the respective macromolecular compositions were calculated by solving the system of linear equations (Eq. (1) - (4)). When residual water content was neglected by taking $n_6=0$, the resulting values of macromolecular composition were not realistic (Table 2, case a). Variations in the chemical compositions of macromolecular fractions did not improve the results. The results of the calculations with water included in the balance equations and the nucleic acids content fixed (5% DNA and 10% RNA), are presented in Table 2, (case b). These results are much more reasonable and indicate that most of the samples indeed contained water up to 10% of their dry weight. However, as the elemental composition data presented in Table 2 are not supplied with the corresponding experimentally estimated macromolecular compositions the results of the calculations can be evaluated only qualitatively.

Balancing of experimental data on elemental and macromolecular composition of *E. coli*. The *E. coli* cells were cultivated in the chemostat at nitrogen limiting conditions at various dilution rates from 0.05 to 0.54 h^{-1} in order to obtain considerable changes in biomass composition, depending on the dilution rate (Herbert, 1961, 1976). The data set of directly measured biomass components included protein, RNA, DNA, polysaccharide, C. H, N, and ashes. The total mutually balanced biomass composition was calculated, taking the C, H, O, N, and contents of nucleic acids as fixed parameters while solving the system of balance equations (1) - (4) The oxygen content of biomass was calculated as follows:

$$c = 1 - a - b - d - 1/2$$
 ashes, (5)

assuming that the ashes consist mainly of P_2O_5 , K_2O , SO_3 , MgO and the weight of the minor elements (P, K, S, Na, Mg) forming approximately 50% (w/w) of the total amount of ashes.

1.0

Biomass macromolecular compositions (% of dry weight) calculated in two different ways from the data on chemical composition

Table 2

(Roels 1980; Ishikawa, Shoda 1983): a - dry biomass was supposed to consist only of nucleic acids (n. a.), protein (prot.), lipid (lip.), and polysaccharide (p. sacch.); b - residual water in biomass was also included in balance Eqs. (1)-(4)

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SHEET 255 ALADER	Water not included (a)				Water included (b)				
Microorganism	Biomass formula	п. а.	prot.	lip.	p. sacch.	prot.	lip.	p. sacch.	water
Candida utilis Klebsiella	CH1.83O0.54N0.10	-271	300	-110	159	19.3	22.2	39.2	5.9
aerogenes Kl. aerogenes	$\begin{array}{c} CH_{1.73}O_{0.43}N_{0.24}\\ CH_{1.75}O_{0.47}N_{0.24} \end{array}$	$-52 \\ -59$	132 137	43 —53	56 —67	66.9 64.5	12.0 8.2	2.8 7.9	6.1 6.8
Saccharomyces cerevisiae S. cerevisiae	$\begin{array}{c} CH_{1.64}O_{0.52}N_{0.16}\\ CH_{1.83}O_{0.56}N_{0.17} \end{array}$	$-38 \\ -358$	92 406	$-17 \\ -163$	61 184	39.4 40.6	7.5 10.4	38.9 28.3	1.1 7.7
Paracoccus denitriticans Escerichia coli	CH _{1.81} O _{0.51} N _{0.20} CH _{1.77} O _{0.49} N _{0.24}	$-80 \\ -67$	145 145	$-62 \\ -61$	88 76	51.1 64.0	16.6 6.8	11.4 9.4	8.7 7.5
Aerobacter aerogenes	CH _{1.83} O _{0.55} N _{0.25}		163		96 73	63.4 62.3	0.6	14.0	9.3 9.5
E. coli E. coli E. coli	CH1.73O0.53N0.235 CH1.78O0.51N0.235 CH1.78O0.51N0.237	$-48 \\ -69$	122 144	$-52 \\ -64$	71 80	60.5 61.8	0 5.3	20.9 12.6	5.7
E. coli E. coli	CH _{1.81} O _{0.49} N _{0.234} CH _{1.74} O _{0.464} N _{0.26}	-84 -56	168 141	-78 -56	85 64	61.9 70.9	11.0 3.2	6.0 6.8	8.9 6.5

The experimental as well as the calculated compositions of the biomass samples are shown in Table 3. The values of the water contents in the samples determined in our experiments (12-15%) were in good agreement with the moisture content in *E. coli* dried biomass, estimated by using only the moisture uptake kinetics (O'Toole, 1983). In order to control the water content estimation in the biomass through element balancing, the samples were additionally dried (cf Methods) and the decrease in the weight of each sample (5-8%) was measured. After carrying out C, H and N analyses of the additionally dried samples, the water contents were calculated again. The directly measured losses in sample weight and the calculated changes of water contents were in a good agreement, the corresponding ratio being 1.07 ± 0.17 .

The indirectly derived protein and polysaccharide contents in Table 3 can be compared to the experimental values. The ratio of the experimentally determined protein contents to the calculated levels is 1.11 ± 0.06 . It is known from the literature (Peterson, 1977, 1979) that while using the Folin method the extinction coefficients for various protein standards may differ significantly. As we used the Folin method using bovine serum albumin for the calibration we probably overestimated the protein content of *E. coli*. Judging by the data from the literature, the amino acid composition of total protein fraction of cells and, therefore, the element composition of the latter could vary considerably. However, on the basis of the results of the measurements and calculations carried out by ourselves, we showed that in our experimental conditions the amino acid composition of the protein fraction of the bacteria (and therefore also its element composition) was constant with rather great accuracy (the data not shown).

According to our data, the lipid contents calculated vary between 10 and 13.5% of dry weight exceeding somewhat the claimed 9% given in the literature (Neidhardt, 1963). The ratio of the experimentally determined polysaccharide content to that obtained from the calculations was 1.12 ± 0.15 . This could be explained by the great "sensitivity" of the calculated values of polysaccharides to experimental errors (see the text below).

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Macromolecular and elemental compositions of E. coli biomass (% of dry weight) at different dilution rates (D)

	Z	$\begin{array}{c} 10.6\\111.6\\111.8\\112.3\\12.7\\12.7\\12.7\end{array}$
mass	0	$\begin{array}{c} 31.8\\ 31.9\\ 30.2\\ 28.7\\ 29.2\\ 29.2\\ 28.0\\ 28.4\\ 28.4\\ 28.4\end{array}$
ree bio	Н	6.6 6.5 6.5 6.5 6.7 6.7 6.7
water-fi	U	49.5 49.7 50.1 50.0 49.9 49.7 49.2
jo no	Poly- glu- cose	27.8 21.4 22.6 17.5 13.5 13.5 13.5 13.6 13.6
mpositi	Lipid	9.9 10.7 10.3 13.5 13.0 13.0 12.3 12.3 12.3 12.3
ated co	Prot- tein	$\begin{array}{c} 48.1 \\ 51.1 \\ 51.1 \\ 52.2 \\ 53.6 \\ 53.3 \\ 51.2 \\ 51.2 \end{array}$
Calcula	DNA	3.6 4.7 4.5 4.5 4.5 4.2 4.2
1	RNA	10.5 12.1 13.4 13.6 15.1 16.1 16.1 18.9
	Water	
1	Ashes	3.8 5.7 5.5 5.5
	N	$\begin{array}{c} 9.30\\ 9.74\\ 9.74\\ 10.22\\ 10.77\\ 11.11\\ 11.11\\ 11.02\\ 10.98\end{array}$
) water	H	7.19 7.33 7.17 7.18 7.18 7.18 7.18 7.18 7.18 7.18
orackets	C	43.33 43.77 41.99 43.44 43.86 43.86 43.80 42.81 42.63
en in l	lcose	$\begin{array}{c} (23.1) \\ (12.7) \\ (16.9) \\ (10.7) \\ (10.7) \\ (10.7) \\ (13.0) \end{array}$
are giv	Polygli	24.3 18.8 19.2 15.1 13.3 11.9 11.9 11.2 11.2
alues		() () () () () () () () () () () () () (
ot of ited v	otein	$\begin{array}{c} 1 & (42. \\ 1 & (45. \\ 1 & (41. \\ 45. \\ 1 & (44. \\ 45. \\ 47. \\ 1 & (44. \\ 1 & (44. \\ 44. \\ 1 & (44. \\ 1 $
calcula	Pr	43. 54. 56. 50. 53. 53.
))	DNA	3.2 3.9 3.7 3.7 3.7
	RNA	9.2 10.6 11.4 11.8 13.2 13.2 14.1 15.0 16.4
	D, h ⁻¹	0.05 0.12 0.19 0.26 0.34 0.34 0.39 0.54

Table 4

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Changes in calculated biomass components' values as a result of variations of initial data

7_{DSe} Lipid Protein Water .5 -42.0 -2.0 -0.6 .4 9.5 -2.7 -0.4 .3 2.5 -0.9 -0.2 .4 -4.4 2.8 0.3 .9 17.7 0 -9.2 .0 26.3 0 9.3
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.0 26.3 0 9.3

In order to obtain compositions of water-free biomass, the values given in Table 3 were correspondingly recalculated, using the water contents data. Table 3 shows the compositions of water-free biomass where all the values of the components are mutually consistent. After recalculations the oxygen content of water-free biomass is 28—32% (instead of 36—38% in samples containing residual water), which is also in agreement with data in the literature (Herbert, 1976; Roels, 1980; Ishikawa, Shoda, 1983).

In order to evaluate the range of variation of the calculated values due to the experimental errors of the initial data, biomass composition at $D=0.34h^{-1}$ was taken as an example. The experimentally obtained values of dry weight, macromolecular and element compositions were varied in the range of their errors of estimate, as shown in Table 4. The corresponding changes of the calculated values of other components are also given in Table 4. It is seen from Table 4 that the most "sensitive" fractions to the errors in experimental determination are polysaccharides and lipids. This could explain, as noted, rather high variation of the calculated lipid and polysaccharide values in Tables 2 and 3, and suggests that at least one of them should be measured experimentally. Errors in calculations of protein contents are less than 5%, which allows to use nitrogen balancing for indirect protein estimation. The error in water content estimation is caused mainly due to the errors of elemental composition estimations. It is important to notice that the value of the calculated water content is affected very little by the value of the contents of nucleic acid. Even when varying RNA contents from 5 to 25% of dry weight, water content varies only from 12.8 to 11.4% of biomass. This means that the biomass water contents could be calculated on the basis of C, H, and N analyses, assuming the nucleic acid level to be in the physiological range.

The results show that while using direct experimental values of biomass composition the data do not match. The main reason for this is the residual water in the dry biomass samples and overestimation of protein content. On the basis of the results obtained we suggest, as a practical recommentation, to determine experimentally RNA, DNA, C, H, N, and polysaccharides (or lipid) from which the other components: protein, lipid (or polysaccharide), oxygen and water could be then calculated, using Eq. (1)-(5). In fact only parallel determination of elemental composition of biomass and at least some of its macromolecular fractions with subsequent check of the consistency of the data, using element balance equations, enable to obtain reliable and quantitatively fitting description of the processes of microbiological synthesis. The chemical composition of the macromolecular fractions given in Table 1 should be adjusted to the corresponding organism, remembering that the lipid fraction is the most variable of them in different organisms.

In this paper the experimental problems of matching element composition of biomass with its macromolecular composition were solved and the suggestions for the practical use of the approach developed were given. The analysis carried out should be useful in cases when the quantitative description of growth processes, processes of microbiological synthesis, is used for the optimization of production processes or for the quantitative optimization of intracellular metabolism.

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MIKROOBSE BIOMASSI ELEMENT- JA MAKROMOLEKULAARSE KOOSTISE KVANTITATIIVSE VASTAVUSE KONTROLLI MEETOD

Võttes aluseks biomassi makromolekulaarsete iraktsioonide (nukleiinhapped, valk, polüsahhariidid, lipiidid) keskmised elementkoostised, on seatud omavahel vastavusse biomassi makromolekulaarne ja elementkoostis. *E. coli* rakke kultiveeriti kemostaatses režiimis lämmastiku limitatsioonil eri läbivoolukiirustel, et saada erineva koostisega biomassi proove. Analüüsides saadud tulemusi on leitud, et elementide bilansi võrrandite kasutamisel on võimalik biomassi element- ja makromolekulaarne koostis omavahel koos-kõlla viia, kui võtta arvesse jääkvee sisaldus biomassis ning korrigeerida Folini meeto-dil leitud valgusisalduse väärtusi vastavalt N bilansile. Selgus, et lipiidide ja polüsahhariidide kaudse määramise viga on vaadeldud komponentide puhul suurim ja 'seetõttu tuleb vähemalt üks nendest määrata eksperimentaalselt. Jääkvee sisaldust on võimalik määrata *ca* 10% täpsusega, kasutades ainult elementkoostise andmeid.

Калью ВАНАТАЛУ, Райво Вилу

МЕТОД КОНТРОЛЯ КОЛИЧЕСТВЕННОЙ СВЯЗИ МЕЖДУ ЭЛЕМЕНТНЫМ И МАКРОМОЛЕКУЛЯРНЫМ СОСТАВОМ МИКРОБНОЙ БИОМАССЫ

На базе усредненных данных элементных составов главных макромолекулярных компонентов (нуклеиновые кислоты, белок, полисахариды, липиды) проведено сопоставление макромолекулярных и элементных составов биомассы. Для получения проб с различными составами биомассы клетки *E. coli* культивировали в хемостатном режиме при азотном лимите. При анализе полученых данных выяснили, что с помощью уравнений баланса макромолекулярные и элементные составы биомассы можно связать количественно только в случае, если в пробах биомассы учитывается содержание остаточной воды и корригируется определенное методом Фолина содержание белка в соответствии с балансом азота. Выяснилось, что из рассмотренных компонентов самые большие ошибки отмечены у липидов и полисахаридов при косвенном определении, и один из этих двух параметров необходимо определить экспериментально. Содержание остаточной воды можно определить с точностью 10%, используя только данные элементного состава.