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Escherichia coli high-cell-density culture: carbon mass balances and release of outer membrane components

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Abstract Carbon mass balances were calculated in fed-batch cultures of *E. coli* W3110, using mineral medium with glucose as the limiting substrate. The carbon recovery, based on biomass, CO_2, and acetate was ~90% at the end of the culture (25 h, 27 g L^{-1} dw). The missing carbon remained as soluble organic compounds in the medium. Outer membrane (OM) constituents, such as lipopolysaccharides (LPS), phospholipids (PL), and carbohydrates (each at ~1 g L^{-1}) contributed to 63% of the extracellular carbon. The amount of released LPS and PL equaled the total amount of OM bound to the cells in the culture. Small amounts of DNA and protein detected in the medium indicated that no cell lysis had occurred. Acetate, lactate, ethanol, formate, succinate and amino acids (Glu, Gln, Asp, Asn, Ala, Gly, Ser) were detected in the culture medium, but made up only a few percent of the extracellular carbon mass. The remaining 30% was not identified, but was assumed to constitute complex carbohydrates.

Keywords Carbon balance, *Escherichia coli*, Fed-batch culture, Outer membrane, Lipopolysaccharide

List of symbols

C_{CO2}	CO_2 carbon (g)
C_{EtOH}	ethanol carbon (g)
$C_{EtOH,samp}$	ethanol carbon in sample (g)
C_{Glc}	glucose carbon (g)
$C_{Glc,feed}$	glucose carbon fed into bioreactor (g)
$C_{Glc,samp}$	glucose carbon in sample (g)
C_{HAc}	acetate carbon (g)
$C_{HAc,samp}$	acetate carbon in sample (g)
$C_{metabolites}$	sum of the carbon in acetate, formate, lactate, and succinate (g)
$C_{metabolites,samp}$	sum of carbon in acetate, formate, lactate, and succinate in sample (g)
C_P	product carbon (g)
C_{rec}	carbon recovery (%)
C_S	substrate carbon (g)
C_{samp}	carbon in sample (g)
C_X	biomass carbon (g)
$C_{X,samp}$	biomass carbon in sample (g)
$CO_{2,in}$	CO_2 concentration in inlet gas (%)
$CO_{2,out}$	CO_2 concentration in exit gas (%)
CPR	CO_2 production rate (mol h^{-1})
$[C_{citr}]$	carbon concentration of citrate (g L^{-1})
$[C_{culture}]_{calculated}$	calculated carbon concentration of culture broth (g L^{-1})
$[C_{culture}]_{measured}$	measured carbon concentration of culture broth (g L^{-1})
$[C_m]$	carbon concentration in medium (g L^{-1})
F^*	feed solution fed into bioreactor (g)
Glc^*	glucose (g)
Glc^*_{feed}	glucose fed into bioreactor (g)
Glc^*_{samp}	glucose in sample (g)
$O_{2,in}$	O_2 concentration in inlet gas (%)
$O_{2,out}$	O_2 concentration in exit gas (%)
OUR	oxygen utilisation rate (mol h^{-1})
Q_{in}	inlet gas flow rate (L h^{-1})
Q_{out}	exit gas flow rate (L h^{-1})
V	culture volume (L)
V_{base}	volume of base fed into bioreactor (L)
V_{feed}	volume of feed solution fed into bioreactor (L)
V_m	molar volume of gas (L mol^{-1})
V_{samp}	sampling volume (L)
X	dry cell weight (g L^{-1})
X^*	dry cell weight (g)
X^*_{samp}	dry cell weight in sample (g)
$Y^*_{X/Glc}$	overall biomass yield on glucose (g g^{-1})
ρ	density of feed solution (g L^{-1})

Subscripts

0	at 0 h
1	at first moment
2	at second moment
i	at any moment
0–i	from 0 h to any moment

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1 Introduction

The high-cell-density fed-batch culture mode is frequently utilised by industry for recombinant protein production in *Escherichia coli* (*E. coli*) processes. A high cellular yield coefficient ($Y_{X/S}$) is essential for obtaining high cell densities, given that biomass formation rate is limited by the substrate feed rate, which in turn is limited by the oxygen

transfer rate. A decrease in $Y_{X/S}$ is associated with increased oxygen consumption for nonbiomass purposes. The maximum value of $Y_{X/S}$ for *E. coli* grown in a glucose-limited fed-batch culture is around 0.5 g g^{-1}, but $Y_{X/S}$ declines at higher cell densities. The explanation for this behaviour is an increased requirement for maintenance energy at low growth rates, provided that no cell lysis occurs [1], and it is generally assumed that no by-products are formed during glucose limitation. However, such conclusions may be erroneous if not supported by proper mass-balance calculations. Such a study has not been made, as far as we are aware, on *E. coli* high-cell-density fed-batch cultures. This may be explained by the complexity and the dynamics of this culture mode. It involves changes in culture volume caused by the substrate feed, by base addition for pH control, and by samples taken from the bioreactor, resulting in continuous changes in the concentrations of all solutes and biomass. Moreover, the accuracy of the gas analyser and the gas flow meter are critical, because CO_2 evolution from the culture is an important part of the carbon recovery calculation.

This study presents carbon mass-balance calculations and analyses of various extracellular metabolites in a bioreactor fed-batch culture of *E. coli* using glucose mineral medium. The results show that a significant amount of consumed glucose was converted to products, which were released into the culture medium; the dominant compounds originated from the outer membrane. Release of the outer membrane (OM), including the lipopolysaccharide layer (LPS), was reported as early as the 1960s [2, 3]. Issues such as mechanisms [4] and function [5] of the release, factors affecting the release [6, 7], and the concomitant changes of cell surface morphology [8] are still actively studied. However, no attention has been paid to carbon loss in bioprocesses caused by OM release, and the phenomenon seems to have been overlooked by biotechnologists.

2
Materials and methods

2.1
Strain and medium

Escherichia coli K-12 W3110 (F^-, IN(rrnD-rrnE)1; λ^-) [9] was stored at –80°C in LB medium containing glycerol (15%). The culture was reactivated by inoculating a frozen aliquot (50 μL) into an Erlenmeyer flask with 100 mL mineral medium, which was incubated at 37°C on a rotary shaker until the culture had reached an OD_{610} of 1 (approx. 16 h).

The mineral medium contained per litre: Na_2SO_4, 2.0 g; NH_4Cl, 4.3 g; $(NH_4)_2SO_4$, 2.0 g; $Na_2HPO_4{\cdot}2H_2O$, 3.0 g; KH_2PO_4, 7.0 g; $(NH4)_2$-H-citrate, 1.0 g; thiamine·HCl, 0.1 g; 2 mL $MgSO_4$ solution (1 mol L^{-1}); 2 mL trace-element solution [10]; and glucose, 2.5 g. The feeding solution contained 250 g L^{-1} glucose, and the other components at double concentration.

2.2
Fed-batch culture

A 12-L bioreactor (Belach Bioteknik, Sweden, supported by KENT BS-4, GENESIS computer process-control program) was used. The gas flow meter was newly calibrated by the manufacturer. The gas analyser (Industrial Emissions monitor Type 1311, Brüel and Kjær, Denmark) was calibrated before each cultivation.

The inoculum, 0.5 L at $OD_{610}\sim1$, was added to 4.5 L mineral medium in the bioreactor. The culture was started with a 4.55-h batch phase. This was followed by an 8-h exponential feeding phase with an initial feed rate of $\sim$10 mL h^{-1} and a final feed rate of $\sim$100 mL h^{-1}; the feed rate constant was 0.26. Thereafter, the feeding rate was kept constant at $\sim$100 mL h^{-1}. The culture was run for 25 h at 37°C. The culture pH was controlled at 6.8 by 25% of ammonia solution. The dissolved oxygen tension was never below 40%. The gas flow rate, CO_2, and O_2 concentrations in the outgoing gas were automatically recorded on-line.

Samples were taken every hour as well as at the moment when the culture was switched from batch to fed-batch phase. The sampling device was washed by the culture before the formal sampling, and the withdrawn volumes were carefully recorded. An aliquot of the culture broth was frozen directly in liquid N_2. The remaining sample was centrifuged, and a part of the supernatant was filtered through a 0.45 μm cellulose acetate filter, while the other part was kept unfiltered. All samples were kept at –20°C for later analyses.

2.3
Analysis

Dry weight of biomass was determined by centrifuging 3×5 mL of cell suspension samples in preweighed tubes, washing the cells with saline, and drying overnight at 105°C before weighing.

The carbon concentration in the cell-free spent medium, both filtered and unfiltered, and in whole culture broth as well as the carbon content in dried biomass were analysed by a carbon analyser (TOC-5000, Shimadzu). In brief, total carbon was decomposed into CO_2 by catalytical oxidation at 680°C, and the formed CO_2 was detected by a nondispersive infrared gas analyser.

For the determination of lipopolysaccharide (LPS), also known as endotoxin, the unfiltered samples were used. LPS was quantitated by a chromogenic Limulus amebocyte lysate (LAL) assay (test kits from Charles River Endosafe). LAL is a reagent prepared from washed blood cells (amebocytes) of *Limulus polyphemus*, the horseshoe crab. The LAL contains an enzyme system that is activated in the presence of endotoxin. The activated enzyme splits off para-nitro aniline (pNA) from the chromogenic substrate S-2423, which produces a yellow colour. The pNA release is continuously measured at 405 nm [11].

Reducing sugar was measured by using the Somogyi–Nelson colorimetric method. Free reducing sugars were measured before, and total reducing sugars were measured after hydrolysis in 3 mol L^{-1} of HCl at 100°C for 40 min. Glucose was used as the standard, and the absorbance was measured at 560 nm after the colour reaction. Total phospholipids (PL) in the medium were extracted by chloroform and determined according to the method of Bligh and Dyer [12]. Both filtered and unfiltered medium

samples were used for analyses of reducing sugars and phospholipids.

The concentration of DNA in the medium was determined by measuring fluorescence (DyNA Quant 200, Hoefer Pharmacia Biotech) using dye binding (Hoechst 33258) to DNA [13]. The concentration of protein in the medium was analysed by determining the adsorption of Coomassie Brilliant Blue G-250 to protein [14]. The unfiltered cell-free medium samples were used for the DNA and protein analyses.

The filtered medium samples were used for analysis of small metabolites. The concentrations of glucose, acetic acid, ethanol, formic acid, D- or L-lactic acid, succinic acid, and citrate were measured enzymatically (test kits from Boehringer Mannheim). Evaporation of ethanol from the bioreactor was estimated in a cell-free control 'culture'. Amino acids were analysed by HPLC (Waters) using the Pico-Tag system, and a C18 reversed-phase column [15].

2.4 Calculations and data processing

The strategy to calculate carbon balances in a fed-batch culture is based on the mass of carbon in the total reactor volume. Volume changes due to feeding and sampling were included in the calculations, as was the mass of carbon in the samples. The data presented in Figs. 2b and 3 are overall data, i.e. are calculated from the beginning of the culture to each time-point represented. The total carbon recovery is

$$C_{\mathrm{rec}_{0-i}} = \frac{\sum_{0}^{i} C_{\mathrm{P}}}{\sum_{0}^{i} C_{\mathrm{S}}} 100. \tag{1}$$

The carbon recovery in Fig. 2b was calculated as

$$C_{\mathrm{P}_i} = (C_{X_i} - C_{X_0}) + (C_{\mathrm{HAc}_i} - C_{\mathrm{HAc}_0}) + C_{\mathrm{CO2}_{0-i}} + C_{X,\mathrm{samp}_{0-i}}, \tag{2}$$

and

$$C_{\mathrm{S}_i} = C_{\mathrm{Glc}_0} - C_{\mathrm{Glc}_i} + C_{\mathrm{Glc,feed}_{0-i}} - C_{\mathrm{Glc,samp}_{0-i}}, \tag{3}$$

where

$$C_{X_i} = X_i \cdot V_i \cdot 0.48. \tag{4}$$

The carbon content in dried biomass is 0.48 g g^{-1}.

The culture volume was calculated according to

$$V_2 = V_1 + (V_{\mathrm{feed2}} - V_{\mathrm{feed1}}) + (V_{\mathrm{base2}} - V_{\mathrm{base1}}) - V_{\mathrm{samp1}}, \tag{5}$$

where

$$V_{\mathrm{feed}_{0-i}} = \frac{F^*_{0-i}}{\rho}. \tag{6}$$

The feed solution was placed on a balance, and F^* was continuously recorded. The density of feed solution ρ was measured by weighing (at room temperature), in triplicate, 5.00-mL aliquots of the feed solution.

The concentration of soluble carbon in the medium (Fig. 3a) was calculated as:

$$[C_{\mathrm{m}}]_i = \frac{C_{\mathrm{Glc}_0} + C_{\mathrm{Glc,feed}_{0-i}} - C_{\mathrm{samp}_{0-i}} - C_{X_i} - C_{\mathrm{CO2}_{0-i}}}{V_i} + \frac{C_{\mathrm{EtOH}_{0,\mathrm{corr}}} + C_{\mathrm{metabolites}_0}}{V_i} + [C_{\mathrm{citr}}]_i, \tag{7}$$

where

$$C_{\mathrm{samp}_{0-i}} = C_{\mathrm{Glc,samp}_{0-i}} + C_{X,\mathrm{samp}_{0-i}} + C_{\mathrm{EtOH,samp}_{0-i}} + C_{\mathrm{HAc,samp}_{0-i}} + C_{\mathrm{metabolites,samp}_{0-i}}, \tag{8}$$

and $C_{\mathrm{EtOH0,corr}}$ is the amount of ethanol carbon at time 0, corrected for the evaporation until time i. The quantity $C_{\mathrm{metabolites}}$ is the sum of the amount of carbon in acetate, formate, lactate, and succinate. Citrate is not metabolised, and the amount of carbon can therefore be calculated directly from the concentration.

The carbon recovery in Fig. 3b was calculated on the basis of the measured carbon content in the whole culture broth

$$C_{\mathrm{rec}_{0-i}} = \frac{[C_{\mathrm{culture}_i}]_{\mathrm{measured}}}{[C_{\mathrm{culture}_{0-i}}]_{\mathrm{calculated}}} \cdot 100, \tag{9}$$

where

$$[C_{\mathrm{culture}_{0-i}}]_{\mathrm{calculated}} = \frac{C_{\mathrm{Glc}_0} + C_{\mathrm{Glc,feed}_{0-i}} - C_{\mathrm{samp}_{0-i}} - C_{\mathrm{CO2}_{0-i}}}{V_i} + \frac{C_{\mathrm{EtOH}_{0,\mathrm{corr}}} + C_{\mathrm{metabolites}_0}}{V_i} + [C_{\mathrm{citr}}]_i. \tag{10}$$

The CO_2 production and oxygen utilization rates, *CPR* and *OUR*, respectively, were calculated according to the following equations:

$$CPR = \frac{Q_{\mathrm{out}} \cdot CO_{2,\mathrm{out}} - Q_{\mathrm{in}} \cdot CO_{2,\mathrm{in}}}{100 \cdot V_{\mathrm{m}}}, \tag{11}$$

and

$$OUR = \frac{Q_{\mathrm{in}} \cdot Q_{2,\mathrm{in}} - Q_{\mathrm{out}} \cdot Q_{2,\mathrm{out}}}{100 \cdot V_{\mathrm{m}}}, \tag{12}$$

where Q_{out} was calculated from the mass balance of nitrogen in the ingoing and outgoing gas.

The overall biomass yield $Y^*_{X/S}$ was calculated from the total amount of biomass (g) in the reactor, and from the amount of glucose (g) consumed

$$Y^*_{X/\mathrm{Glc}_i} = \frac{X^*_i - X^*_0 + X^*_{\mathrm{samp},0-i}}{Glc^*_0 + Glc^*_{\mathrm{feed},0-i} - Glc^*_i + Glc^*_{\mathrm{samp},0-i}}. \tag{13}$$

Calculation of the specific rates for growth μ, substrate uptake q_s, and OM production q_{OM} was based on mass-balance equations.

All calculations were performed using commercially available software (Microsoft Excel 98, Kaleidagraph 3.08,

Synergy Software). The kinetic analyses were made by curve-fitting techniques.

3
Results

3.1
Fed-batch culture

The growth profile, the feeding rate, and the change in culture volume throughout the fed-batch culture were determined (Fig. 1a). The culture volume, which initially was 5.0 L, first decreased because of sampling, but eventually increased to 5.9 L because of feeding. At 25 h, 27 g L^{-1} dry weight of biomass had formed. In total, 162 g of biomass was produced, including that contained in the withdrawn samples. The kinetic parameters μ, q_{Glc}, q_{CO2}, and q_{O2} dropped upon transition from batch to fed-batch (Fig. 1b). Although μ remained relatively constant at 0.26 h^{-1} during the exponential feeding phase, q_{Glc}, q_{CO2}, and q_{O2} increased slightly. After that, all rates decreased throughout the culture. The respiratory quotient *RQ* was almost constant during the whole fed-batch, at around a value of 1.1 (not shown).

3.2
Yield coefficients and carbon recovery

The overall yield coefficient $Y^*_{X/Glc}$ decreased from a level around 0.50 g g^{-1} during the first 10 h of culture, to 0.42 g g^{-1} at the end of the culture (Fig. 2a). This might be explained by an increased maintenance energy demand in relation to the total energy demand at lower growth rates in the fed-batch culture. However, as the momentary yield μ/q_{Glc} decreased to about 0.17 g g^{-1} at the end of the culture, and the increases in momentary $Y_{CO2/Glc}$ and $Y_{O2/Glc}$ were comparatively small, there may be other explanations as well.

To further analyse this situation the carbon balance during the entire culture was calculated. To do this, the carbon content in the dried biomass was first determined to be 0.47–0.48 g g^{-1}. By summing up the carbon in biomass, CO_2, and acetate it became clear that only ~90% of the substrate carbon was recovered in these products at the end of the culture (Fig. 2b). Obviously, other products than biomass and CO_2 must have been formed.

In order to identify the remaining products, the carbon concentration in the cell-free spent medium and in whole culture broth (including cells) was determined. Indeed, soluble carbon compounds accumulate in the culture medium as growth proceeds in the fed-batch culture (Fig. 3a). The extracellular carbon concentration was also calculated from the added glucose and citrate included in the medium, formed biomass, and carbon dioxide, and the calculated data were found to fit well to measured data (Fig. 3a). This calculation also included the metabolites present at time 0. The basal level of soluble carbon in the culture at 5–10 h originated mainly from medium citrate. At the end of the culture (25 h), organic compounds (citrate excluded) corresponding to 2.5 g L^{-1} carbon, or to 6.3 g L^{-1} glucose, had accumulated in the medium. In total, in the whole culture the amount of produced, unidentified organic compounds corresponded to 15 g glucose carbon, or to 37.5 g glucose.

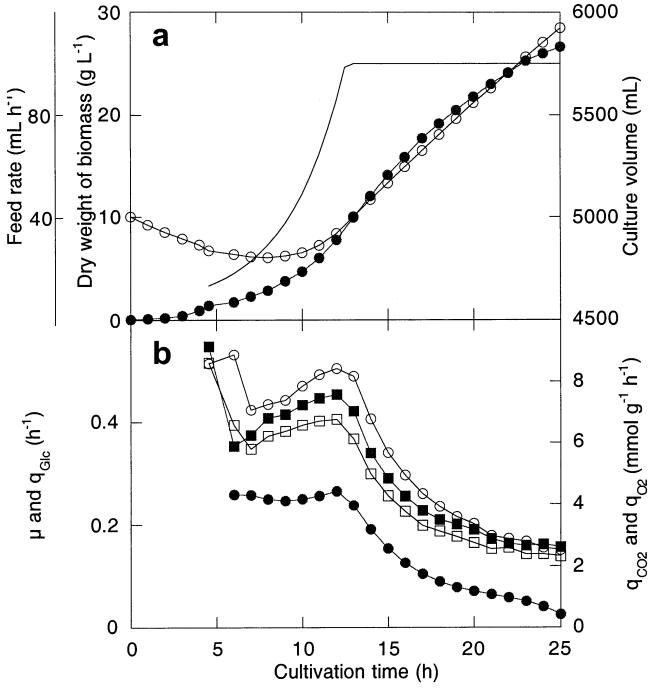

Fig. 1a, b. Kinetic characteristics of an *E. coli* fed-batch culture: **a** dry weight of biomass (-●-), culture volume (-○-), and substrate feed profile (—); **b** specific rates for growth μ (-●-), glucose consumption q_{Glc} (-○-), CO_2 production q_{CO2} (-■-), and O_2 consumption q_{O2} (-□-)

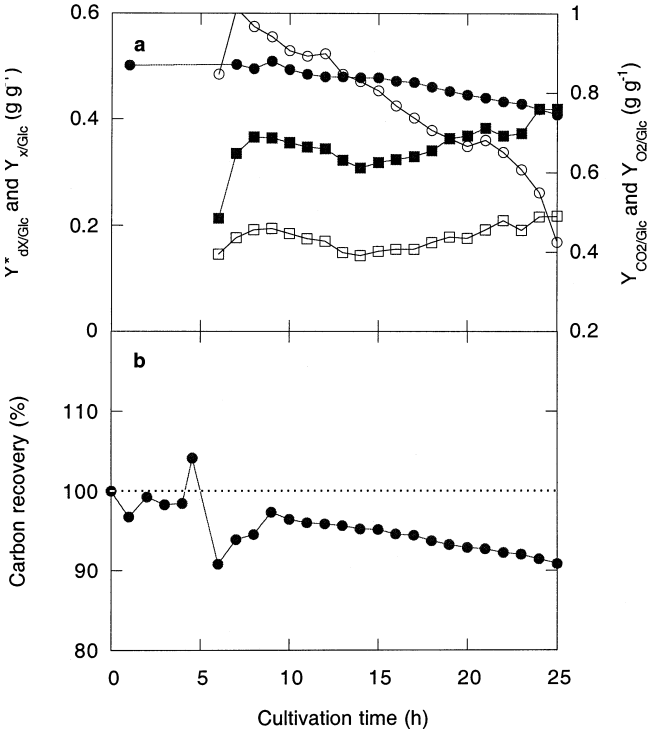

Fig. 2a, b. Yield coecients and carbon recovery in the fed-batch culture: **a** accumulated yield of biomass on glucose $Y^*_{X/Glc}$ (-●-), and momentary yields $Y_{CO2/Glc}$ (-■-), $Y_{O2/Glc}$ (-□-), and $Y_{X/Glc}$ (-○-); **b** glucose carbon recovered in biomass, CO_2, and acetate (-●-) from Eqs. (2) and (3)

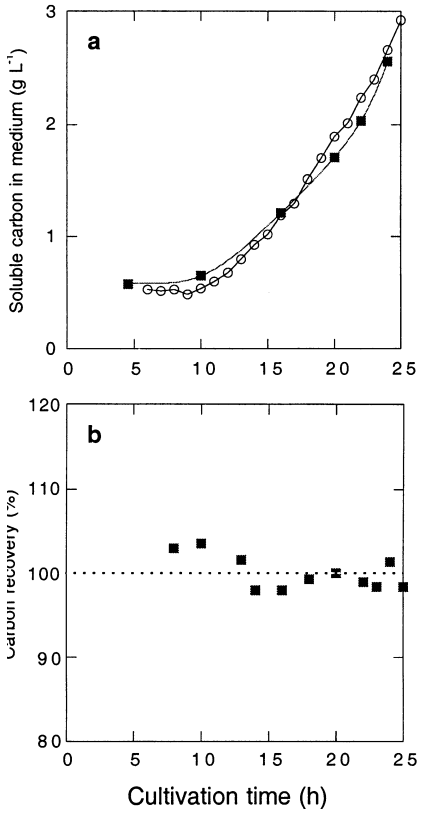

Fig. 3. **a** Soluble carbon concentration in the culture medium as determined by measurement (-■-), and by calculation (-○-). **b** Carbon recovery calculated from the measured carbon content in the culture broth including cells (-■-) using Eqs. (9) and (10)

The carbon recovery was then calculated from the measured carbon content in the culture broth including cells (Fig. 3b). The results show that the carbon balances are close to 100% during the constant feeding phase. Thus, we conclude that the missing carbon compounds have been found, and remain in the medium.

3.3
Analysis of extracellular components in the medium

An analytical program, aimed at identifying the extracellular compounds and understanding the metabolism of *E. coli* during fed-batch conditions, was undertaken. To investigate whether cell lysis was a problem, the presence of protein and nucleic acids were first examined. Almost no DNA was found in the medium, and the profile of the small amounts of protein that accumulated followed the growth curve well (Fig. 4). This indicates that the extracellular compounds did not originate from lysed cells.

The production of metabolites such as lactate, formate, succinate, ethanol, and acetate, which frequently occur in *E. coli* cultures, could only account for a minor part of the unidentified compounds (Fig. 5a, b). At the end of the culture, the sum of lactate, formate, ethanol, acetate, and succinate was 36 mg L^{-1}. Figure 5a2 also shows the residual glucose concentration, which increased to 84 mg L^{-1} during the fed-batch phase, a phenomenon also observed by others [16, 17, 18]. A comparison of the ethanol

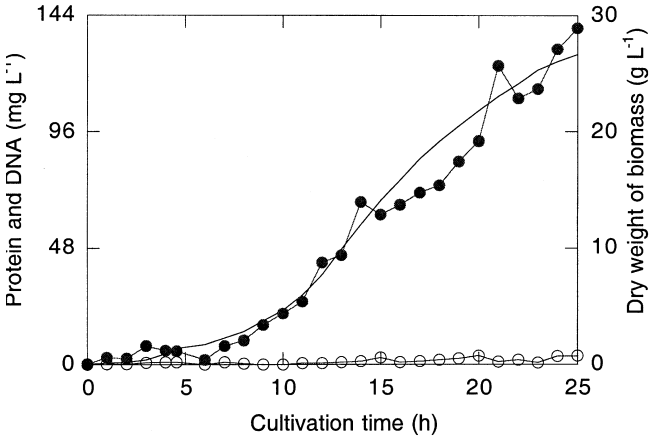

Fig. 4. Concentration of extracellular DNA (-○-), protein (-●-), as well as biomass (—)

concentration in the fed-batch culture and in a cell-free fed-batch 'culture' shows that the decrease in ethanol concentration does not depend on evaporation, although some evaporation/dilution occurs (Fig. 5b). The accumulation of citrate from medium feeding is also shown in Fig. 5b.

Leakage, or production of the amino acids glutamate, glutamine, aspartate, asparagine, alanine, glycine, and serine did occur, but to a small extent; at the end of the culture 131 mg L^{-1} had accumulated (Fig. 5c). Other amino acids were not detected in the medium.

3.4
Outer membrane components

In contrast to the small metabolites discussed in Sect. 3.3, we found that macromolecules such as lipopolysaccharide (LPS), phospholipids (PL), and carbohydrates, which are the main components of the *E. coli* outer membrane (OM), accumulated to significant levels in the medium during the latter part of the culture (Fig. 6a). The average content of LPS and PL in *E. coli* is 3.4% and 9.1% w/w, respectively [19]. Based on this, the proportion of released OM components relative to that contained in the cell was calculated. As discussed in Sect. 4, it is anticipated that a complete OM is retained on the cell surface in spite of the shedding of OM. At the end of the culture, the amount of released LPS was 95% of the LPS in the cells, and the amount of PL in the medium corresponded to 40% of that in the cell (Fig. 6b). Since the *E. coli* cell envelope contains one layer of LPS in the outer leaflet of the OM and in total three layers of PL (the cytoplasmic membrane and the inner leaflet of the OM), the amount of LPS and PL shed into the medium is approximately equal to the OM of the cells in the culture.

The specific production rates of OM components q_{OM} are shown in Fig. 6c. LPS and PL have a similar profile, while the carbohydrate profile is somewhat different. Nevertheless, it is evident that the specific production rates remain relatively low until the end of the culture. When μ approaches 0.06 h^{-1} (at 20 h), a tremendous increase in production can be seen (inset, Fig. 6c). In fact, at the last sampling point when μ is 0.026 h^{-1}, the sum of the

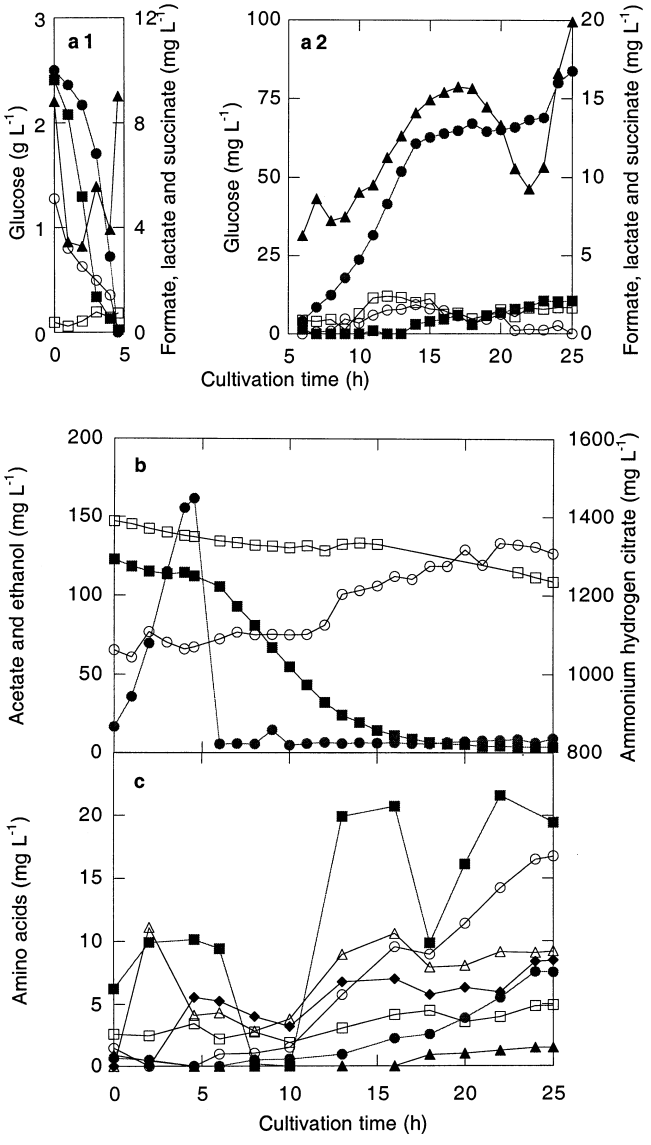

Fig. 5a–c. Concentration of metabolites and substrates in the medium: **a** glucose (-●-), formate (-○-), D-lactate (-■-), L-lactate (-□-), and succinate (-▲-); **b** acetate (-●-), ammonium hydrogen citrate (-○-), ethanol (-■-), and ethanol in a cell-free control 'culture' (-□-). **c** Amino acids: glycine (-●-), aspartate (-○-), glutamate (-■-), asparagine (-□-), serine (-▲-), glutamine (-△-), and alanine (-◆-)

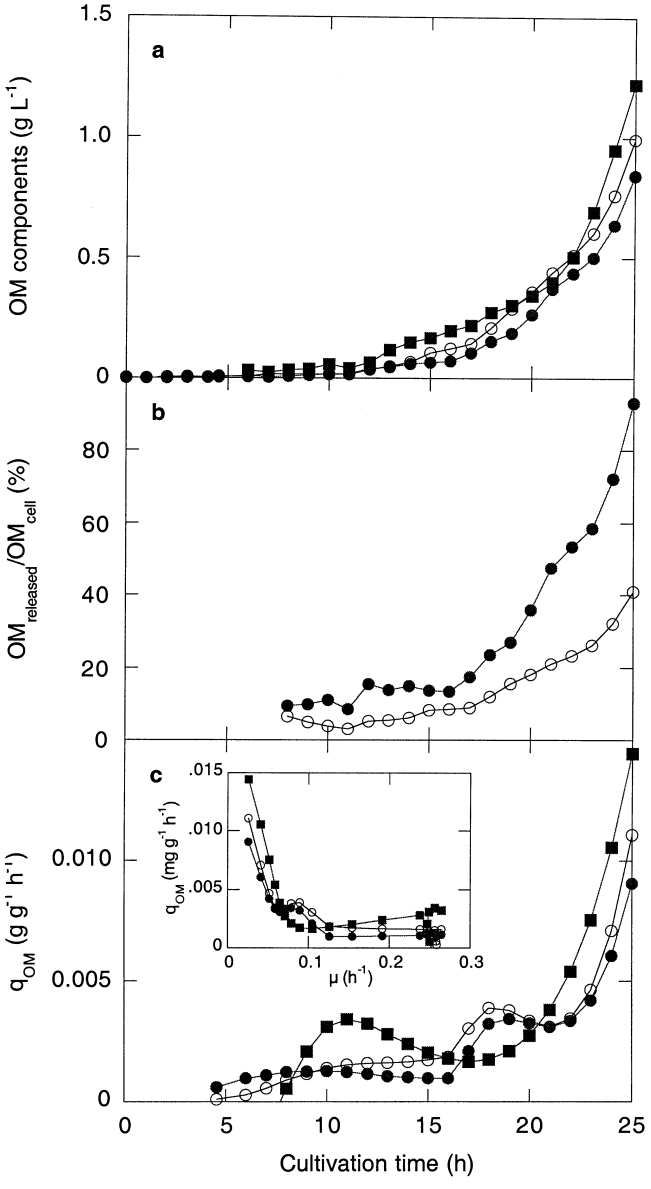

Fig. 6a–c. Release characteristics of outer membrane (OM) components: **a** medium concentration of OM components; **b** proportions between released and cell-bound OM components; **c** specific OM release rate q_{OM} (*inset:* q_{OM} versus specific growth rate μ). Symbols: LPS (-●-), phospholipids (-○-), and total carbohydrates (-■-). The concentration of complex carbohydrates was obtained by subtracting the amount of core oligosaccharide in LPS, and residual glucose from the measured total carbohydrate by the Somogyi–Nelson method

three q_{OM} is 0.034 h^{-1} (0.014+0.011+0.009, Fig. 6c). Thus, at this culture stage only 40% of the cellular biosynthetic capacity is directed to biomass formation, while 60% is used for synthesis of OM components shed into the environment.

3.5
Distribution of glucose carbon in cells and extracellular compounds

The (accumulated) distribution of glucose carbon in cells and products at 25 h culture time is given in Fig. 7. Although LPS, PL, and complex carbohydrates clearly make up 62.6% of the extracellular organic compounds, about 30% (on a carbon basis) still remains to be identified. During the last culture hour, about 50% of the consumed glucose carbon was diverted to CO_2 formation, about 25% to extracellular compounds, and the rest to biomass (data not shown).

4
Discussion

This work demonstrates the importance of carbon recovery calculations. Our results show that a significant part of the cell's biosynthetic capacity is used for purposes other than biomass formation, as justified by the identification and quantification of OM components in the culture medium (Fig. 6). In fact, at the end of the culture (25 h), more extracellular OM compounds than biomass were formed,

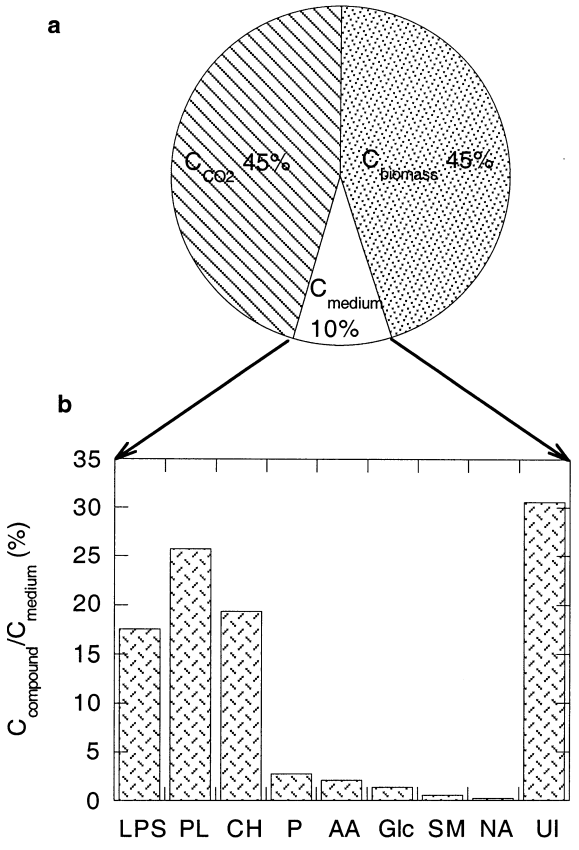

Fig. 7a, b. Substrate carbon distribution and distribution of carbon in various compounds in the medium at 25 h: **a** percentages of glucose carbon converted to biomass, CO_2, and extracellular compounds; **b** percentages of soluble medium carbon (citrate excluded), where LPS, lipopolysaccharide; PL, phospholipids; CH, carbohydrates (nonglucose, non-LPS core oligosaccharide); P, protein; AA, amino acids; Glc, glucose; SM, small metabolites: acetate, lactate, succinate, ethanol, and formate; NA, nucleic acids DNA and RNA (RNA was deduced from the amount of DNA, using a conversion factor of RNA/DNA=20.5/3.1 [19]); UI, unidentified compounds

while small metabolites and amino acids contributed insignificantly to the extracellular carbon (Fig. 5). The momentary yield $Y_{X/S}$, which decreased from above 0.6 g g^{-1} at the beginning of the fed-batch phase to 0.17 g g^{-1} at the end of the culture, clearly shows that a dramatic change in cell physiology takes place during the course of the culture (Fig. 2a). However, the marginal increase in the amount of substrate converted to CO_2 and the amount of oxygen consumed per unit substrate (Fig. 2a) indicate that the energy metabolism remains relatively unaffected by the massive production of extracellular OM components.

4.1
Release of OM components

Lipopolysaccharide (LPS), phospholipids (PL), and carbohydrates (Fig. 6), which are the main components of the Gram-negative cell's OM, were found to be released into the medium by *E. coli* W3110 grown in a glucose-limited fed-batch culture. At the end of the culture significant amounts (about 1 g L^{-1}) of each species had accumulated. The release of OM constituents by growing *E. coli*, including strain W3110, is a normal phenomenon [20, 21]. The shed material may occur as membrane vesicles and as membrane fragments [20, 22]. It has been proposed that OM vesicles are formed when the OM expands faster than the underlying peptidoglycan layer [4]. Thus, the cell-attached OM remains intact, and cell lysis is avoided as the OM release is accompanied by continuous synthesis of new OM components [23, 24]. The release of LPS and PL were synchronous (Fig. 6), which indicates that a close relation between these species exists. The small amounts of extracellular DNA and protein (corresponding to a maximum of 0.12–0.25 g L^{-1} dw) detected (Fig. 4) confirm that almost no cell lysis occurred. Therefore, the OM components in the medium represent shed cell material.

The reason for the rapid release of OM components late in the fed-batch culture is not clear. It is known that the extent of OM release is strain specific [23] and is affected by the environment. For example, EDTA, tris-buffer, or saline promote OM release [7, 25, 22]. The presence of antibiotics [26], phage infection [6, 27], increased temperature [28], and amino acid depletion [20] also enhance the release. However, LPS synthesis was shown to be regulated by the stringent control mechanism, and amino acid deprivation stimulated the rate of LPS release in *relA* mutants but not in *relA*$^+$ strains [21]. Given that *E. coli* W3110 is *relA*$^+$, the possibility still exists that, at the low glucose utilisation rate (0.15 h^{-1}) at the end of the culture, cell proliferation is severely hampered due to energy or precursor limitation, and the consumed glucose is instead used for OM synthesis. In the natural environment OM release may offer protection against external stress or may contribute to pathogenesity. For example, release of LPS delays antibiotic-induced lysis [29] and increases the resistance to EDTA-induced membrane permeability [30]. The released OM vesicles are associated with penicillinase release [31], with heat-labile enterotoxin and Shiga toxin release, and with transfer of virulence gene [8, 5]. The OM vesicles may also be the site for phage attachment [4].

4.2
Unidentified extracellular matter

About 30% of the extracellular material remains unidentified. We suggest that this mainly depends on the underestimation of the amount of extracellular carbohydrates, as we explain in the following. *E. coli* produces two main types of complex carbohydrates in addition to the core oligosaccharide of LPS and the O-antigen, namely the enterobacterial common antigen (ECA), and capsular polysaccharides [32]. ECA consists of repeats of a trisaccharide made up of 4-acetamido-4,6-dideoxy-D-galactose, N-acetyl-D-mannosaminuronic acid, and N-acetyl-D-glucosamine, the latter also being O-acetylated on 70% of the sugar moieties. ECA is either anchored in the OM by covalent linkage to a phosphoglyceride, or by covalent linkage to the core oligosaccharide of LPS. Laboratory strains of *E. coli* such as K-12 and B completely lack the O–antigen polysaccharide region [33]. Capsular polysaccharides are acidic polymers of different composition, containing colanic acid, amino sugar, sialic acid, KDO, glucuronic acid, N-acetylmannosamine, and phosphate. Some of these are also linked to a diacylglycerol and are anchored in the OM. Thus, the *E. coli* polymers are complex, containing numerous

substitutions and building blocks other than hexoses. This complexity cannot be resolved by the analytical method used (acid hydrolysis, reducing sugar) in which glucose was used as standard.

Released OM vesicles also contain embedded proteins, albeit at a different composition than the cell-attached OM [4, 34]. It is possible that part of the protein that is exposed on the outer surface of the vesicles is detected in the protein assay, and is thereby included in the protein measurements. On the other hand, if the embedded segments do not react with the dye, they may remain undetected. Therefore, some fraction of the unidentified carbon compounds could be due to this.

4.3 Process implications

Release of OM components to the medium could create several problems in recombinant protein production processes. It directly decreases the yield of the protein product and may do so also indirectly by decreasing the oxygen transfer rate. The presence of endotoxin in the medium complicates downstream processing and decreases the product quality. In addition, the presence of endotoxin in exhaust gas aerosols constitutes a health hazard, and calls for special treatment of the outlet gas. A better understanding of the factors influencing OM release in industrial bacterial cultures is clearly needed.

References

1. Andersson L, Strandberg L, Häggström L, Enfors S-O (1994) Modeling of high cell density fed batch cultivation. FEWS Microbiol Rev 14:39–44
2. Leive L (1965) Release of lipopolysaccharide by EDTA treatment of *Escherichia coli*. Biochem Biophys Res Commun 21:290–296
3. Knox KW, Vesk M, Work E (1966) Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*. J Bacteriol 92:1206–1271
4. Wensink J, Witholt B (1981) Outer-membrane vesicles released by normally growing *Escherichia coli* contain very little lipoprotein. Eur J Biochem 116:331–335
5. Kolling GL, Matthews KR (1999) Export of virulence genes and Shiga toxin by membrane vesicles of *Escherichia coli* O157:H7. Appl Environ Microbiol 65:1843–1848
6. Taniai K, Wago H, Yamakawa M (1997) In vitro phagocytosis of *Escherichia coli* and release of lipopolysaccharide by adhering hemocytes of the silkworm, *Bombyx mori*. Biochem Biophys Res Commun 231:623–627
7. Pelletier C, Bourlioux P, van Heijenoort J (1994) Effects of sub-minimal inhibitory concentrations of EDTA on growth of *Escherichia coli* and the release of lipopolysaccharide. FEMS Microbiol Lett 117:203–206
8. Wai SN, Takade A, Amako K (1995) The release of outer membrane vesicles from the strains of enterotoxigenic *Escherichia coli*. Microbiol Immunol 39:451–456
9. Smith MW, Neidhardt FC (1983) Proteins induced by anaerobiosis in *Escherichia coli*. J Bacteriol 154:336–343
10. Holme T, Arvidson S, Lindholm B, Pavlu B (1970) Enzymes: laboratory-scale production. Process Biochem 5:62–66
11. Mattsby-Baltzer I, Lindgren K, Lindholm B, Edebo L (1991) Endotoxin shedding by enterobacteria: free and cell-bound endotoxin differ in Limulus activity. Infect Immun 59:689–695
12. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
13. Moe D, Garbarsch C, Kirkeby S (1994) The protein effect on determination of DNA with Hoechst 33258. J Biochem Biophys Methods 28:263–276
14. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
15. Bidlingmeyer BA, Cohens SA, Tarvin TL (1985) Rapid analysis of amino acids using precolumn derivation. J Chromatogr 336:93–104
16. Lin HY, Mathiszik B, Xu B, Enfors S-O, Neubauer P (2001) Determination of the maximum specific uptake capacities for glucose and oxygen in glucose-limited fed-batch cultivation of *Escherichia coli*. Biotechnol Bioeng 73:347–357
17. Castan A, Enfors S-O (2002) Formate accumulation due to DNA release in aerobic cultivations of *Escherichia coli*. Biotechnol Bioeng 77:324-328
18. Xu B, Jahic M, Blomsten G, Enfors S-O (1999) Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with *Escherichia coli*. Appl Microbiol Biotechnol 51:564–571
19. Neidhardt FC, Umbarger HE (1996) Chemical composition of *Escherichia coli*. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella* cellular and molecular biology. American Society for Microbiology, Washington, pp 13–16
20. Hoekstra D, van der Laan JW, de Leij L, Witholt B (1976) Release of outer membrane fragments from normally growing *Escherichia coli*. Biochimi Biophys Acta 455:889–899
21. Ishiguro EE, Vanderwel D, Kusser W (1986) Control of lipopolysaccharide biosynthesis and release by *Escherichia coli* and *Salmonella typhimurium*. J Bacteriol 168:328–333
22. Irvin RT, MacAlister TJ, Chan R, Costerton JW (1981) Citrate-tris(hydroxymethyl)aminomethane-mediated release of outer membrane sections from the cell envelope of a deep-rough (heptose-deficient lipopolysaccharide) strain of *Escherichia coli* O8. J Bacteriol 145:1386–1396
23. Marvin HJP, ter Beest MBA, Witholt B (1989) Release of outer membrane fragments from wild-type *Escherichia coli* and from several *E. coli* lipopolysaccharide mutants by EDTA and heat shock treatments. J Bacteriol 171:5262–5267
24. Tesh VL, Duncan RL Jr, Morrison DC (1986) The interaction of *Escherichia coli* with normal human serum: the kinetics of serum-mediated lipopolysaccharide release and its dissociation from bacterial killing. J Immunol 137:1329–1335
25. Tsuchido T, Katsui N, Takeuchi A, Takano M, Shibasaki I (1985) Destruction of the outer membrane permeability barrier of *Escherichia coli* by heat treatment. Appl Environ Microbiol 50:298–303
26. Bucklin SE, Fujihara Y, Leeson MC, Morrison DC (1994) Differential antibiotic-induced release of endotoxin from gram-negative bacteria. Eur J Clin Microbiol Infect Dis 13 Suppl 1:S43–51
27. Loeb MR, Kilner J (1978) Release of a special fraction of the outer membrane from both growing and phage T4-infected *Escherichia coli* B. Biochim Biophys Acta 514:117–127
28. Mackowiak PA (1984) Relationship between growth temperature and shedding of lipopolysaccharides by gram-negative bacilli. Eur J Clin Microbiol 3:406–410
29. van den Berg C, de Neeling AJ, Schot CS, Hustinx WNM, Wemer J, de Wildt DJ (1992) Delayed antibiotic-induced lysis of *Escherichia coli* in vitro is correlated with enhancement of LPS release. Scand J Infect Dis 24:619–627
30. Voll MJ, Leive L (1970) Release of lipopolysaccharide in *Escherichia coli* resistant to the permeability increase induced by ethylenediaminetetraacetate. J Biol Chem 245:1108–1114
31. Aono R (1989) Release of penicillinase by *Escherichia coli* HB101 (pEAP31) accompanying the simultaneous release of outer-membrane components by Kil peptide. Biochem J 263:65–71
32. Rick PD, Silver RP (1996) Enterobacterial common antigen and capsular polysaccharides. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella* cellular and molecular biology. American Society for Microbiology, Washington, pp 104–122
33. Nikaido H (1996) Outer membrane. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella* cellular and molecular biology. American Society for Microbiology, Washington, pp 29–47
34. Mug-Opstelten D, Witholt B (1978) Preferential release of new outer membrane fragments by exponentially growing *Escherichia coli*. Biochim Biophys Acta 508:287–295