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C. F. Mignone · C. Avignone-Rossa

Analysis of glucose carbon fluxes in continuous cultures of *Bacillus thuringiensis*

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Abstract The glucose carbon fluxes in continuous cultures of *Bacillus thuringiensis* grown in a complex medium have been studied as a function of the growth rate. The results are discussed in the light of a growth model. From reduced nicotinamide adenine dinucleotide (NADH) and carbon balances it was determined that the fraction of glucose consumed for biomass synthesis decreased with the growth rate, while the glucose flux through the tricarboxylic acid (TCA) cycle diminished after a threshold value of $D = 0.34\ h^{-1}$, where D = dilution rate. At the highest growth rate tested, glucose was used almost exclusively as the energy source, via fermentative pathways, which indicates that the yeast extract was used as the carbon source. The specific rate of oxygen consumption increased with growth even after the beginning of the accumulation of acids, indicating that the respiratory chain was not saturated. The results suggest that there is a mismatch between glycolysis and TCA cycle capacity, depending on the growth rate. Furthermore, values of (P/O) ratio and m_{ATP} are presented, where (P/O) is mole of ATP formed per gram atom oxygen consumed by the respiratory chains and m_{ATP} is the maintenance requirement for ATP.

Introduction

Bacillus thuringiensis is a sporulating bacteria able to synthesize a crystalline glycoprotein (δ-endotoxin) that is highly toxic against a wide range of insect larvae. The metabolic pattern of *B. thuringiensis* is closely related to that of *B. cereus*. In batch cultures glucose is degraded through the Embden-Meyerhoff-Parnas (EMP) pathway to yield pyruvate and acetate as the main products. Once glucose is exhausted, the acids are oxidized via the tricarboxylic acid (TCA) cycle (Lüthy et al. 1982), this process being closely associated with the onset of sporulation (Bulla et al. 1971; Benoit et al. 1990). Acetate is partially converted into poly-β-hydroxybutyrate (PHB), which is consumed during sporulation (Benoit et al. 1990). Regarding nitrogen metabolism, it has been known for a long time that ammonia is assimilated through the enzymes alanine dehydrogenase and glutamate dehydrogenase (Aronson et al. 1975).

In spite of the fact that batch cultures are not the best way of studying growth parameters, the majority of the work has been performed batchwise. The use of continuous cultures of *B. thuringiensis* has been limited to a study of spore production (Freiman and Chupin 1973; Khovrychev et al. 1990; Kang et al. 1993). Information about the physiology of *B. thuringiensis* growing in nutrient limited cultures is not available. So, we examined this bacterium for ammonia assimilation, as well as for pyruvate, acetate, and PHB production in glucose-limited continuous cultures. The results have been analysed using carbon, reduced nicotinamide adenine dinucleotide (NADH), and adenosine 5-triphosphate (ATP) balances.

Materials and methods

Microorganism and culture medium

B. thuringiensis var. *israelensis* (serotype H-14) was obtained from the World Health Organization's International Entomopathogenic Bacillus Centre (IEBC Collection, Institut Pasteur, France). The microorganism was maintained on nutritive agar, under vaseline, at 4 °C. Solid medium subcultures were made periodically, incubated at 30 °C for 96 h to obtain complete sporulation of the microorganism, and kept at 4 °C until they were used to inoculate the bioreactor.

C. F. Mignone (✉) · C. Avignone-Rossa
Centro de Investigación y Desarrollo en Fermentaciones Industriales, CINDEFI, Facultad de Ciencias Exactas, UNLP, Calle 47 y 115, (1900) La Plata, Argentina.
Fax: + 54–21–25 4533
E-mail:⟨mignone@nahuel.biol.unlp.edu.ar⟩

Inocula and culture conditions

Continuous cultures were carried out using a medium containing ($g \cdot l^{-1}$): glucose, 3.5; yeast extract, 3; $(NH_4)_2SO_4$, 1.5; K_2HPO_4, 0.75; KH_2PO_4, 0.75; $MgSO_4 \cdot 7H_2O$, 0.25; $MnSO_4 \cdot H_2O$, 0.025; $CaCl_2 \cdot 2H_2O$, 0.04, silicone antifoaming agent (Dow Corning 1520), 0.14. Medium components were sterilized separately by autoclaving at 121 °C for 45 min. Sporulated solid cultures were suspended with sterile distilled water and the suspension was used to inoculate the medium. The 2-l bioreactor (LH Inceltech), with a working volume of 660 ml, was equipped with pH and temperature controls. Initially, the bioreactor was operated batchwise until the cells reached mid-exponential growth. At that moment, feeding of fresh medium was initiated at the selected feed rate by means of a peristaltic pump (Masterflex). The pH was maintained at 6.7 by the automatic addition of 1 N KOH or 1 N H_2SO_4, and the temperature was controlled at 30 °C. Dissolved oxygen was continuously monitored by means of a polarographic dissolved oxygen electrode (Ingold). The agitation speed was fixed at 650 rpm, and the air flow was 0.6 vvm; these conditions promoted dissolved oxygen values above 30% of saturation, which ensured fully aerobic conditions (Avignone-Rossa et al. 1992). Foam formation was prevented by automatic addition of a 10% suspension of a silicone antifoaming agent by means of a peristaltic pump which was intermittently activated for 1 s every 100 s. No variations in dissolved oxygen, optical density (at 625 nm) or oxygen consumption rate were observed after five retention times. Samples were taken after at least six retention times, ice-cooled, and centrifuged at 4 °C. Supernatants and pellets were frozen until analysis. After sampling, the feeding rate was changed to the predetermined value, until the new steady-state was reached.

Analytical procedures

Biomass dry weight was determined as previously described (Avignone-Rossa and Mignone 1993). Total nitrogen content of the biomass was determined by means of the Kjeldahl method employing a Tecator Kjeltec Auto 1030 Analyzer. Ammonium concentration was determined by the phenol-hypochlorite reaction (Weatherburn 1967). Glucose concentration was determined with a glucose oxidase enzymatic kit (Wiener, Argentina). Acetic acid was steam-distilled from the supernatants (Dawes et al. 1971), and its concentration was determined by the method of Hutchens and Kass (1949). Pyruvate was determined using lactate dehydrogenase following the method of Czok and Lamprecht (1974). PHB was determined using the method of Slepecky and Law (Herbert et al. 1971).

Oxygen and carbon dioxide concentrations in the emitted gas were determined using a paramagnetic oxygen analyser (Rosemount Analytical, Model 755 R), and an infrared carbon dioxide analyser (Horiba PIR 2000). Gas flow rates were evaluated with a bubble flowmeter. Oxygen uptake rates and carbon dioxide production rates were determined by a mass balance method according to Cooney et al. (1977).

Results

Results from steady-states reached at different dilution rates (D, see Appendix) are presented in Fig. 1. Glucose concentrations remained at very low values ($< 15\ mg \cdot l^{-1}$) up to $D = 0.63\ h^{-1}$, increasing thereafter, which is in concordance with the observed decrease in biomass concentration. PHB accumulation in the biomass was observed when D values were higher than $0.14\ h^{-1}$, while at $D > 0.34\ h^{-1}$, glucose oxidation

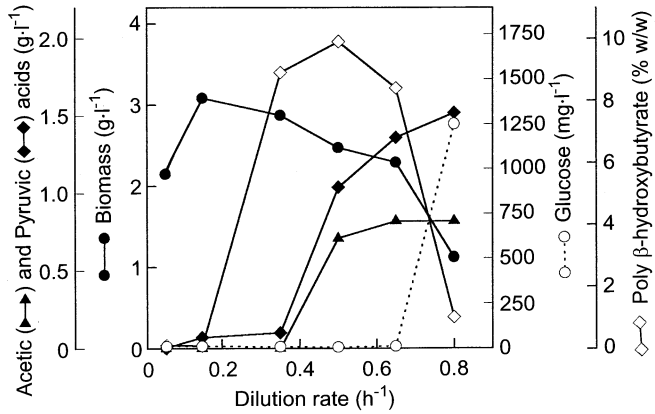

Fig. 1 Raw data from continuous cultures of *B. thuringiensis* in a glucose–yeast extract medium

was incomplete, as can be seen from the significant accumulation of acetate and pyruvate. Specific rates of carbon dioxide production and oxygen consumption increased steadily with D, while the specific rate of organic acid production increased with D after a threshold value of $0.34\ h^{-1}$ (Table 1). Dissolved oxygen dropped from 91% ($D = 0.054\ h^{-1}$) to 30% ($D = 0.63\ h^{-1}$) and then increased to 40% ($D = 0.79\ h^{-1}$).

Analysis of the biomass nitrogen content gave values of $11.2 \pm 0.35\%$ w/w for all growth rates tested. The culture medium contained two nitrogen sources: ammonium sulphate and yeast extract; the latter may act also as a carbon source. It can be seen (Table 1) that at low values of D, both nitrogen sources were used in almost the same proportion ($f_{NH_3} = 0.42$–0.54), while at higher values of D, *B. thuringiensis* preferentially assimilated nitrogen (and carbon) from the yeast extract ($f_{NH_3} = 0.07$).

Growth model

Aerobic growth can be described by a series of simple equations accounting for the assimilation of the carbon source into biomass, oxidation (glycolysis + TCA cycle), product formation and respiration (Roels 1983; de Hollander 1991). Using this set of equations it is possible to obtain a relationship between growth and product formation with internal metabolic fluxes, which, in turn are linked to production (or consumption) of ATP and reducing power (NADH).

In Fig. 2 a simplified scheme of the growth of *B. thuringiensis* in glucose-yeast extract medium is proposed. Glucose is used as a carbon and energy source. Moreover, it is assumed that yeast extract components are incorporated into the biomass with no previous chemical modification, and are therefore not being used as an energy source. According to this scheme, there are two ways for biomass synthesis to occur:

Table 1 Specific rates of glucose (q_S) and O_2 (q_{O_2}) consumption and product (q_A, q_P, q_H) formation, and ammonium assimilation (f_{NH_3}) in a glucose-limited continuous culture of *Bacillus thuringiensis* at different growth rates. q_{O_2} and q_{CO_2} are expressed in mmol·C-mol^{-1}·h^{-1}; specific production rates of acetate (q_A), pyruvate (q_P), and poly-β-hydroxybutyrate (q_H), and specific consumption rate of glucose (q_S) in mC-mol·C-mol^{-1}·h^{-1}. f_{NH_3} is the ratio of assimilated ammonium nitrogen to total assimilated nitrogen ($N_{NH_4^+\,inlet} - N_{NH_4^+\,outlet}$)·(total N in the biomass)$^{-1}$

D (h^{-1})	q_S	q_{O_2}	q_{CO_2}	q_A	q_P	q_H	f_{NH_3}
0.054	71.5	53.1	47.7	0	0	0	0.54
0.141	139.0	80.2	81.0	0	0	0	0.42
0.343	364.0	189	186.0	9.8	48	36.6	0.24
0.513	631.6	235	225.7	127	190	61.2	0.17
0.633	865.0	276	249.0	195	327	64.0	0.18
0.791	1455.0	400	330.0	473	844	9.5	0.07

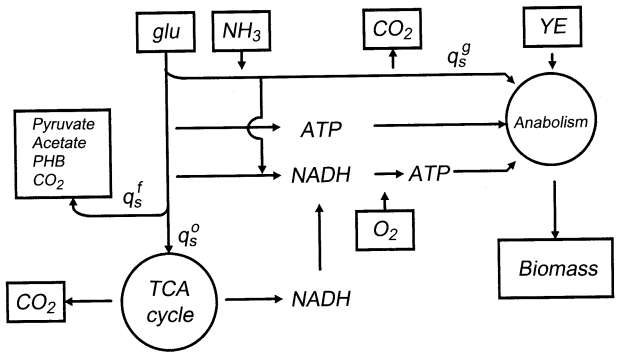

Fig. 2 Simplified metabolic scheme for *B. thuringiensis* growth in a glucose-yeast extract medium. Yeast extract (YE) is used only as a biosynthetic precursor, while glucose provides energy through fermentative and oxidiative (TCA cycle) pathways, as well as being used as a biosynthetic precursor. *Glu* glucose, *YE* yeast extract, *PHB* poly-β-hydroxybutyrate, q_s^f glucose carbon flux to acid synthesis, q_S^o glucose carbon flux through TCA cycle, q_s^g glucose carbon flux to biomass synthesis

Biomass synthesis from glucose

According to Dekkers et al. (1981), the synthesis of one C-mole of biomass from glucose and ammonium can be described by the following equation:

$$\sigma CH_2O + aNH_3 + C_1\ ATP \rightarrow CH_{1.8}O_{0.5}N_{0.2} + (\sigma - 1)CO_2 + \frac{\sigma\gamma_S - \gamma_x}{2}\ NADH \quad (1)$$

The value of σ depends on the assimilation pathway of glucose. When *B. thuringiensis* is grown in a medium containing glucose and yeast extract, glucose is assimilated via the EMP pathway (Nickerson et al. 1974). In this case, σ equals 1.19 (Babel and Müller 1985). Coeficient C_1 represents the number of moles of ATP needed for the synthesis of one C-mole of biomass from glucose and ammonium. γ_S and γ_x represent the degree of reduction of glucose and of biomass, respectively (Erickson et al. 1978). As no data are available on the elemental composition of *B. thuringiensis*, we assumed the standard ash-free biomass composition given by the formula $CH_{1.8}O_{0.5}N_{0.2}$ accounting for the 95% of biomass. Therefore, one C-mole of biomass equals to 25.8 g and $\gamma_x = 4.2$ (Roels 1983).

Biomass synthesis from yeast extract

Yeast extract is a complex mixture obtained from yeast autolysis (Peppler 1982). It contains all the precursors needed for microbial biomass synthesis, such as amino acids and nucleotides. If these precursors are incorporated into the biomass without any chemical modification, the equation that represents the synthesis of one C-mole of biomass can be written as follows:

$$YE + C_2ATP \rightarrow CH_{1.8}O_{0.5}N_{0.2} \quad (2)$$

where YE represents the amount of yeast extract containing 1 gram-equivalent of carbon and C_2 the number of moles of ATP needed for the synthesis of one C-mole of biomass from the yeast extract precursors.

According to Stouthamer and van Verseveld (1985), the amount of ATP needed for the synthesis of one C-mole of biomass from glucose and ammonium is similar to that needed for the synthesis of one C-mole of biomass from precursors (i.e. amino acids and nucleotides). Thus, we can assume that $C_1 \approx C_2 = C$.

Bearing this in mind, the global equation which represents the synthesis of one C-mole of biomass from glucose, ammonium and yeast extract can be expressed as a combination of Eqs. 1 and 2.

$$f\sigma CH_2O + (1-f)YE + faNH_3 + C\,ATP \rightarrow CH_{1.8}O_{0.5}N_{0.2} + f(\sigma - 1)CO_2 + f\,\frac{\sigma\gamma_S - \gamma_x}{2}\,NADH \quad (3)$$

where f and $(1-f)$ represent the biomass carbon fractions coming from glucose and yeast extract, respectively, and C represents the amount of ATP needed for the synthesis of one C-mole of biomass ($1/y_{x/ATP}$).

According to Eq. 3, the ratio of assimilated ammonium nitrogen to total nitrogen assimilated, f_{NH_3}, is $f\,a/0.2$. As, according to Eq. 1, a equals 0.2, it follows that:

$$f = f_{NH_3} \quad (4)$$

which means that glucose assimilation is linked to ammonium incorporation.

The reactions corresponding to glucose catabolism are:

1. Oxidation

$$CH_2O \rightarrow CO_2 + 2\ NADH + \frac{2}{3}\ ATP \quad (5)$$

(in this and the following reactions, no distinction is made between ATP and GTP, or NADH, NADPH and FADH2).

2. Acetate production

$$CH_2O \rightarrow \frac{2}{3}\,CH_2O + \frac{1}{3}\,CO_2 + \frac{2}{3}\,NADH + \frac{1}{3}\,ATP \quad (6)$$

3. Pyruvate production

$$CH_2O \rightarrow CH_{1.33}O + \frac{1}{3}\,NADH + \frac{1}{3}\,ATP \quad (7)$$

4. PBH production

$$CH_2O \rightarrow \frac{2}{3}\,CH_{1.5}O_{0.5} + \frac{1}{3}\,CO_2 + \frac{1}{2}\,NADH + \frac{1}{3}\,ATP \quad (8)$$

5. Respiration

$$NADH + \frac{1}{2}\,O_2 \rightarrow H_2O + (P/O)\,ATP \quad (9)$$

In Eq. 6 it has been assumed that acetate is formed from pyruvate via the 2,3-butanediol cycle, which means that the same amount of ATP is obtained in the pathway to pyruvate as in the pathway to acetate (Benoit et al. 1990).

6. Glucose, NADH and ATP balances

A simple, general method to construct the balances will be introduced here. Let Eq. 3 be multiplied by a parameter α_1, Eq. 5 by α_2, and so on. If it is assumed that one C-mole glucose is consumed, it can be derived that:

$$f_{NH_3}\,\sigma\alpha_1 + \alpha_2 + \alpha_3 + \alpha_4 + \alpha_5 = 1 \quad (10)$$

So, coefficients α_i are related to macroscopic yields:

$$\alpha_1 = y_{x/S} \quad (11)$$

$$\frac{2}{3}\alpha_3 = y_{A/S} \quad (12)$$

$$\alpha_4 = y_{P/S} \quad (13)$$

$$\frac{2}{3}\alpha_5 = y_{H/S} \quad (14)$$

$$\frac{1}{2}\,\alpha_6 = b \quad (15)$$

Carbon dioxide is produced in the reactions represented by Eqs. 3, 5, 6 and 8, so CO_2 yield can be written as:

$$y_{CO_2/S} = \alpha_1(\sigma - 1)f_{NH_3} + \alpha_2 + \frac{1}{3}\,\alpha_3 + \frac{1}{3}\alpha_5 \quad (16)$$

The ATP balance will be:

$$\alpha_1 C = \frac{2}{3}\,\alpha_2 + \frac{1}{3}(\alpha_3 + \alpha_4 + \alpha_5) + (P/O)\,\alpha_6 \quad (17)$$

and the NADH balance:

$$\frac{1}{2}f_{NH_3}(\sigma\gamma_s - \gamma_x)\,\alpha_1 + 2\,\alpha_2 + \frac{2}{3}\,\alpha_3 + \frac{1}{3}\,\alpha_4 + \frac{1}{2}\,\alpha_5 = \alpha_6 \quad (18)$$

Introducing Eqs. 11–16 into Eq. 18 the NADH balance can be expressed as:

$$\frac{y_{x/S}f_{NH_3}\left[\frac{1}{2}(\sigma\gamma_S - \gamma_x) - 2(\sigma - 1)\right] + 2y_{CO_2/S} + \frac{1}{3}\,y_{P/S} - \frac{1}{4}\,y_{x/S}}{2b} = 1 \quad (19)$$

As macroscopic yields can be expressed as quotients of specific rates (i.e. $b = q_{O_2}/q_s\; y_{x/s} = \mu/q_s$, etc.), the NADH balance now transforms to:

$$\frac{\mu f_{NH_3}\left[\frac{1}{2}(\sigma\gamma_S - \gamma_x) - 2(\sigma - 1)\right] + 2q_{CO_2} + \frac{1}{3}\,q_P - \frac{1}{4}\,q_H}{2q_{O_2}} = 1 \quad (20)$$

By introducing Eqs. 11–16 into Eq. 17, Eq. 21 is obtained:

$$y_{x/S}\,C = \frac{2}{3}\,[y_{CO_2/S} - y_{x/S}(\sigma - 1)f_{NH_3}] + \frac{1}{6}y_{A/S} + \frac{1}{6}y_{H/S} + \frac{1}{3}\,y_{P/S} + 2(P/O)b \quad (21)$$

The product $y_{x/S}\,C$ equals to $y_{ATP/s}$, then and following the same procedure as done for Eq. 19, the specific rate of ATP production is obtained:

$$q_{ATP} = 2(P/O)\,q_{O_2} + \frac{2}{3}\,[q_{CO_2} - \mu(\sigma - 1)f_{NH_3}] + \frac{1}{6}q_A + \frac{1}{6}q_H + \frac{1}{2}q_P \quad (22)$$

Alternatively, an equivalent expression can be obtained by introducing Eqs. 11–15 and Eq. 18 into Eq. 17:

$$q_{ATP} = 2(P/O)\,q_{O_2} + \frac{2}{3}q_{O_2} - \frac{1}{6}f_{NH_3}(\sigma\gamma_S - \gamma_x)\,\mu + \frac{1}{6}q_A + \frac{2}{9}q_P + \frac{1}{4}q_H \quad (23)$$

Eq. 10 represents the glucose carbon balance and α_i values ($i = 1$–5) are related to the glucose fraction consumed in the reaction i. Thus, the fraction of carbon from glucose consumed for biomass synthesis, $f_{NH_3}\sigma\alpha_1$, can be expressed as:

$$R_G = \frac{f_{NH_3}\,\sigma\,\mu_C}{q_S} \tag{24}$$

On the other hand, the fraction which is oxidized, α_2, can be obtained from Eq. 16:

$$R_O = \frac{q_{CO_2} - \mu_C(\sigma - 1)f_{NH_3} - \frac{1}{2}q_A - \frac{1}{2}q_H}{q_S} \tag{25}$$

Finally, $\alpha_3 + \alpha_4 + \alpha_5$ represents the fraction which is converted into acids, so:

$$R_F = \frac{\frac{3}{2}q_A + q_P + \frac{3}{2}q_H}{q_S} \tag{26}$$

It is obvious that $R_G + R_O + R_F = 1$.

As PHB is an intracellular product, for the sake of the carbon balance calculation, the value of μ was corrected for PHB content:

$$\mu_C = \mu(1 - \text{C-mole}_{PHB}/\text{C-mole}_{biomass}) \tag{27}$$

Apart from checking for carbon balances, Eqs. 24–26 allow for the calculation of the corresponding fluxes:

$$q_S^G = q_S\,R_G \tag{28}$$

$$q_S^O = q_S\,R_O \tag{29}$$

$$q_S^F = q_S\,R_F \tag{30}$$

where q_S^G, q_S^O and q_S^F are the glucose carbon fluxes to biomass, through oxidative and through fermentative metabolisms, respectively.

The ATP requirement for growth and maintenance is given by Stouthamer and Bettenhaussen (1973):

$$q_{ATP} = \frac{\mu}{y^{max}_{x/ATP}} + m_{ATP} \tag{31}$$

Combining Eqs. 31 and 22 the following is obtained:

$$\frac{\mu}{y^{max}_{x/ATP}} - L = 2q_{O_2}(P/O) - m_{ATP} \tag{32}$$

where

$$L = \frac{2}{3}[q_{CO_2} - \mu(\sigma - 1)f_{NH_3}] + \frac{1}{6}q_A + \frac{1}{6}q_H + \frac{1}{3}q_P \tag{33}$$

Discussion

Table 2 shows the values of R_G, R_O, and R_F calculated for different growth rates. It can be seen that the fraction of glucose consumed for biomass synthesis decreases with the growth rate, being only 4% at the highest growth rate tested. The same trend is observed for the fraction of glucose that is oxidized through the TCA cycle. This decrease is compensated by an increase in the fraction of glucose that is converted into acids. At $D = 0.79\ h^{-1}$, almost all glucose is dissimilated to provide energy (ATP, NADH) through acid formation, the contribution of the TCA cycle being negligible. As the dissolved oxygen level during cultivation was above 30% of saturation, acid production cannot be attributed to an oxygen limitation. Figure 3 shows that the flux of carbon through the TCA cycle reached the maximum at $D = 0.34\ h^{-1}$, diminishing thereafter, while the flux to acid formation strongly increased. These results suggest that the decreasing activity of this cycle is responsible for acid formation at high growth rates. Reiling et al. (1985) found that, for *E. coli*, q_{O_2} remained constant after the onset of acetate production, suggesting that a saturation of the respiratory chain would be the responsible for that accumulation. This is not seemingly the case for *B. thuringiensis*, because q_{O_2} increased with the specific growth rate even after the onset of acid accumulation (see Table 1). According to these results, the TCA cycle would be strongly inhibited at the maximum specific growth rate,

Table 2 Fractional conversion of glucose into biomass (R_G), through oxidative (R_O) and fermentative (R_F) metabolism at different growth rates

D (h^{-1})	R_G	R_O	R_F	GCR[a]	NB[b]
0.054	0.48	0.59	0	1.07	0.87
0.141	0.51	0.50	0	1.01	0.97
0.343	0.24	0.45	0.32	1.01	0.98
0.513	0.15	0.18	0.75	1.08	1.04
0.633	0.14	0.11	0.83	1.08	1.05
0.791	0.04	0.05	1.08	1.17	1.17

[a] Glucose carbon recovery = $R_G + R_O + R_F$
[b] NADH balance Eqs. 19 and 20

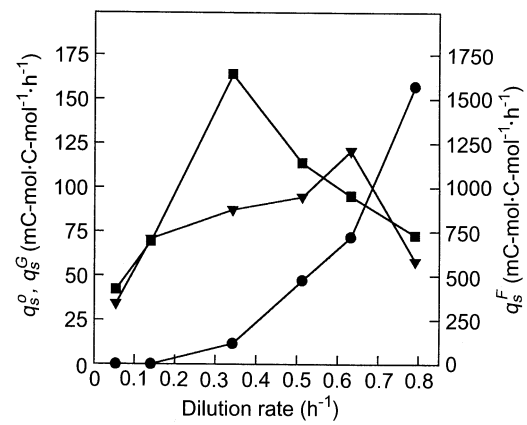

Fig. 3 Glucose carbon fluxes to biomass (q_S^G, ▼) and through oxidative (q_S^O, ■) and fermentative (q_S^F, ●) metabolism at different growth rates

thus explaining the acid formation observed in batch cultures.

The behaviour of the TCA cycle in *B. thuringiensis* differs significantly from that observed for *B. subtilis* growing in continuous cultures (Goel et al. 1993). These authors found that the flow through the TCA cycle increased with the specific growth rate, and inhibition of this cycle was observed only at very high growth rates such as in exponentially growing cells in batch cultures. For *E. coli* growing in a glucose–yeast extract medium, a rather different phenomenon has been described (Han et al. 1992). q_S^O increased with D up to $0.7\ h^{-1}$, remaining constant thereafter when acid formation is observed. These results suggest a saturation of the TCA cycle.

NADH balances shown in Table 2 are very close to 1, except for the extreme values. At $D = 0.05\ h^{-1}$ it is possible to explain this by measurement errors due to low oxygen consumption and low carbon dioxide production. At $D = 0.79\ h^{-1}$, NADH and glucose carbon recovery (GCR) values are significantly higher than 1. It is therefore likely that yeast extract components have been oxidized. Thus, the proposed model could not be completely valid for this growth condition.

It is well known that while the composition of biomass in terms of protein, RNA, etc., can vary considerably, the elemental composition and thus the degree of reduction, γ_x, remains fairly constant whatever the cell species, substrate or growth rate (Erickson et al. 1978). Values of 6.4% (Andrews 1989) and of 5% (Solomon and Erickson 1981) for the coefficient of variation were reported for γ_x and for carbon content, respectively. Moreover, it was found that variations in the macromolecular composition of the microbial biomass have only a slight effect on σ, amounting to maximally 3% (Babel and Müller 1985). Thus, these variations are too small to affect significantly the NADH and glucose carbon balances reported in Table 2.

To correlate Eq. 32 with experimental data, either $y_{x/ATP}^{max}$ or (P/O) must be known. Unfortunately, no information related to these parameters is available for *B. thuringiensis*. Bulthuis et al. (1991) obtained a value of $y_{x/ATP}^{max}$ of $12.9\ g\cdot(mol\ ATP)^{-1}$ [= 0.524 C-mole·(mol ATP)[1]] in anaerobic continuous cultures of *B. licheniformis*. Assuming a similar value for *B. thuringiensis*, it is found (Fig. 4) that $(P/O) = 1.76$ and $m_{ATP} = 0.104$ mol ATP·(C-mol biomass·h)$^{-1}$. Similar results are obtained if Eq. 23 instead of Eq. 2 is combined with Eq. 31. The (P/O) ratio and m_{ATP} are close to those obtained by Bulthuis et al. (1993) for aerobic cultures of *B. licheniformis*. According to the (P/O) ratio found for *B. thuringiensis*, the main source of ATP production should be the respiratory chain, with only a minor contribution of substrate-level phosphorylation.

The results presented here suggest that there is a mismatch between glycolysis and TCA cycle capacity, depending on the growth rate. This must be taken into account in order to minimize acid formation in *B. thuringiensis* cultures. This can be achieved by optimizing either the values of μ in fed-batch or continuous cultures, or the carbon flux through the TCA cycle. *B. thuringiensis* lacks α-ketoglutarate dehydrogenase, this metabolic step is circumvented by the γ-aminobutyric acid pathway (Aronson et al. 1975), which requires glutamate as a substrate. This could be one of the reasons for the low "performance" of the TCA cycle, and this is currently being studied by the authors.

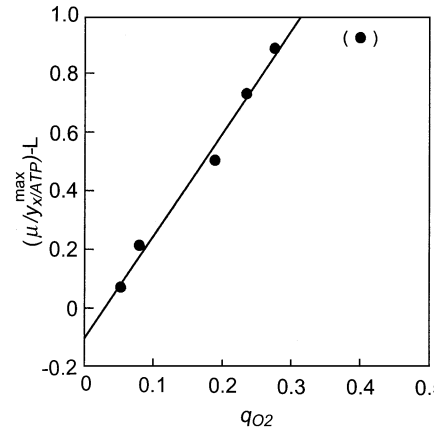

Fig. 4 Specific rate of ATP production corrected for substrate level phosphorylation versus O_2 consumption rate. The point in *parentheses* was not considered for regression analysis

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Appendix

D	Dilution rate (h^{-1})
f_{NH_3}	Ratio of assimilated ammonium nitrogen to total assimilated nitrogen (−)
f	Biomass carbon fraction coming from glucose (−)
a	Stoichiometric coefficient (mol NH_3·C-mol biomass^{-1})
C	Stoichiometric coefficient (mol ATP·C-mol biomass^{-1})
b	Stoichiometric coefficient (mol O_2·C-mol glucose^{-1})
$y_{i/S}$	Yield factor for compound i on glucose (C-mol i·C-mol glucose^{-1})
q_i	Specific rate of production (or consumption) for component i (C-mol i·C-mol biomass^{-1}·h^{-1} or mol i·C-mol biomass^{-1}·h^{-1})
(P/O)	ATP formed per gram atom oxygen consumed by the respiratory chain (mol ATP·gram atom oxygen^{-1})
$y_{x/ATP}^{max}$	Maximum growth yield based on ATP (C-mol biomass·mol ATP^{-1})
m_{ATP}	Maintenance requirement for ATP (mol ATP·C-mol biomass^{-1}·h^{-1})

Subscripts:

x	Biomass
S	Glucose
A	Acetic acid
P	Pyruvic acid
H	Poly-β-hydroxybutyrate

Greek letters:

σ	Amount of glucose required for the formation of one C-mol of biomass (C-mol glucose · C-mol biomass^{-1})
μ	Specific growth rate (h^{-1})
γ	Degree of reduction (equiv available electrons · C-mol^{-1})

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