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Efficiency Analysis of Fermentation Process on Available Electron Balance

Lee, Kyung-Hee*, Woo-Kyu Kang and Byung-Woo Kim

Department of Pharmacy, College of Pharmacy, Pusan National University Pusan 609-735, Korea

Available Electron Balance에 의한 발효과정의 에너지효율

李京熙*·姜宇奎·金昞祐

釜山大学校 薬学大学 薬学科

Energy efficiency of bacterial cell mass and product formation from cellulose using *Ruminococcus albus* and *Ruminococcus flavefaciens* with application of available electron balance were discussed. Values of true growth yield, η_{max} and η_{th}^{max}, and maintenance coefficient, m_e were estimated using experimental data, and the results were compared with estimates obtained from theoretical approach. Experimental values were similar in magnitude to theoretical values in Y_{ATP}^{max} = 10.5 g cells/mole ATP. Therefore, Y_{ATP}^{max} values of *Ruminococcus albus* and *Ruminococcus flavefaciens* were considered similar to 10.5 g cells/mole ATP.

The microbial conversion of cellulosic materials to useful compounds is necessary for the effective use of waste cellulosics. Therefore, extensive studies have been done to decompose cellulosic materials with cellulase from such aerobic microorganisms as molds, fungi, and Actinomyces (1-4).

Some rumen anaerobes such as *Ruminococcus albus* or *Ruminococcus flavefaciens* have been reported to be more potent than other fungi since they directly ferment cellulosic materials to ethanol and organic acids(5,6).

The contribution of these organisms to the large scale bioconversion of cellulose to organic acids has emphasized the need for systematic methods of evaluating microbial growth and conversion yields.

The mass and energy balance regularities have been suggested by Minkevich and Eroshin (7,8). Erickson and co-workers (9-15) used these regularities widely in the analysis of experimental data describing cell growth, substrate utilization, and product formation.

The mass and energy balance approach has been used for evaluating the efficiency of conversion of cellulose into bacterial cell mass and products using *Ruminococcus albus* and *Ruminococcus flavefaciens*.

Methods for the estimation of yield and maintenance parameters in anaerobic microbial fermentation processes were also considered.

Theory

In most anaerobic fermentation, ATP generation is well coupled to product formation. In these fermentations, microorganisms can meet their ATP requirements for growth and maintenance by only producing products. If the available electrons that are transferred from organic substrate to products are viewed as being expended to provide energy for growth and maintenance, true growth yield, η_{max}, can be defined as (9,10)

$$\eta_{max} = \frac{\frac{\sigma_b \gamma_b}{12} Y_{ATP}^{max}}{\frac{\sigma_b \gamma_b}{12} Y_{ATP}^{max} + \delta} \quad (1)$$

Key words: *Ruminococcus albus, R. flavefaciens,* available electron, true growth yield, maintenance coefficient, reductance degree
* Corresponding author

where δ is number of equivalents of available electrons transferred to products to produce 1g mole ATP from ADP, σ_b is weight fraction of carbon in bacterial cell mass, and γ_b is reductance degree of bacterial cell mass.

True growth yield, η_{max}, obtained from equation 1 gives the fraction of available electrons of substrate that can be incorporated into microbial cell mass when maintenance is neglected.

Based on the available electron concept, an available electron balance for anaerobic processes can be written as follows (16):

$$\eta + \xi_p = 1.0 \tag{2}$$

where η and ξ_p are the fraction of available electrons of organic substrate which are incorporated into bacterial cell mass and products, respectively. Equation 2 can be used to check the consistancy of experimental data.

If available electrons that are transferred from substrate to product are viewed as being expended to provide energy for microbial growth and maintenance, the following equation can be written to estimate maintenance parameter, m_e, and true growth yield, η_{max}, obtained from the experimental data (16).

$$\frac{1}{\eta} = \frac{1}{\eta_{max}} + \frac{m_e}{\mu} \tag{3}$$

where μ is the specific growth rate.

Equation 3 follows Pirt's model (17) for growth and maintenance.

Erickson and Oner (16) have introduced the concepts associated with yield and maintenance parameters based on free energy.

For anaerobic growth,

$$\eta_{th} = \frac{g_b \eta}{g_s - g_p \xi_p} \tag{4}$$

$$\eta_{th}^{max} = \frac{g_b \eta_{max}}{g_s - g_p(1 - \eta_{max})} \tag{5}$$

where the subscript *th* indicates a free energy and g is free energy per equivalent of available electrons relative to ammonia, carbon dioxide, and water.

From these equations, free energy equivalent values of η, η_{max}, and m_e can be calculated.

Materials and Methods

Microorganisms

Ruminococcus albus ATCC 27210 and *Ruminococcus flavefaciens* ATCC 19208, representative cellulytic anaerobes in rumen, were used. These bacteria were obtained from American Type Culture Collection

Ball-milled cellulose (BMC)

Pure cellulose from Toyo filter paper No. 2 was ball-milled in a 2 % suspension for 3 days.

Medium

Medium was made anaerobically by bubbling with oxygen-free carbon dioxide which was obtained by passing carbon dioxide into a glass cylinder with reduced shot copper at 500 degrees (18). The composition of rumen fluid medium was Mineral I (0.6% K_2HPO_4), Mineral II (0.6% KH_2PO_4, 1.2% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% $MgSO_4.7H_2O$, and 0.12% $CaCl_2$), Mineral III (1% $FeSO_4.7H_2O$, 1% $ZnSO_4.7H_2O$, 1% $MnSO_4.6H_2O$ and 1% $CaCl_2.6H_2O$), rumen fluid, trypticase, yeast extract, and carbon source. The details are shown in Table 1.

A mixed solution of 75 m*l* of Mineral I and II, and 4 m*l* of Mineral III was stirred well together with carbon source (BMC or cellobiose), resazurin, yeast extract, trypticase, and rumen fluid, adjusted to pH 6.8 with NaOH, and kept at about 50 degrees for 30 minutes after adding 50 m*l* of 8% sodium carbonate solution. Then medium was bubbled with oxygen-free carbon dioxide gas for 30 minutes or more until its color turned into pink.

10 m*l* of 2.5% cysteine solution was added, keeping the volume at one liter, and 10 m*l* of 2.5% Na_2S solution was finally added.

Analysis

Residual Cellulose: The cellulose in 1 m*l* of culture broth was washed twice with 8.5% Tween 80 solution (in 7.5% of Mineral 1, and Mineral 11) and

Table 1. Composition of medium

Mineral I[a]	75 m*l*
Mineral II[b]	75 m*l*
Mineral III[c]	4 m*l*
0.1% Resazurin-Na	1 m*l*
Yeast extract	1g/*l*
Rumen fluid[d]	300 m*l*
Trypticase (BBL)	0.2 g
8% Sodium carbonate	50 m*l*
2.5% Cysteine-HCl	10 m*l*
2.5% Na_2S. $9H_2O$	10 m*l*
Cellulose (or cellobiose)	variable

[a] Mineral I: 0.6% K_2HPO_4

[b] Mineral II: 0.6% KH_2PO_4, 1.2% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% $CaCl_2$, and 0.12% $MgSO_4.7H_2O$

[c] Mineral III: 1% $FeSO_4.7H_20$, 1% $ZnSO_4.7H_20$, 1% $MnSO_4.6H_2O$, and $CoCl_2.6H_2O$

[d] Rumen fluid: Obtained contents from the rumen (first stomach or paunch) of a cow at a local slaughter house. Squeezed contents through 2 layers of cheese-cloth. Siphoned fluid into bottles being flushed with oxygen-free CO_2. Stoppered, placed in press, and autoclaved. Stored at 4 °C. Before use, centrifugated at 10,000 × g for 20 minutes and used clear supernatant.

centrifuged at 200 × g for 15 minutes. The cellulose precipitate was suspended in 40 m*l* of distilled water and boiled for 1 hr in a water bath. The precipitate was separated by centrifugation at 100 × g for 20 minutes. 20 m*l* of 60% (v/v) sulfuric acid was added to the precipitate to solubilize the cellulose overnight.

Keeping the volume at 25 m*l* with 60% of sulfuric acid, 0.5% of this solution was mixed with 5 m*l* of anthrone reagent, boiled for 10 minutes, and cooled quickly. After 15 minutes, optical density of reaction mixture was read at 620 nm.

Bacterial Cell Mass: Bacterial cell mass was determined from the nitrogen content of cells, which was measured with Nessler's reagent (19) after digestion of the cells in a Kjeldahl flask with 98% sulfuric acid. (20)

Products: Ethanol and acetic acid were determined from the supernatant of each culture broth by gas chromatography (Hitachi Gas Chromatograph 163). Operating conditions were as follows: column temperature, 153 C; injection block temperature: 200 C, detector: FID, column: stainless steels column 3 mm by 1 m packed with 80-100 mesh chromosorb 101, air flow rate: 300 m*l*/min, hydrogen flow rate: 250 m*l*/min, nitrogen flow rate: 25 m*l*/min. (20)

Results and Discussion

Bacterial cell mass and product formation

The results of cultivation are shown in Fig. 1-2.

As shown in Fig. 1, *Ruminococcus albus* was allowed to grow for 50 hrs. During 48 hrs, amount of cellulose digested was measured to be 9.6 g/*l* and that of dry cell mass, 1.5 g/*l*. The major fermentation products were ethanol, 3.0 g/*l* and acetic acid, 4.2 g/*l*. In Fig. 2, *Ruminococcus flavefaciens* was allowed to grow for 72 hrs. During 72 hrs, amount of cellulose digested was measured to be

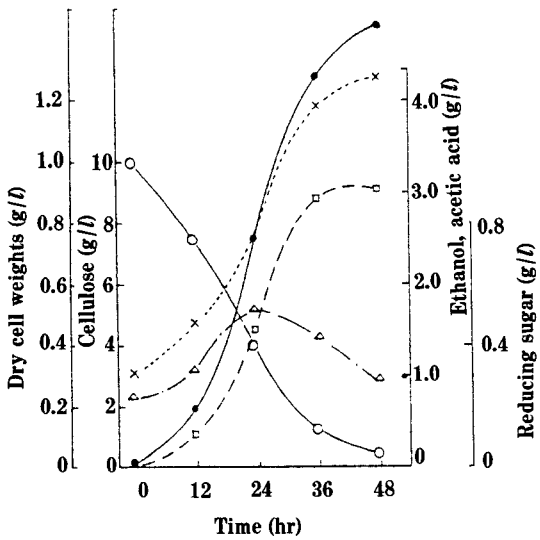

Fig. 1. Time course of cellulose digestion in a jar fermentor cultivation of *Ruminococcus albus* at 37 °C, pH 6.0.

symbol: cellulose (○—○), cell mass (●—●), ethanol (□--□), acetic acid (×···×), reducing sugar (△···△)

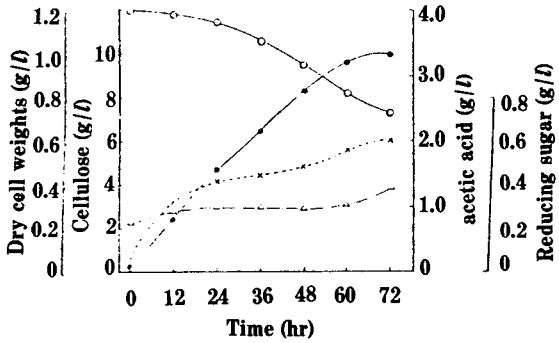

Fig. 2. Time course of cellulose digestion in a jar fermentor cultivation of *Ruminococcus flavefaciens* at 37°C, pH 6.8.
symbol: cellulose (○—○), cell mass (●—●), acetic acid (×---×), reducing sugar (△-·-△).

4.7 g/*l*, that of dry cell mass 1.0 g/*l*, and acetic acid, major sole product, 2.1 g/*l*.

Energy efficiency of baterial cell mass and product formation

The values of specific growth rate, specific glucose consumption rate, specific acetic acid production rate, and specific ethanol production rate calculated from experimental data are shown in Table 2 and 4.

Data obtained from using available electron balance in these values are presented in Table 3 and 5.

Table 3 shows values of $\eta + \xi_p$ to be very close to theoretical value 1, but in Table 5 the corresponding values are much lower than 1. This error is due to misconception that all cellulose convert into glucose.

Calculation of the maintenance coefficient, m_e, and the true growth yield parameters, η_{max} and η_{th}^{max}

Plotting η *vs.* μ from equation 3, values of m_e and η_{max} were determined.

Equation 5 was used to calculate the values of η_{th}^{max}.

Numerical values of free energy per equivalent of available electrons of substrate and product may usually be found from tabulated literature data (21). For substrates such as glucose, ethanol, and acetic acid that do not contain nitrogen, free energy of combusion per equivalent of available electrons should be used to find g_s and g_p.

g_s value of glucose was 119.8 KJ/equiv. e^-. g_p value of acetic acid and ethanol were 111.8 KJ/equiv. e^- and 109.9 KJ/equiv. e^-, respectively.

g_p balue of *Ruminococcus albus* was 110.85 KJ/eqiuv. e^-.

g_p value of *Ruminococcus flavefaciens* was 111.8 KJ/equiv. e^-.

Minkevich (22) reported value for free energy of formation of biomass to be 114.7 KJ/ equiv. e^-.

For example, η_{th}^{max} value is obtained from g_b, 114.7 KJ/equiv. e^-, g_s, 119.7 KJ/equiv. e^-, g_p, 110.85 KJ/equiv. e^-, and η_{max} value, 0.181 in *Ruminococcus albus*.

$\eta_{th}^{max} = 114.7 \times 0.178 / [(119.7\text{-}110.85(1\text{-}0.181)]$
$= 0.722$

The maintenance coefficient, m_e, and the true

Table 3. Data based on instantaneous available electron balance for the batch growth of *Ruminococcus albus*

θ	η	ξ_{p1}^*	ξ_{p2}^{**}	$\eta + \xi_{p1} + \xi_{p2}$
12	0.092	0.375	0.554	1.021
24	0.194	0.217	0.845	1.256
36	0.193	0.344	0.870	1.407
48	0.283	0.250	9.546	1.089

* acetate, ** ethanol

Table 2. Calculated variables for the batch fermentation of *Ruminococcus albus*

θ (hr)	μ (1/hr)	q_s (g substrate/g dry cell. hr)	q_{p_1} (g acetate/g dry cell. hr)	q_{p_2} (g ethanol/g dry cell. hr)
12	0.144	1.918	0.719	0.401
24	0.085	0.538	0.117	0.172
36	0.020	0.125	0.043	0.041
48	0.006	0.028	0.007	0.006

Table 4. Calculated variables for the batch fermentation of *Ruminococcus flavefaciens*

σ	μ	q_s	q_p
12	0.083	0.158	0.025
24	0.049	0.124	0.019
36	0.024	0.139	0.021
48	0.012	0.144	0.020
60	0.007	0.086	0.021
72	0.004	0.069	0.016

Table 5. Data based on instantaneous available electron balance for the batch growth of *Ruminococcus flavefaciens*

θ	μ	ξ_p	$\eta+\xi_p$
12	0.647	0.237	0.884
24	0.493	0.230	0.723
36	0.210	0.227	0.437
48	0.110	0.209	0.319
60	0.189	0.366	0.555
72	0.115	0.348	0.460

growth yield parameters, η_{max} and η_{th}^{max} are shown in Table 6.

Theoretical calculation of η_{max} and η_{th}^{max}

In *Ruminococcus albus,* when glucose hydrolysed from cellulose converts into products, theoretically estimated equation can be written as follows:

Glucose + 3 ADP + 3 Pi + 2 NAD^+ ⟶
3 ATP + 2 NADH + CO_2 + C_2H_5OH + CH_3COOH

$\delta = 24/3 = 8$

The Y_{ATP}^{max} values of 10.5, 28.8 and 31.9 g cells/mole ATP were used for true growth yield on available electron basis and free energy.

Bauchop and Elsden (23) reported that average values of Y_{ATP}^{max} obtained from *Streptococcus faecalis, Saccharomyces cerevisiae,* and *Zymomonas mobilis* growing anaerobically in complex media to be all 10.5 g cells/mole ATP. The value of Y_{ATP}^{max} reported by Stouthamer (24) for anaerobic ethanol production on glucose and mineral salts was 28.8 g cells/mole ATP. When yeast extract was used to provide amino acids and other biochemicals for cell growth, Stouthamer (24) reported that Y_{ATP}^{max} = 31.9 g cells/mole ATP was estimated theoretical maximum value based on biochemical pathway analysis.

Table 6. Values of true growth yield on an avilable electron basis and a free energy, and maintenance parameters from Table 3 and 5

m_e	η_{max}	η_{th}^{max}	microorganisms	comments
−0.011	0.181	0.722	*Ruminococcus albus*	Substrate & bacterial cell mass date
0.011	0.184	0.741	*Ruminococcus flavefaciens*	"

Table 7. Comparison of theoretical values of true growth yields on an available electron basis and a free energy for given values of δ and Y_{ATP}^{max} of *Ruminococcus albus*

δ	Y_{ATP}^{max}	η_{max}	η_{th}^{max}
8	10.5	0.178	0.712
	28.8	0.373	0.852
	31.9	0.397	0.861

Using these values and $\delta = 8$, equation (1) gives η_{max} for $\sigma_b = 0.462$, $\gamma_b = 4.291$, and $Y_{ATP}^{max} = 10.5$ cells/mole ATP.

$$\eta^{max} = \frac{\frac{\sigma_b \gamma_b}{12} Y_{ATP}^{max}}{\frac{\sigma_b \gamma_b}{12} Y_{ATP}^{max} + \delta} = \frac{\frac{0.462 \times 4.291}{12} \times 10.5}{\frac{0.462 \times 4.291}{12} \times 10.5 + 8}$$

$$\eta_{max} = 0.178$$

That is, neglecting maintenance, the maximum fraction of available electrons of glucose that can be incorporated into biomass under these anaerobic conditions was 0.178.

For $\delta = 8$ and $Y_{ATP}^{max} = 10.5$, 28.8, and 31.9g cells/mole ATP, η_{max} and η_{th}^{max} values are shown in Table 7.

In *Ruminococcus flavefaciens,* theoretically estimated equation can be written as follows;

Glucose + 4 ADP + 4 Pi + 4 NAD^+ ⟶
4 ATP + 4 NADH + 2 CO_2 + Acetic acid

$\delta = 24/4 = 6$

For $\delta = 6$ and $Y_{ATP}^{max} = 10.5$, 28.8, and 31.9 g cells/mole ATP, η_{max} and η_{th}^{max} values were calculated and shown in Table 8.

Comparison of experimental values with theoretical values

When Y_{ATP}^{max} is 10.5 g cells/mole ATP, the theoretical η_{max} and η_{th}^{max} values shown in Table 9,10, and 11 were similar in magnitude to experimental values shown in Table 8.

Therefore, Y_{ATP}^{max} values of *Ruminococcus albus, Ruminococcus flavefaciens* and mixed culture res-

Table 8. Comparison of theoretical values of true growth yields on an available electron basis and a free energy for given values of δ and Y_{ATP}^{max} of *Ruminococcus flavefaciens*

δ	Y_{ATP}^{max}	η_{max}	η_{th}^{max}
6	10.5	0.224	0.763
	28.8	0.442	0.876
	31.9	0.468	0.884

pectively, were considered to 10.5 g cells/molc ATP.

This experiment has been proved by Hopgood and Walker (25) using values of Y_{ATP} for *Ruminococcus flavefaciens* growing anaerobically in complex media with glucose equal to 10.6 g cells/mole ATP, while Hungate (26) assumed Y_{ATP} equal to 11.3 g cells/mole ATP for *Ruminococcus albus* anaerobically in minimum media with cellobiose.

요 약

*Ruminococcus albus*와 *Ruminococcus flavefaciens* 두 균주를 사용하여 기질 cellulose로부터 균체량과 생성물 형성에 대해서 available electron balance를 적용한 에너지 효율이 검토되었다.

실험 결과로부터 진정수율(true growth yield) 값인 η_{max}, η_{th}^{max} 두 값과 유지 계수값인 m_e 값이 이론적 접근으로부터 얻어진 값들과 비교 검토되어졌다. 실험치는 Y_{ATP}^{max}=10.5g cells/mole ATP 값을 적용한 이론치와 비슷하므로 *Ruminococcus albus*, *Ruminococcus flavefaciens*의 Y_{ATP}^{max} 값은 10.5와 비슷한 값을 갖는 것으로 사료되어진다.

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Nomenclature

g_b : Amounts of free energy in bacterial cell mass per gram equivalent of available electron in biomass (KJ/g equiv. electron)

g_p : Amounts of free energy in products per gram equivalent of available electron in products (KJ/g equiv. electron)

g_s : Amounts of free energy in substrate per gram equivalent of available electron in substrate (KJ/g equiv. electron)

m_e : Rate of organic substrate consumption of maintenance, gram eqivalent of available electron per gram equivalent of available electron in bacterial cell mass (1/hr)

q_p : Specific rate of organic product production [(g/g dry cell (hr)]

q_s : Specific rate of organic substrate consumption [(g/g dry cell (hr)]

Y_{ATP}^{max} : "True" bacterial cell mass yield on ATP (g bacterial cell mass/g mole ATP)

γ_b : Reductance degree of bacterial cell mass (equiv available electron/g atom carbon)

γ_p : Reductance degree of product (equiv available electron/g atom carbon)

γ_s : Reductance degree of substrate (equiv available electron/g atom carbon)

δ : Number of equivalents of available electron transferred to product to produce one gram mole of ATP from ADP

η : Fraction of available electrons in organic substrate which is converted to bacterial cell mass (-)

η_{max} : "True" bacterial cell mass available electron yield (-)

η_{th} : Bacterial cell mass free energy yield (-)

η_{th}^{max} : "True" bacterial cell mass free energy yield (-)

μ : Specific growth rate (1/hr)

ξ_p : Fraction of available electrons in organic substrate which is converted to product (-)

σ_b : Weight fraction of carbon in bacterial cell mass (-)

σ_p : Weight fraction of carbon in products (-)

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