

ESTIMATION OF GROWTH YIELDS
IN AEROBIC AND ANAEROBIC CULTURES

by

MEHMET DURDU ONER

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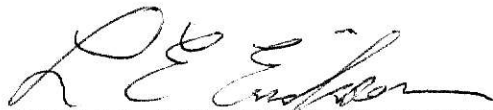
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Major Professor

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TABLE OF CONTENTS

	<u>Page</u>
CHAPTER I INTRODUCTION AND SUMMARY	
Introduction	1-1
References	1-3
CHAPTER II APPLICATION OF STATISTICAL METHODS TO THE ESTIMATION OF TRUE GROWTH AND PRODUCT YIELDS IN AEROBIC CULTURES	
Introduction	2-1
Theory	2-3
Discussion and Results	2-11
Conclusions	2-16
Nomenclature	2-18
References	2-21
Tables	2-23
CHAPTER III ESTIMATION OF MAINTENANCE AND TRUE GROWTH YIELD IN ANAEROBIC CULTURES	
Introduction	3-1
Theory	3-2
Discussion and Results	3-10
Conclusions	3-14
Nomenclature	3-15
References	3-18
Tables	3-20

	<u>Page</u>
CHAPTER IV ETHANOL PRODUCTION BY <u>ZYMOMONAS MOBILIS</u> ON GLUCOSE IN ANAEROBIC BATCH CULTURE	
Introduction	4-1
Theory	4-2
Materials and Methods	4-5
Discussion and Results	4-10
Conclusions	4-14
Nomenclature	4-15
References	4-17
Tables	4-18
APPENDIX AN EMPIRICAL COMPARISON OF THE BOOTSTRAP AND NORMAL THEORY METHODS FOR CONSTRUCTING CONFIDENCE INTERVALS	5-1
References	5-6
Table	5-7

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CHAPTER I

INTRODUCTION AND SUMMARY

INTRODUCTION

This research is primarily concerned with yields and thermodynamic efficiencies of microbial production of useful products, mainly ethanol. Both, aerobic and anaerobic fermentation processes are studied. Some statistical methods, to obtain better estimates of thermodynamic efficiencies, are developed. Some literature data and experimental results of this research on ethanol production are used to demonstrate the application of these methods. This work is divided into three chapters. Tables, nomenclature and references are located at the end of each chapter.

Overview of Chapters

Chapter II is mainly concerned with the development and application of some statistical methods to obtain better estimates of yield and maintenance parameters from all available experimental data. Equations, derived by Erickson [1] for aerobic cultures, are used to estimate the maintenance coefficient, true growth and product yields for the data of Nishizawa et al. [2] and de Hollander et al. [3].

In Chapter III, equations for anaerobic fermentation processes are derived. These equations are used to estimate the maintenance coefficient and true growth yield. Data of Dekkers et al. [4] and Lee et al. [5] are analyzed to demonstrate the application of the equations developed for anaerobic fermentation processes. In both cases ethanol is the main product.

Chapter IV is an experimental study of ethanol production by anaerobic fermentation of Zymomonas mobilis, ATCC 10988, on glucose in batch culture. Glucose, biomass and ethanol concentrations and carbon dioxide evolution rate were recorded and used to check the consistency of the data. The

results of the experiment are used to estimate the maintenance coefficient and true growth yield.

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CHAPTER II

APPLICATION OF STATISTICAL METHODS
TO THE ESTIMATION OF TRUE GROWTH AND
PRODUCT YIELDS IN AEROBIC CULTURES

INTRODUCTION

Yield is an important industrial consideration in any process. In microbial production of useful products, both the growth yield and product formation yield are important. In earlier work the concept of energetic yield has been introduced and used [1-10]. When maintenance is considered, the true growth yield, $\eta_{\max}$, and the true product yield, $\xi_p^{\max}$, may be found based on the concept that the energy in the organic substrate is allocated for these three functions; that is,

$$\text{Energy Input} = \frac{\text{Energy allocated for biomass production}}{\text{Energy allocated for biomass production}} + \frac{\text{Maintenance energy}}{\text{Maintenance energy}} + \frac{\text{Energy allocated for product formation}}{\text{Energy allocated for product formation}} \quad (1)$$

If Equation (1) is divided by the energy input, it may be written in the form:

$$1 = \frac{\eta}{\eta_{\max}} + \epsilon_m + \frac{\xi_p}{\xi_p^{\max}} \quad (2)$$

where η and ξ_p are the energetic yields of biomass and products respectively based on the energy input, and $\eta_{\max}$ and $\xi_p^{\max}$ are the true energetic yields for biomass and products, respectively, based on the energy allocated for biomass production and product formation, respectively. The fraction of the input energy evolved as heat because of maintenance is ϵ_m . As shown elsewhere [5], Equation (2) can be divided by η and written in the form

$$\frac{1}{\eta} = \frac{1}{\eta_{\max}} + \frac{m_e}{\mu} + \frac{\xi_p}{\eta \xi_p^{\max}} \quad (3)$$

where μ is the specific growth rate and m_e is a maintenance coefficient. The energetic yields η and ξ_p depend on the specific growth rate and vary with the fractions of energy allocated for growth and product formation while the true energetic yields $\eta_{\max}$ and $\xi_p^{\max}$ are better measures of the efficiency of the strain with respect to production of biomass and products,

respectively. Genetic engineering may be used to produce new strains which under optimal growth conditions may exhibit improved yields. Parameter estimation of $\eta_{\max}$, $\xi_p^{\max}$ and m_e and the comparison of these parameters with the estimated maximum values for energetic yields is expected to be helpful in guiding the search for genetic changes which improve yields.

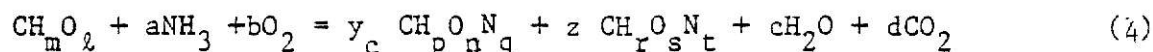
This work is concerned with the estimation of true energetic yield parameters. Several methods are considered, and two different forms of the equations are examined. Some of the statistical methods could be directly used by employing SAS* [11]. In other cases it was necessary to write longer computer programs with subroutines; however, in these cases SAS was also frequently employed. The consistency of the experimental data and the need for methods which allowed all of the data to be simultaneously used in the parameter estimation process are considered in this work. Both point and interval estimates of $\eta_{\max}$, m_e , and $\xi_p^{\max}$ are obtained for several sets of experimental data.

The estimation of true growth yields and true product yields has been considered previously [5-8]; however, interval estimates of the true product yield have not previously been reported. Stouthamer, Roels, and their coworkers [12-15] have considered yields associated with product formation, but they have not estimated $\xi_p^{\max}$. Interval estimates of $\eta_{\max}$ have been reported [8, 9, 16]; however, product formation was not presented in most of the cases which were considered. In earlier work in which products were present, $\xi_p^{\max}$ was fixed and $\eta_{\max}$ and m_e were estimated [6-9].

*SAS or Statistical Analysis System is a system of statistical method subroutines which are available for use in data analysis. Very little computer programming is required for their application.

THEORY

The balance equation of microbial growth and product formation can be written as follows:



where CH_mO_l denotes the elemental composition of organic substrate, $\text{CH}_p\text{O}_n\text{N}_q$ denotes the elemental composition of biomass and $\text{CH}_r\text{O}_s\text{N}_t$ represents the average elemental composition of extracellular products. For this growth process, carbon and available electron balances can be written as [2-4]:

$$y_c + z + d = 1.0 \quad (5)$$

$$\varepsilon + \eta + \xi_p = 1.0 \quad (6)$$

By using Pirt's model [17,18] and the regularities, Erickson [5] has derived the following equations:

Form I:

$$\frac{1}{\eta} = \frac{1}{\eta_{\max}} + \frac{m_e}{\mu} + \frac{\xi_p}{\eta \xi_p^{\max}} \quad (7)$$

$$\frac{\varepsilon}{\eta} = \frac{1 - \eta_{\max}}{\eta_{\max}} + \frac{m_e}{\mu} + \frac{\xi_p}{\eta} \left(\frac{1 - \xi_p^{\max}}{\xi_p^{\max}} \right) \quad (8)$$

$$\frac{d}{\eta} = \frac{\left(1 - \frac{\gamma_s}{\gamma_b} \eta_{\max}\right)}{\eta_{\max}} + \frac{m_e}{\mu} + \frac{\xi_p}{\eta} \left(\frac{1 - \frac{\gamma_s}{\gamma_p} \xi_p^{\max}}{\xi_p^{\max}} \right) \quad (9)$$

Multiplication of each term by μ gives Form II:

$$\frac{\mu}{\eta} = \frac{\mu}{\eta_{\max}} + m_e + \frac{\xi_p \mu}{\eta} \frac{1}{\xi_p^{\max}} \quad (10)$$

$$\frac{\varepsilon \mu}{\eta} = \frac{1 - \eta_{\max}}{\eta_{\max}} \cdot \mu + m_e + \frac{\xi_p \mu}{\eta} \left(\frac{1 - \xi_p^{\max}}{\xi_p^{\max}} \right) \quad (11)$$

$$\frac{d\mu}{\eta} = \frac{(1 - \frac{\gamma_s}{\gamma_b} \eta_{\max})}{\eta_{\max}} \cdot \mu + m_e + \frac{\xi_p \mu}{\eta} \left(\frac{1 - \frac{\gamma_s}{\gamma_p} \xi_p^{\max}}{\xi_p^{\max}} \right) \quad (12)$$

Form I and Form II correspond to the two different forms considered previously in yield studies without products [18,19]. Because of measurement errors, parameter estimates differ for each of these six equations. Equations (7) and (10) give different parameter estimates because the forms of the two equations differ while Equations (7), (8) and (9) give different parameter estimates because different experimental data are used. Biomass, product, organic substrate, and specific growth rate measurements are used with Equations (7) or (10). Oxygen, biomass, product and specific growth rate measurements are used with Equations (8) or (11). Carbon dioxide, biomass, product, and specific growth rate measurements are used with Equations (9) or (12).

Several different methods of statistical treatments of the data may be employed to obtain point and interval estimates of m_e , $\eta_{\max}$, and $\xi_p^{\max}$. Three different methods are employed in this work and the results are compared.

Method I:

Equations (7) through (12) can be rearranged in the following form:

$$Y_{1i} = \frac{1}{\eta_{\max}} + \frac{m_e}{X_{1i}} + X_{2i} \frac{1}{\xi_p^{\max}} + e_{1i} \quad (13)$$

$$Y_{2i} = \frac{1 - \eta_{\max}}{\eta_{\max}} + \frac{m_e}{X_{1i}} + X_{2i} \frac{1 - \xi_p^{\max}}{\xi_p^{\max}} + e_{2i} \quad (14)$$

$$Y_{3i} = \frac{1 - \frac{\gamma_s}{\gamma_b} \eta_{\max}}{\eta_{\max}} + \frac{m_e}{X_{1i}} + X_{2i} \frac{1 - \frac{\gamma_s}{\gamma_p} \xi_p^{\max}}{\xi_p^{\max}} + e_{3i} \quad (15)$$

$$Y_{4i} = \frac{1}{\eta_{\max}} X_{1i} + m_e + X'_{2i} \frac{1}{\xi_p^{\max}} + e_{4i} \quad (16)$$

$$Y_{5i} = \frac{1 - \eta_{\max}}{\eta_{\max}} X_{1i} + m_e + X'_{2i} \frac{1 - \xi_p^{\max}}{\xi_p^{\max}} + e_{5i} \quad (17)$$

$$Y_{6i} = \frac{1 - \frac{\gamma_s}{\gamma_b} \eta_{\max}}{\eta_{\max}} X_{1i} + m_e + X'_{2i} \frac{1 - \frac{\gamma_s}{\gamma_p} \xi_p^{\max}}{\xi_p^{\max}} + e_{6i} \quad (18)$$

where

$$Y_{1i} = \frac{1}{\eta_i}, \quad Y_{2i} = \frac{\varepsilon_i}{\eta_i}, \quad Y_{3i} = \frac{d_i}{\eta_i}$$

$$Y_{4i} = \frac{\mu_i}{\eta_i}, \quad Y_{5i} = \frac{\varepsilon_i \mu_i}{\eta_i}, \quad Y_{6i} = \frac{d_i \mu_i}{\eta_i}$$

$$X_{1i} = \mu_i, \quad X_{2i} = \frac{\xi_{pi}}{\eta_i}, \quad X'_{2i} = \frac{\xi_{pi} \mu_i}{\eta_i}$$

$$i = 1, 2, \dots, N$$

where N is the number of data points. The values of reductance degree γ_s , γ_b and γ_p , are constants for specified substrate, biomass and extracellular products, respectively [1-5].

The last term in Equations (13) through (18), e_{ji} , is an error term. It can be defined as:

$$e_{ji} = Y_{ji, \text{obs}} - Y_{ji, \text{cal}} \quad (19)$$

One can find the estimates of parameters m_e , n_{max} and ξ_p^{max} by using a non-linear least square technique which is based on minimizing the quantity [20].

$$\sum_{i=1}^N [e_{ji}]^2 \quad \text{for } j = 1, 2, \dots, 6 \quad (20)$$

Also, it is possible to obtain combined estimates of parameters from M of Equations (7) through (9) or (10) through (12);

$$\sum_{j=1}^M \sum_{i=1}^N (e_{ji})^2 \quad (21)$$

for M equations each with N data points.

For the case of correlated Y_{ji} and $Y_{j'i}$ ($j \neq j'$), Box and Draper [23] have derived a determinant criterion which, when minimized with respect to the parameters, m_e , n_{max} and ξ_p^{max} , gives estimates of these unknown parameters in the multiresponse models (13) through (15) or (16) through (18); that is, the determinant of the following matrix is minimized.

$$\begin{bmatrix} \sum_{i=1}^N (e_{1i})^2 & \sum_{i=1}^N (e_{1i})(e_{2i}) & \dots & \sum_{i=1}^N (e_{1i})(e_{Mi}) \\ \sum_{i=1}^N (e_{2i})(e_{1i}) & \sum_{i=1}^N (e_{2i})^2 & \dots & \sum_{i=1}^N (e_{2i})(e_{Mi}) \\ \vdots & \vdots & \ddots & \vdots \\ \sum_{i=1}^N (e_{Mi})(e_{1i}) & \sum_{i=1}^N (e_{Mi})(e_{2i}) & \dots & \sum_{i=1}^N (e_{Mi})^2 \end{bmatrix} \quad (22)$$

in this work, the Hooke and Jeeves Pattern Search Technique [24] was used to estimate parameters by minimizing the above determinant.

For 95% confidence intervals of parameters, the Smooth Bootstrap Method was used [25,26].

Basically, N sets of data are generated based on the assumed model and the observed data using Monte Carlo simulation techniques. From the N sets of generated data, N estimates of the parameters are obtained. These estimates are arranged in ascending order as for example,

$$\hat{\eta}_{\max}^1 \leq \hat{\eta}_{\max}^2 \leq \dots \leq \hat{\eta}_{\max}^N \quad (23)$$

Then a 95% confidence interval for $\eta_{\max}$ is $[\eta_{\max}^r, \eta_{\max}^{N-r}]$ where r is the integral part of 0.025 N. Of course, the larger the value of N the more precise the estimated interval is.

Method II:

As a first step, nonlinear regression was applied to each of Equations (13) through (15) or (16) through (18) to obtain least squares estimates of errors ($\hat{e}_{ji}$) by using SAS.

These estimates of errors were used to estimate the variance covariance matrix (Σ) as follows:

$$\hat{\Sigma} = \frac{1}{N-P} \begin{bmatrix} \sum_{i=1}^N (\hat{e}_{1i})^2 & \sum_{i=1}^N (\hat{e}_{1i})(\hat{e}_{2i}) & \dots & \sum_{i=1}^N (\hat{e}_{1i})(\hat{e}_{Mi}) \\ \sum_{i=1}^N (\hat{e}_{2i})(\hat{e}_{1i}) & \sum_{i=1}^N (\hat{e}_{2i})^2 & \dots & \sum_{i=1}^N (\hat{e}_{2i})(\hat{e}_{Mi}) \\ \vdots & \vdots & \ddots & \vdots \\ \sum_{i=1}^N (\hat{e}_{Mi})(\hat{e}_{1i}) & \sum_{i=1}^N (\hat{e}_{Mi})(\hat{e}_{2i}) & \dots & \sum_{i=1}^N (\hat{e}_{Mi})^2 \end{bmatrix} \quad (24)$$

where P is the number of parameters to be estimated.

Combined point estimates were obtained by minimizing the following quantity:

$$\sum_{i=1}^N (e_{1i}, e_{2i}, \dots, e_{Mi}) \hat{\Sigma}^{-1} \begin{Bmatrix} e_{1i} \\ e_{2i} \\ \vdots \\ e_{Mi} \end{Bmatrix} \quad (25)$$

where e_{ji} is given by Equation (19); that is

$$e_{ji} = y_{ji, \text{obs}} - F_j(X_{1i}, X_{2i} \text{ (or } X'_{2i}), m_e, n_{\max}, \xi_p^{\max}) \quad (26)$$

Again, the Hooke and Jeeves Pattern Search Technique [24] was used.

This method is principally weighted least squares based on the estimated covariance matrix. The 95% confidence intervals were estimated with the same procedure of Method I.

Method III:

This is a method of analysis of growth curves which utilizes the technique of analysis of covariance [27].

To be able to apply this method Equations (7) through (12) are linearized as follows:

for Form I

$$Y_{ji} = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} \quad j = 1, 2, 3 \quad (27)$$

for Form II

$$Y_{ji} = \beta_0' + \beta_1' X'_{1i} + \beta_2' X'_{2i} \quad j = 4, 5, 6 \quad (28)$$

where;

$$\begin{aligned} Y_{1i} &= \frac{1}{n}, & Y_{2i} &= \frac{\varepsilon}{n} + \frac{\xi_p}{n} + 1, & Y_{3i} &= \frac{d}{n} + \frac{\gamma_s}{\gamma_b} + \frac{\gamma_s}{\gamma_p} \frac{\xi_p}{n} \\ Y_{4i} &= \frac{\mu}{n}, & Y_{5i} &= \mu \left(\frac{\varepsilon}{n} + \frac{\xi_p}{n} + 1 \right), & Y_{6i} &= \mu \left(\frac{d}{n} + \frac{\gamma_s}{\gamma_b} + \frac{\gamma_s}{\gamma_p} \frac{\xi_p}{n} \right) \\ X_{1i} &= \frac{1}{\mu}, & X_{2i} &= \frac{\xi_p}{n}, & X'_{1i} &= \mu, & X'_{2i} &= \frac{\xi_p \mu}{n} \\ \beta_0 &= \frac{1}{n_{\max}}, & \beta_1 &= m_e, & \beta_2 &= \frac{1}{\xi_p^{\max}} \end{aligned}$$

$$\beta'_0 = m_e, \quad \beta'_1 = \frac{1}{\max}, \quad \beta'_2 = \frac{1}{p}$$

The variables, Y_{ji} , may be transformed to new variables, Z_{ji} , as follows:

$$Z_{1i} = \frac{1}{3} (Y_{1i} + Y_{2i} + Y_{3i}) \quad (29)$$

$$Z_{2i} = Y_{1i} - 2Y_{2i} + Y_{3i} \quad (30)$$

$$Z_{3i} = Y_{1i} + Y_{3i} \quad (31)$$

For the equations of Form II, Y_{1i} , Y_{2i} , Y_{3i} are replaced by Y_{4i} , Y_{5i} , Y_{6i} , respectively.

Coefficients of Equations (26) and (27) should be orthogonal to coefficients of Equation (25).

By using multiple linear regression procedures (in this study SAS was used for this purpose) maximum likelihood estimates of β_0 , β_1 and β_2 (or β'_0 , β'_1 and β'_2) can be obtained by minimizing

$$\sum_{i=1}^N (Z_{1i, \text{obs}} - Z_{1i, \text{calc}})^2$$

where

$$Z_{1i, \text{calc}} = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \alpha_1 Z_{2i} + \alpha_2 Z_{3i} \quad (32)$$

In this expression α_1 and α_2 are parameters associated with the new variables Z_{2i} and Z_{3i} . The parameters β_0 , β_1 and β_2 are replaced with β'_0 , β'_1 and β'_2 , respectively, for the equations of Form II. The application of this technique was studied in detail by Yang et al., [28].

The 95% confidence intervals for $\hat{\beta}_k$ can be estimated by using the estimated standard errors of parameters, $\hat{\sigma}_{\beta_k}$, which are directly obtained

from computer outputs.

The confidence interval is

$$\hat{\beta}_k \pm t_{0.025} (N-5) \cdot \hat{\sigma}_{\beta_k} \quad k = 0, 1, 2 \quad (33)$$

DISCUSSION AND RESULTS

In microbial growth processes with product formation the energetic yield parameters, m_e , $\eta_{\max}$ and $\xi_p^{\max}$, can be estimated by using one of Equations (7) through (12). Since the errors are different for each group of data, usually estimated values of these parameters depend on the data used and the form of the equations which are used. When sufficient data exists so that more than one of Equations (7), (8) or (9) can be used, statistical techniques to get the best estimates of parameters are needed. In this work, three different statistical methods are used to estimate the true energetic yield parameters under conditions where sufficient data exists to use two or more of Equations (7) through (9). Methods I and II are used with Equations (7) through (9) but Method III is designed for linear equations. Equations (27) and (28) are used with Method III. Methods I and II require utilization of a non-linear regression search technique. Method III is easily applied using SAS.

Data of Nishizawa et al. [24] was previously analyzed by Erickson [7]. For the glucose limited and oxygen limited data sets, values of ξ_p and z were calculated by utilizing available electron and carbon balances [Equations (6) and (5)], respectively. For some part of the oxygen limited growth data set, experimental values of product formation data were reported, and reasonably

good consistency was observed [7].

Since the extracellular product data was obtained from the carbon and available electron balances [7], only Equations (7) and (9) were used for each of these data sets when Form I was used.

Erickson [7] has reported theoretical maximum values of $\eta_{\max}$ and $\xi_p^{\max}$ as 0.88 and 0.95, respectively. Also, it is known that due to thermodynamic limitations, values of $\eta_{\max}$ and $\xi_p^{\max}$ must lie between zero and one and m_e should be positive.

Results of analysing the data of Nishizawa et al. [29] are shown in Tables 1 and 2. For both data sets, the results from Form I appear to be more reasonable than the results obtained using Form II. The point estimates of $\xi_p^{\max}$ are considerably greater than 1.0 when Form II is used. Point estimates are also presented in Tables I and II using the individual equations such as Equation (4) with Form I and the appropriate data for that equation. Parameter estimates using individual equations differ considerably from one another; this shows the need to use statistical methods which allow the data to be combined to obtain point and interval estimates based on all the data.

The results in Table I using Form I give relatively high values of $\eta_{\max}$ and $\xi_p^{\max}$; however, the point estimates are very reasonable compared to the maximum theoretical values. The values for the true growth yield, $\eta_{\max}$, have a relatively narrow 95% confidence interval. Method III consistently yields a larger 95% confidence interval than Methods I and II. The Bootstrap technique [25, 26; also see Appendix] was used to obtain 95% confidence intervals for Methods I and II.

In Table II, the estimates of the true growth yield, $\eta_{\max}$, under oxygen limited growth are even larger than the values in Table I. The average of the six interval estimates is [0.70, 0.78]. While these values are possible, the values are larger than those normally reported elsewhere in the literature.

The estimated values for $\xi_p^{\max}$ in Tables I and II are relatively large and the confidence intervals are very wide. The point estimates in Table I for Form I are less than the theoretical maximum value of 0.95; however, in all other cases, the point estimates of $\xi_p^{\max}$ exceed 0.95. Since the quantity of products is smaller than the quantity of biomass, and since much of the data did not include direct measurements of products, it is not surprising that the confidence intervals are large for $\xi_p^{\max}$. Because of biochemical and thermodynamic limitations, the actual values of $\xi_p^{\max}$ must be less than 0.95 and the actual confidence interval may be shortened to reflect this limitation.

In Table I, the estimated values of the maintenance coefficient are very small. All of the point estimates are positive, but some of the interval estimates include negative values. It is clear that only the positive values have a physical meaning associated with them. It is relatively common for the 95% confidence interval to include zero when maintenance parameters are estimated.

The results in Table II for oxygen limited conditions differ considerably from those in Table I for glucose limited conditions. The estimated value of the true biomass energetic yield, $\eta_{\max}$, is significantly larger under oxygen limited conditions. The estimated value of the maintenance coefficient is also larger under oxygen limited conditions; however, values of the maintenance coefficient are relatively small in both Tables I and II. The confidence intervals for the true product energetic yield, $\xi_p^{\max}$, are so large that significant conclusions can not be obtained by comparing these estimates.

The data of de Hollander et al. [30] has been previously analyzed by Erickson and Hess [8] using linear regression and fixed values of the parameter $\xi_p^{\max}$. An analysis of data consistency showed poor data consistency at the highest dilution rate. Table III shows the parameter estimates using all of the data of de Hollander et al. [30] while Table IV presents similar results, but based on all the data except that at the highest dilution rate. In this analysis Equations (4), (5), (7), and (8) are used; the carbon dioxide data needed for Equations (6) and (9) is not available.

The results in Table III show that the point estimates of the true biomass energetic yield $\eta_{\max}$, are very consistent for Methods I, II, and III for both Form I and Form II, but that the estimates for Form I are larger than those for Form II. The interval estimates for $\eta_{\max}$ differ considerably; Method II gives the shortest interval for Form I while Method I gives the shortest interval for Form II. These two intervals are similar. Method III gives the largest 95% confidence intervals.

The point estimates for the true product energetic yield, $\xi_p^{\max}$, are very similar in Table III for Methods I, II, and III for Form I and also for Form II. The point estimates of $\xi_p^{\max}$ are smaller for Form I than Form II. The interval estimates include an interval which exceeds the maximum theoretical value of 0.93 [8] except for Form I, Method II. The interval of interest is from the lower limit to 0.93 for those five cases. The shortest interval $0.61 \leq \xi_p^{\max} \leq 0.89$ from Form I, Method II appears to be a realistic interval. It is a 95% confidence interval; however, the other intervals are also 95% confidence intervals.

The point estimates for the maintenance coefficient, m_e , are all relatively small for all methods and both forms. The values for Form II

are smaller than those for Form I. All of the interval estimates include an interval where m_e is negative; however, this can be removed because m_e must be positive based on thermodynamic considerations. The interval estimates show that m_e is small for this organism.

The results in Table IV show the results of parameter estimation without using the data at the highest dilution rate. The point estimates for the maintenance coefficient are negative for four of the six cases in Table IV. The point estimates of $\xi_p^{\max}$ exceed the theoretical maximum value of 0.93 for all six cases. It is clear that the parameter estimates in Table IV are not as acceptable as those in Table III. It is also clear that removal of the data at one dilution rate has a significant effect on the parameter estimates. The data at the highest dilution rate appears to include several good measurements and one measurement with significant error.

When products are present and the three parameters, $\xi_p^{\max}$, $\eta_{\max}$ and m_e , are estimated, the data requirements are greater than when only two parameters are being estimated. The results in Table III appears to indicate that there is sufficient data to estimate these parameters from the experimental results of de Hollander et al. [30].

When one obtains several 95% confidence intervals by using three methods and two forms of the equations, the broadest interval estimate from the lowest limit to the highest limit should clearly include the actual value with at least 95% confidence. Method III appears to consistently yield larger intervals than Methods I and II. Methods I and II utilize the Bootstrap method which does not require any assumptions with regard to the model of the data. Because of the simulation approach which is used

to obtain the confidence intervals using Methods I and II, the interval may deviate slightly from a 95% confidence interval. However, even larger deviations may be present in Method III because of the transformation and linearization of the equations.

The point estimates from Methods I, II, and III are generally in good agreement. Since Method III is simplest to use it might be considered as the preferred method for point estimates. When both point and interval estimates are desired, Methods I and II are recommended. Since Method II involves the estimation of the covariance matrix, this method should give very reliable results whenever it is possible to obtain good estimates of the covariance matrix. Normally, the estimates of the covariance matrix are better when more data is available.

CONCLUSIONS

Three different methods are presented and applied to obtain point and interval estimates of true energetic yield and maintenance parameters. Good point and interval estimates of $\eta_{\max}$, $\xi_p^{\max}$ and m_e are obtained for the data of de Hollander et al.[30]. Methods I, II, and III give similar point estimates; however, Methods I and II usually give shorter interval estimates than Method III.

The results show that the parameter estimates depend on the form of the equations. Forms I and II give results which differ some. Utilization of both forms is desirable because this gives a clear indication of the dependence of the estimates on the form of the equations.

For the data considered in this work, the best results are obtained when all of the data is utilized. Data which does not satisfy the consistency test appears to make a positive contribution to the parameter estimates for the three methods which are used in this work.

NOMENCLATURE

a	Moles of ammonia per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
b	Moles of oxygen per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
c	Moles of water per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
d	Moles of carbon dioxide per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
e_{ji}	Error of i^{th} data point in j^{th} equation.
$\hat{e}_{ji}$	Estimate of error of i^{th} data point in j^{th} equation.
$\bar{e}_j$	Mean of estimates of errors of j^{th} equation
λ	Atomic ratio of oxygen to carbon in organic substrate, dimensionless.
M	Number of equations.
MLE	Maximum likelihood estimate.
m	Atomic ratio of hydrogen to carbon in organic substrate, dimensionless.
m_e	Rate of organic substrate consumption for maintenance, g equiv. of available electrons/g equiv of available electrons in biomass (hr)
N	Number of data points.
n	Atomic ratio of oxygen to carbon in biomass, dimensionless.
P	Number of parameters to be estimated.
p	Atomic ratio of hydrogen to carbon in biomass, dimensionless.

r	Atomic ratio of hydrogen to carbon in products, dimensionless.
s	Atomic ratio of oxygen to carbon in products, dimensionless.
t	Atomic ratio of nitrogen to carbon in products, dimensionless.
$t_{0.025}^{(N-5)}$	Students' t distribution with $(N-5)$ df. and 0.95 probability (for one tailed table).
y_c	Biomass carbon yield (fraction of organic substrate carbon in biomass), dimensionless.
z	Fraction of organic substrate carbon in products, dimensionless.
Z_i	Generated normal random number for i^{th} data point ($\sim N(0,1)$).
$\hat{\beta}_k$	Estimate of k^{th} parameter
γ_D	Reductance degree of biomass, equivalents of available electrons per gram atom carbon
γ_P	Reductance degree of products, equivalents of available electrons per gram atom carbon.
γ_S	Reductance degree of organic substrate, available electrons per gram atom carbon.
ϵ	Fraction of energy in organic substrate which is evolved as heat, dimensionless.
ϵ_m	Fraction of energy in organic substrate that is evolved as heat because of cell maintenance, dimensionless.
η	Fraction of energy in organic substrate which is converted to biomass or biomass energetic yield, dimensionless.
η_{max}	"True" biomass energetic yield, dimensionless.
μ	Specific growth rate, hr^{-1}

ξ_p	Fraction of energy in organic substrate which is converted to products, dimensionless
$\xi_p^{\max}$	"True" product energetic yield, dimensionless
$\hat{\sigma}_{\beta_k}$	Estimate of variance of k^{th} parameter.
$\hat{\sigma}_j^2$	Estimate of variance of errors from j^{th} equation.

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Table I. Point and 95% confidence interval estimates of true energetic yield parameters of *Rhodopseudomonas sphaeroides* S in dark and aerobic chemostat cultures under glucose limitation.*

		m_e, hr^{-1}		η_{max}		$\xi_p \text{ max}$	
		point	interval	point	interval	point	interval
<u>Equations are in Form I</u>							
N. Lin. Regression of Eqn. 4	0.00359	-	-	0.63766	-	0.93736	-
N. Lin. Regression of Eqn. 6	0.00545	-	-	0.70169	-	0.78368	-
Method I	0.00419	[-0.00034, 0.00922]		0.65546	[0.62737, 0.68198]		[0.71324, 1.21529]
Method II	0.00414	[0.00094, 0.00814]		0.65551	[0.65199, 0.68493]		[0.71875, 1.05599]
Method III	0.00415	[-0.00589, 0.01418]		0.65554	[0.60722, 0.71222]		[0.56819, 1.11748]
<u>Equations are in Form II</u>							
N. Lin. Regression of Eqn. 7	0.00588	-	-	0.63158	-	1.12152	-
N. Lin. Regression of Eqn. 9	0.00214	-	-	0.64105	-	1.02658	-
Method I	0.00399	[-0.00201, 0.00349]		0.63437	[0.60929, 0.66055]		[0.75995, 1.20996]
Method II	0.00630	[-0.00184, 0.00880]		0.63038	[0.61895, 0.65937]		[0.86519, 1.41736]
Method III	0.00439	[-0.01234, 0.02114]		0.63531	[0.58293, 0.69802]		[0.63646, 4.46982]

*Data of Nishizawa et al. [29]; number of data points = 8.

Table II. Point and 95% confidence interval estimates of true energetic parameters of *Rhodospseudomonas sphaeroides* S in dark and aerobic chemostat cultures under oxygen limitation.*

	m, hr^{-1}		η_{max}		$\xi_{\text{p}}^{\text{max}}$	
	point	interval	point	interval	point	interval
<u>Equations are in Form I</u>						
N. Lin. Regression of Eq. 4	0.01951	-	0.75845	-	1.00984	-
N. Lin. Regression of Eq. 6	0.01349	-	0.77330	-	0.87515	-
Method I	0.01979	[0.01429, 0.02613]	0.75769	[0.72952, 0.78879]	1.01779	[0.87181, 1.16099]
Method II	0.01978	[0.01439, 0.02549]	0.75752	[0.72752, 0.79352]	1.01776	[0.86776, 1.23635]
Method III	0.01979	[0.00696, 0.03261]	0.75777	[0.69186, 0.83754]	1.01718	[0.74208, 1.61642]
<u>Equations are in Form II</u>						
N. Lin. Regression of Eq. 7	0.02029	-	0.72763	-	1.28369	-
N. Lin. Regression of Eq. 9	0.01745	-	0.76221	-	1.07732	-
Method I	0.02059	[0.01367, 0.02610]	0.72276	[0.69550, 0.74799]	1.31924	[1.05847, 1.62199]
Method II	0.02067	[0.01388, 0.02756]	0.72343	[0.69185, 0.74624]	1.31524	[1.06371, 1.80956]
Method III	0.02065	[0.00669, 0.03462]	0.72353	[0.66798, 0.78916]	1.30066	[0.89701, 2.36482]

*Data of Nishizawa et al. [29]; number of data points = 10.

Table III. Point and 95% confidence interval estimates of true energetic yield parameters of chemostat cultures of *Rizobium Trifolii*.*

	m_p, hr^{-1}		η_{max}		$\xi_{\text{p}}^{\text{max}}$	
	point	interval	point	interval	point	interval
Equations are in Form I.						
N. Lin. Regression of Eq. 4	0.02355	-	0.89966	-	0.45138	-
N. Lin. Regression of Eq. 5	0.00809	-	0.64943	-	0.73500	-
Method I	0.00715	[-0.00335, 0.01715]	0.63849	[0.45705, 0.80305]	0.77195	[0.52394, 1.12168]
Method II	0.00665	[-0.00093, 0.01425]	0.63306	[0.52832, 0.68386]	0.78166	[0.61453, 0.89365]
Method III	0.00658	[-0.01322, 0.02637]	0.63220	[0.47382, 0.94986]	0.78315	[0.50885, 1.69900]
Equations are in Form II						
N. Lin. Regression of Eq. 7	0.00989	-	0.70756	-	0.51924	-
N. Lin. Regression of Eq. 8	0.00410	-	0.59963	-	0.83510	-
Method I	0.00409	[-0.00670, 0.01329]	0.59789	[0.53422, 0.68911]	0.85395	[0.68143, 1.10871]
Method II	0.00401	[-0.00514, 0.01375]	0.59709	[0.52428, 0.70353]	0.85571	[0.67555, 1.14526]
Method III	0.00382	[-0.02017, 0.02782]	0.59529	[0.44524, 0.89793]	0.86011	[0.53638, 2.16943]

*Data of de Hollander et al. [30]; number of data points = 11.

Table IV. Point and 95% confidence interval estimates of true energetic yield parameters of chemostat cultures of Rhizobium Trifolii.*

m, hr^{-1}		η_{max}		ξ_p^{max}	
point	interval	point	interval	point	interval
Equations are in Form I					
N. Lin. Regression of Eq. 4	0.00315	-	-	0.45151	-
N. Lin. Regression of Eq. 5	0.00820	-	-	0.73501	-
Method I	-0.00218	[-0.01050, 0.00760]	0.55822 [0.37022, 0.60893]	1.02420	[0.51416, 1.14014]
Method II	-0.00200	[-0.10010, 0.00650]	0.55990 [0.41250, 0.62730]	1.01728	[0.58256, 1.08410]
Method III	-0.00221	[-0.03259, 0.02816]	0.55805 [0.39084, 1.02533]	1.02435	[0.49427, 5.29382]
Equations are in Form II					
N. Lin. Regression of Eq. 7	0.02696	-	-	0.51763	-
N. Lin. Regression of Eq. 8	0.00454	-	-	0.83499	-
Method I	0.00047	[-0.01038, 0.00729]	0.57224 [0.52980, 0.61424]	0.93599	[0.81699, 1.11441]
Method II	0.00046	[-0.10385, 0.01099]	0.57094 [0.49908, 0.65925]	0.94028	[0.74723, 1.32736]
Method III	-0.00002	[-0.03458, 0.03454]	0.56738 [0.39236, 1.02427]	0.95397	[0.48394, 33.1920]

*Data of de Hollander et al. [30]

†Data of highest dilution rate is excluded; number of data points = 10.

CHAPTER III

ESTIMATION OF MAINTENANCE AND TRUE GROWTH YIELD IN ANAEROBIC CULTURES

INTRODUCTION

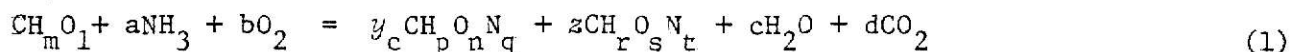
In aerobic microbial processes involving growth and product formation the biomass energetic yield, η , and the product energetic yield, ξ_p , have been determined by assuming that these values are equal to the corresponding fraction of available electrons incorporated into biomass and products, respectively [1 - 8]. For aerobic processes, this assumption has been shown to be appropriate and reasonable [1,9,10].

In this work, consideration is given to anaerobic processes as well as aerobic growth processes. Roels and co-workers [10 - 13] have considered the energetics of anaerobic growth processes and pointed out the need to consider the differences of the energy content per equivalent of available electrons of the substrate and the product. Roels [10,11] presents some results in which the thermodynamic efficiency associated with anaerobic growth has been calculated.

In earlier works [5,9,14,15] the yield parameters, $\eta_{\max}$ and $\xi_p^{\max}$ and the maintenance coefficient, m_e , have been defined and introduced. As attention is directed to anaerobic processes, it should be clearly understood that the available electron balance is exact and that the energy balance differs significantly from the available electron balance. It is also important to carefully consider the basic definitions of quantities and parameters as new concepts are introduced.

THEORY

Consider a stoichiometric equation associated with growth and product formation of the form



where CH_mO_1 , $\text{CH}_p\text{O}_n\text{N}_q$, and $\text{CH}_r\text{O}_s\text{N}_t$ give the elemental compositions of the carbon, hydrogen, oxygen, and nitrogen in the organic substrate, biomass, and extracellular product, respectively. Using the valences $\text{C} = 4$, $\text{H} = 1$, $\text{O} = -2$, and $\text{N} = -3$, the available electron balance is [1 - 4]

$$\frac{4b}{\gamma_s} + y_c \frac{\gamma_b}{\gamma_s} + z \frac{\gamma_p}{\gamma_s} = 1.0 \quad (2)$$

or

$$\epsilon + \eta + \xi_p = 1.0 \quad (3)$$

where γ is the reductance degree [1 - 4] and the subscripts s , b , and p refer to substrate, biomass and product, respectively. The carbon balance is

$$y_c + z + d = 1.0 \quad (4)$$

Equation (1) describes many aerobic processes and some anaerobic processes ($b = 0$ for the anaerobic processes).

Free Energy Yields

A free energy balance may also be written for equation (1). If the free energy level is taken as zero for NH_3 in aqueous solution, H_2O liquid, and CO_2 gas, only the free energy of the substrate, biomass, and product need to be considered in equation (1). That is,

$$\Delta G_s = y_c \Delta G_b + z \Delta G_p + \Delta G_D \quad (5)$$

where ΔG_s , ΔG_b , and ΔG_p are the free energies of the substrate, biomass, and product per g mole of carbon, respectively, and ΔG_D is the free energy dissipated in the process. Let us define a free energy yield for growth as

$$\eta_{th} = y_c \frac{\Delta G_b}{\Delta G_s} \quad (6)$$

and a free energy yield for product formation as

$$\xi_{th} = z \frac{\Delta G_p}{\Delta G_s} \quad (7)$$

Equation (5) may be written in the form

$$\epsilon_{th} + \eta_{th} + \xi_{th} = 1.0 \quad (8)$$

where

$$\epsilon_{th} = \frac{\Delta G_D}{\Delta G_s} \quad (9)$$

Free energy is used in the microbial process for growth, maintenance and product formation. Conceptually one may define

$$\eta_{th}^{max} = \frac{\text{free energy incorporated into biomass}}{\text{free energy allocated for biomass production}} \quad (10)$$

and

$$\xi_{th}^{max} = \frac{\text{free energy incorporated into products}}{\text{free energy allocated for product formation}} \quad (11)$$

The allocation of free energy may now be written in the form

$$\begin{array}{l} \text{Free} \\ \text{energy} \\ \text{input} \end{array} = \begin{array}{l} \text{Free energy} \\ \text{allocated for} \\ \text{biomass production} \end{array} + \begin{array}{l} \text{Free energy} \\ \text{for maintenance} \end{array} + \begin{array}{l} \text{Free energy} \\ \text{allocated for} \\ \text{product forma-} \\ \text{tion} \end{array}$$

or dividing by the free energy input

$$1 = \frac{\eta_{th}}{\eta_{th}^{max}} + \epsilon_m + \frac{\xi_{th}}{\xi_{th}^{max}} \quad (12)$$

where the fraction of free energy consumed for maintenance is

$$\epsilon_m = \frac{m_{th} \eta_{th}}{\mu} \quad (13)$$

where m_{th} is the maintenance coefficient in free energy units; that is, kJ free energy consumed/kJ free energy in biomass per hour. The specific growth rate, μ , divided by the biomass free energy yield, η_{th} , is the specific rate of free energy consumption of organic substrate. Equation (12) may be written in the form

$$\frac{1}{\eta_{th}} = \frac{1}{\eta_{max}} + \frac{m_{th}}{\mu} + \frac{\xi_{th}}{\eta_{th} \xi_{th}^{max}} \quad (14)$$

If the free energy per equivalent of available electrons is g_s , g_b , and g_p for the substrate, biomass, and products, respectively, then Equations (6) and (7) become

$$\eta_{th} = y_c \frac{\gamma_b g_b}{\gamma_s g_s} = \frac{g_b}{g_s} \eta \quad (15)$$

and

$$\xi_{th} = z \frac{\gamma_p g_p}{\gamma_s g_s} = \frac{g_p}{g_s} \xi_p \quad (16)$$

Equation (14) may be written in the form [5,14,15]

$$\frac{1}{\eta} = \frac{1}{\eta_{max}} + \frac{m_e}{\mu} + \frac{\xi_p}{\eta \xi_p^{max}} \quad (17)$$

where

$$\eta_{th}^{max} = \frac{g_b}{g_s} \eta_{max} \quad (18)$$

$$\xi_{th}^{max} = \frac{g_p}{g_s} \xi_p^{max} \quad (19)$$

and

$$m_{th} = \frac{g_s m_e}{g_b} \quad (20)$$

where η_{max} , ξ_p^{max} and m_e are yield and maintenance parameters associated with the available electron yields η and ξ_p [5,14,15]. From Equations (18), (19) and (20), it is clear that the new free energy yield and maintenance parameters are related to the corresponding available electron parameters. The methods of parameter estimation which have been presented previously, [7,8] to estimate the values of η_{max} , ξ_p^{max} and m_e may be employed to obtain point and interval parameter estimates of these parameters from substrate, biomass, product, specific growth rate, oxygen, and carbon dioxide measurements. From these results and Equations (18), (19), and (20), point and interval estimates of η_{th}^{max} , ξ_{th}^{max} and m_{th} may be obtained.

The numerical values of the free energy per equivalent of available electrons for the substrate and product may usually be found from tabulated literature data [10,16]. For substrates such as glucose and ethanol which do not contain nitrogen, the free energy of combustion per equivalent of available electrons should be used to find g_s and g_p . For biomass, the value of Roels [10] of -67.1 kJ/C mole for the free energy of formation of biomass may be used to obtain a value of

$$g_b = 113 \text{ kJ/equivalent}$$

for the free energy of the biomass relative to aqueous NH_3 , CO_2 gas and liquid H_2O . This value is in good agreement with the value of $114.7 \text{ kJ/equivalent}$ reported by Minkevich [17,18].

Equation (14) should be valid for both anaerobic and aerobic processes which follow equation (1). To illustrate the application of these equations, let us examine the ethyl alcohol fermentation with glucose as the organic substrate. Under conditions of excess oxygen, aerobic cell growth and maintenance are important. Under oxygen limited conditions, ethanol is produced and both substrate level phosphorylation and oxidative phosphorylation are important. Under strictly anaerobic conditions, ethanol is the electron acceptor and ATP production from substrate level phosphorylation provides all the energy for rearrangement of substrate available electrons into biomass.

Under strictly anaerobic conditions, two moles of ATP are formed from ADP when one mole of glucose is converted to ethyl alcohol. The available electrons in the glucose are transferred to ethyl alcohol and stoichiometrically, $\xi_p^{\max} = 1.0$; that is, the available electrons allocated for ethyl alcohol production are all incorporated into ethanol. The available electron balance for Equation (1) becomes

$$\eta + \xi_p = 1.0 \tag{21}$$

Equation (21) implies that available electrons which are not transferred to alcohol will be incorporated into biomass; that is, Equation (1) does not consider any loss of available electrons because of other products or biochemicals which might be present in small quantities. Equation (21) implies that $\eta_{\max} = 1.0$; that is, all the available electrons allocated for biomass production are incorporated into biomass. These results also have implications for the free energy balance; that is,

$$\epsilon_{th} = 1 - \frac{g_b}{g_s} \eta - \frac{g_p}{g_s} \xi_p \quad (22)$$

or because of equation (21)

$$\epsilon_{th} = 1 - \frac{g_b}{g_s} + \xi_p \left(\frac{g_b - g_p}{g_s} \right) \quad (23)$$

In the process of anaerobic growth with alcohol production, the maximum possible thermodynamic efficiency would occur when all of the available electrons are transferred to ethanol and biomass. Under these conditions from equations (18) and (19) and $\eta_{\max} = 1.0$ and $\xi_p^{\max} = 1.0$,

$$\eta_{th}^{\max} = \frac{g_b}{g_s} \quad (24)$$

and

$$\xi_{th}^{\max} = \frac{g_p}{g_s} \quad (25)$$

Consideration of the generation of ATP in product formation for use in growth would increase $\xi_{th}^{\max}$ and decrease $\eta_{th}^{\max}$. The fraction of free energy dissipated must be at least as large as that given in Equation (23). In actual practice, some of the available electrons will not be transferred to ethanol and biomass; and the free energy dissipated will be larger than that given in Equation (23).

Anaerobic Conditions

The ethyl alcohol produced under anaerobic conditions may be viewed as a product which must be generated to produce the ATP which is required for

growth and maintenance. If the available electrons that are transferred from glucose to ethanol are viewed as being expended to provide energy, another set of yield and maintenance parameters may be defined. A prime will be used with these parameters to distinguish them from those used earlier.

Under strictly anaerobic conditions, the fraction of the available electrons incorporated into biomass depends on the mass of cells per mole of ATP, $y_{ATP}^{\max}$, according to the relationship [15].

$$\eta'_{\max} = \frac{\frac{\sigma_b \gamma_b}{12} y_{ATP}^{\max}}{\frac{\sigma_b \gamma_b}{12} y_{ATP}^{\max} + 12} \quad (26)$$

where 12 equivalents of available electrons are transferred to ethanol to produce 1 g mole of ATP from ADP. For $\sigma_b = 0.462$, $\gamma_b = 4.291$, and $y_{ATP}^{\max} = 28.8$ g cells per mole ATP, $\eta'_{\max} = 0.284$. For $y_{ATP}^{\max} = 10.5$ g cells/mole ATP, $\eta'_{\max} = 0.126$. That is, neglecting maintenance, the maximum fraction of available electrons of glucose that can be incorporated into biomass under these anaerobic conditions is 0.284 based on the value of 28.8 g cells/mole ATP of Stouthamer [19]. Using the average value of 10.5 g cells/mole ATP of Bauchop and Elsdén [20], $\eta'_{\max} = 0.126$. To obtain a large fraction of ethanol and a small fraction of cells, a small value of $y_{ATP}^{\max}$ or a large maintenance coefficient appears to be desirable. Based on equation (1), for strictly anaerobic processes, the maintenance process may be viewed as the utilization of ATP, which is generated during ethanol formation and used for maintenance purposes. Growth yield and maintenance parameters for anaerobic growth may be written in the form

$$\frac{\mu}{\eta} = \frac{\mu}{\eta'_{\max}} + m'_e \quad (27)$$

$$\frac{\mu(\eta + \xi_p)}{\eta} = \frac{\mu}{\eta'_{\max}} + m'_e \quad (28)$$

$$\frac{\mu(y_c + d + z)}{\eta} = \frac{\mu}{\eta'_{\max}} + m'_e \quad (29)$$

$$\frac{\mu}{Y_{\text{ATP}}} = \frac{\mu}{Y_{\text{ATP}}^{\max}} + m_{\text{ATP}} \quad (30)$$

and

$$\frac{\mu}{\eta'_{\text{th}}} = \frac{\mu}{\eta'_{\text{th}}^{\max}} + m'_{\text{th}} \quad (31)$$

where $\eta'_{\max}$ and $Y_{\text{ATP}}^{\max}$ are related as shown in equation (26). An equation analogous to equation (26) may be used to relate η and Y_{ATP} . The maintenance coefficients are related according to the relationship

$$m'_e = \frac{144 m_{\text{ATP}}}{\phi_b \gamma_b} \quad (32)$$

where m_{ATP} is the moles of ATP expended per g of cells per hour for maintenance and m'_e is the maintenance coefficient in equivalents of available electrons expended per equivalent of available electrons of biomass per hour. Equation (32) is limited to fermentations in which 12 equivalents of available electrons are transferred per mole of ATP generated. Equations (27), (28), and (29) are similar and follow Pirt's model [21] for growth and maintenance. Equation (27) utilizes organic substrate, biomass, and specific growth rate measurements. Equation (28) utilizes biomass, ethanol and specific growth rate measurements, while equation (29) is designed for biomass, carbon dioxide, ethanol, and specific growth rate measurements. Equations (28) and (29) may be viewed as alternate versions of equation (27) where the available electron and carbon balance, respectively have been inserted to enable other measurements to be used in data analysis. Equations (27), (28), and (29) may also be viewed as the result

of derivations analogous to those presented elsewhere [4,5] in which glucose consumption, ethanol production, and CO_2 production, respectively provide the starting form of the Pirt model.

In Equation (31), the free energy biomass yield, η'_{th} , must take into account the free energy remaining in the ethanol product; that is,

$$\eta'_{th} = \frac{g_b \eta}{g_s - g_p \xi_p} \quad (33)$$

The available electron balance may be used to eliminate ξ_p in equation (33).

The true free energy biomass yield, $\eta'_{th}^{\max}$, is related to the corresponding available electron yield according to the relationship

$$\eta'_{th}^{\max} = \frac{g_b \eta'_{\max}}{g_s - g_p (1 - \eta'_{\max})} \quad (34)$$

The free energy and available electron maintenance coefficients are related; that is,

$$m'_{th} = \frac{(g_s - g_p) m'_e}{g_b} \quad (35)$$

where m'_{th} is the free energy expended for maintenance per unit of biomass free energy per hour. Comparison of m'_{th} and m_{th} as well as $\eta'_{th}^{\max}$ and $\eta_{th}^{\max}$ shows that these parameters are defined the same way in both cases. Thus, the numerical values should be similar. Note, however, that η'_{th} is not the same as η_{th} .

RESULTS AND DISCUSSION

The application of the theory to anaerobic process is illustrated using data from the published literature [13,22]. The anaerobic data of Dekkers et al., [13] for the growth of Saccharomyces cerevisiae CBS 426 on glucose in a chemostat is examined for consistency in Table I. Except for the 12th data point, the data are reasonably consistent. The results of parameter estimation with the 12th data point excluded are presented in Table II. The methods of data analysis have been described previously [7,8,23,24]. Equations (27), (28) and (29) are used with least squares parameter estimation to estimate the true growth yield, $\eta'_{\max}$, and the maintenance coefficient, m_e^i . Linear regression with Equation (27) in the form presented is referred to as Form II. Division of every term of Equation (27) by the specific growth rate, μ , gives Form I. Methods to use all of the measurements simultaneously to obtain maximum likelihood estimates (MLE) have been presented elsewhere [7,23]. Method III with all the covariates included gives the maximum likelihood estimate [23]. Method III with only one covariate included was also applied in this work. When one eigenvalue is much larger than the other, the covariate associated with the largest eigenvalue should be retained. A measure of the goodness of fit is [23]

$$J = \frac{\sigma^2}{N - P - 1}$$

Where σ^2 is a mean square error, N is the number of data points, and P is the number of parameters to be estimated. For additional detail on the statistical methods, please refer to the earlier works [7,8,23,24]. The average values $\sigma_b = 0.462$ and $\gamma_b = 4.291$ are used in the analysis of the data.

For a known value of $Y_{ATP}^{\max}$, the value of $\eta'_{\max}$ can be calculated using Equation (26). Dekkers et al. calculated a value of $Y_{ATP} = 8.6$ g dry wt/mole ATP for their anaerobic data and they reported a value of $Y_{ATP} = 15.1$ g dry wt/mole ATP for their aerobic data. Examination of the results in Table II, together with Equation (26), shows that the values of $\eta'_{\max}$ in Table II correspond to smaller values of $Y_{ATP}^{\max}$ than 8.6 g dry wt/mole ATP if 12 equivalents of available electrons transferred to ethanol produce 1 g mole of ATP. The value of $Y_{ATP}^{\max} = 8.6$ corresponds to $\eta'_{\max} = 0.11$. This value is at the upper end of the 95% confidence interval

$$0.0608 \leq \eta'_{\max} \leq 0.118$$

The results in Table II show that the maintenance coefficient, m'_e , is small. Only the positive portion of the 95% confidence interval is of interest since m'_e must be positive; that is, we have 95% confidence that

$$0 \leq m'_e \leq 0.0866$$

Esener et al., [25] showed that m_{ATP} varies between 0.25 to 0.70 m moles ATP/g(hr) which corresponds to 0.018 to 0.051 hr^{-1} for m'_e . These values are within the 95% confidence interval presented above.

The continuous culture data of Lee et al., [22] for growth of Zymomonas mobilis on sucrose is examined for consistency in Table III. The consistency is reasonably good. The data points in which the feed contained 100 g/l sucrose were combined with those in which the feed contained 150 g/l and analyzed together. Zymomonas mobilis has an absolute requirement for pantothenic acid [26,27]. It produces levan when it grows on sucrose [20]. In this analysis, yeast extract as substrate and levan as side product are included in analyzing the data. Levan is considered as product analogous to

ethanol; that is, ATP is assumed to be produced in the process of converting glucose to levan. The yeast extract is included as a substrate and the values of $\sigma = 0.40$ and $\gamma = 4.0$ are used in estimating the carbon and available electron content of the yeast extract.

The parameter estimation results are presented in Table IV. The values of the maintenance parameter, m_e' , are much larger for this organism while the values of the true growth yield are much smaller when they are compared to the results in Table II. Zymomonas mobilis uses the Entner-Doudoroff pathway and produces 1 mole of ATP/mole of glucose converted to ethanol [26]. This organism has received considerable attention with respect to ethanol production as it is more ethanol tolerant and it can produce ethanol faster than conventional yeasts [28-31]. The high maintenance requirements of this organism have been considered by Cromie et al. [32] and Esener et al., [25]. Esener et al., [25] pointed out that the high maintenance requirement requires rapid ethanol production by the organism to meet this need. Thus, larger values of m_e' should be found for this organism. Esener et al., [25] has estimated that $m_{ATP} = 10.3$ m moles ATP/g (hr) for this organism which corresponds to 1.50 hr^{-1} for m_e' assuming 24 equivalents of available electrons generate 1 ATP. (The coefficient in Equation (32) is 288). This value is within the 95% confidence interval

$$0.937 \leq m_e' \leq 4.10$$

The 95% confidence interval for the true growth yield is

$$0.0305 \leq \eta_{\max}' \leq 0.0539$$

The interval may be compared to $Y_{ATP}^{\max} = 8.3$ which is equivalent to $\eta_{\max}' = 0.054$ found by Forrest [33]. Since 24 equivalents of available electrons yield 1 mole of ATP, Equation (26) becomes

$$\eta'_{\max} = \frac{\frac{\sigma_b \gamma_b}{12} Y_{\text{ATP}}^{\max}}{\frac{\sigma_b \gamma_b}{12} Y_{\text{ATP}}^{\max} + 24}$$

for Zymomonas mobilis

Values of Y_{ATP} ranging from 4.3 to 9.3 have been estimated by Swing and DeLey [26]; however, with the large maintenance requirement for this organism, the corresponding value of $Y_{\text{ATP}}^{\max}$ may be considerably higher.

The true growth yield in available electron units, $\eta'_{\max}$, can be converted to a better thermodynamic measure of growth efficiency by using Equation (34) to obtain the true free energy biomass yield. In Table V values of the true free energy biomass yield, the true free energy product yield, and the corresponding maintenance parameter in free energy units are presented for several sets of data. The close agreement of the true free energy biomass yield from aerobic and anaerobic measurements for the data of Dekkers et al., [13] should be noted. For Zymomonas mobilis and the efficiency of growth is lowest and the maintenance coefficient is largest. Both of these contribute to the high rate of alcohol production by this organism.

CONCLUSIONS

The methods of parameter estimation in which all of the data are considered simultaneously to obtain maximum likelihood estimates of parameters have been extended to anaerobic growth processes. Parameter estimates have been obtained for two sets of literature data. For anaerobic growth where the product is the only electron acceptor, growth and maintenance are coupled with product formation. When product formation is desired, a high maintenance requirement is a desirable property.

NOMENCLATURE

- a Moles of ammonia per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
- b Moles of oxygen per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
- c Moles of water per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
- d Moles of carbon dioxide per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
- ΔG_b free energy of biomass per g mole of carbon in biomass, KJ/mole C.
- ΔG_D free energy dissipated per g mole of carbon in substrate, KJ/mole C.
- ΔG_p free energy in product per g mole of carbon in product, KJ/ mole C.
- ΔG_s free energy in substrate per g-mole of carbon in substrate, KJ/mole C.
- g_b Amount of free energy in biomass per g equiv. of available electron in biomass, KJ/g. equiv. of a.e.
- g_p Amount of free energy in product per g. equiv. of available electron in product, KJ/g. equiv. of a.e.
- g_s Amount of free energy in substrate per g. equiv. of available electron in substrate, KJ/g. equiv. of a.e.
- MLE Maximum likelihood estimate.
- m_e Rate of organic substrate consumption for maintenance, g equiv. of available electron per g equiv. of available electrons in biomass (hr).
- m_{ATP} Moles of ATP expended per g of cells per hour for maintenance, moles ATP/g-hr.
- m'_e Same definition with m_e . Prime denotes anaerobic process.
- m_{th} Amount of free energy consumed per unit of free energy in biomass per hour, KJ free energy consumed/KJ free energy in biomass. (hr).
- m'_{th} Same definition with m_{th} . Prime denotes anaerobic process.
- N Number of data points.
- P Number of parameters to be estimated.
- Y_{ATP} Biomass yield based on ATP, g dry biomass/g mole ATP.

Y_{ATP}^{max}	Maximum biomass yield based on ATP, g dry biomass/g mole ATP.
y_c	Biomass carbon yield (fraction of organic substrate carbon in biomass), dimensionless.
z_i	Fraction of organic substrate carbon in products, dimensionless.
z	Covariates included in Method III.
γ_b	Reductance degree of biomass, equivalents of available electrons per g atom carbon.
γ_p	Reductance degree of products, equivalents of available electrons per g atom carbon.
γ_s	Reductance degree of substrate, equivalents of available electrons per g-atom carbon.
ϵ	Fraction of energy in organic substrate which is evolved as heat, dimensionless.
ϵ_m	Fraction of energy in organic substrate that is evolved as heat because of cell maintenance, dimensionless.
ϵ_{th}	Fraction of free energy in substrate which is evolved as heat dimensionless.
η	Fraction of available electrons in organic substrate which is converted to biomass or biomass energetic yield dimensionless.
η_{max}	"True" biomass available electron yield, dimensionless.
η'_{max}	"True" biomass available electron yield, dimensionless. Prime denotes anaerobic process.
η_{th}	Fraction of free energy in organic substrate which is converted to biomass energetic yield, dimensionless.
η'_{th}	Biomass free energy yield, dimensionless.
η_{th}^{max}	"True" biomass free energy yield, dimensionless.
η'_{th}^{max}	"True" biomass free energy yield, dimensionless. Prime denotes anaerobic process.
μ	Specific growth rate hr^{-1} .
ξ_p	Fraction of available electrons in organic substrate which is converted to products, dimensionless.
ξ_p^{max}	"True" product energetic yield, dimensionless.

ξ_{th} Fraction of free energy in organic substrate which is converted to products, dimensionless.

ξ_{th}^{max} "True" product free energy yield, dimensionless.

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Table I. Data consistency analysis for the growth of *Saccharomyces cerevisiae* CBS 426 on glucose under anaerobic conditions.*

No. of data point	μ	n	ξ_p	$n + \xi_p$	y_c	z	d	$y_c + z + d$
1	0.012	0.0634	0.7697	0.8332	0.0591	0.5269	0.2527	0.8387
2	0.014	0.0931	1.0194	1.1125	0.0867	0.6918	0.3881	1.1667
3	0.014	0.1197	0.8907	1.0105	0.1116	0.5953	0.4721	1.1791
4	0.017	0.1033	0.9245	1.0279	0.0963	0.6262	0.3179	1.0405
5	0.027	0.0916	0.7220	0.8137	0.0854	0.4813	0.3177	0.8844
6	0.030	0.0957	0.8398	0.9355	0.0892	0.5716	0.2885	0.9493
7	0.034	0.0999	0.8087	0.9087	0.0932	0.5495	0.2437	0.8864
8	0.051	0.0639	0.8297	0.8937	0.0596	0.5665	0.2059	0.8320
9	0.053	0.0949	0.8183	0.9132	0.0885	0.5607	0.2804	0.9296
10	0.056	0.0757	0.9148	0.9905	0.0706	0.6229	0.2647	0.9582
11	0.058	0.0932	0.8914	0.9846	0.0868	0.6080	0.2480	0.9428
12	0.058	0.0812	1.4507	1.4981	0.0847	0.9381	0.2971	1.4981
13	0.062	0.0909	0.9862	1.0674	0.0756	0.6708	0.2562	1.0027
14	0.067	0.0627	0.8799	0.9426	0.0584	0.5953	0.3249	0.9787
15	0.064	0.0836	0.8297	0.9133	0.0779	0.5547	0.2537	0.8864

* Data of Dekkers et al., [13].

Table II. Point and 95% confidence interval estimates of the maintenance parameter, m_e , and the true biomass available electron yield, $n_{\max}$, for the data of Table I*.

Equation and Method	Form	Maintenance Parameter			True Biomass Yield			Covariates included	J
		Point Estimate	95% confidence interval		Point Estimate	95% confidence interval			
Eq. (27), L.R. ^a	I	-0.0192	-0.0791, 0.0408		0.0796	0.0661, 0.100			
	II	-0.0431	-0.160, 0.0738		0.0751	0.0635, 0.0917			
Eq. (28), L.R.	I	-0.0181	-0.0737, 0.0341		0.0840	0.0706, 0.104			
	II	-0.0407	-0.154, 0.0731		0.0791	0.0667, 0.0973			
Eq. (29), L.R.	I	-0.0032	-0.0517, 0.0454		0.0876	0.0740, 0.107			
	II	-0.0261	-0.144, 0.0922		0.0823	0.0685, 0.103			
Eq. (27), (28), (29) Method III	I	0.0043	-0.0610, 0.0696		0.0896	0.0723, 0.1181	z_2, z_3	0.524	
		-0.0064	-0.0602, 0.0474		0.0879	0.0721, 0.1125	z_2	0.448	
		-0.0104	-0.0712, 0.0502		0.0838	0.0701, 0.1043	z_3	0.495	
	II	-0.0464	-0.179, 0.0866		0.0766	0.0608, 0.118	z_2, z_3	0.00123	
		-0.0375	-0.159, 0.0841		0.0783	0.0633, 0.102	z_2	0.00103	
		-0.0447	-0.168, 0.0788		0.0774	0.0643, 0.0972	z_3	0.00101	

* The 12th data point is excluded.

^a L.R. refers to linear regression.

For Form I, the eigenvalues are 16.8 and 2.92 for z_2 and z_3 ; for Form II the eigenvalues are 0.0107 and 0.00658 for z_2 and z_3 , respectively.

Table III. Data consistency analysis for the growth of Zymomonas mobilis on sucrose. *†.

No. data point	μ	η	ξ_p	$\eta + \xi_p$	y_c	z	d	$y_c + z + d$
1	0.0367	0.0113	0.9063	0.9176	0.0105	0.6223	0.2838	0.9167
2	0.0836	0.0138	0.7574	0.7712	0.0165	0.5286	0.2286	0.7737
3	0.1313	0.0216	0.8789	0.9005	0.0201	0.6189	0.2599	0.8989
4	0.1859	0.0243	0.9986	1.0230	0.0226	0.7028	0.2958	1.0212
5	0.2508	0.0285	0.9945	1.0230	0.0226	0.7100	0.2847	1.0211
6	0.0680	0.0168	0.8339	0.8509	0.0157	0.5597	0.2742	0.8496
7	0.1000	0.0201	0.8129	0.8329	0.0184	0.5412	0.2605	0.8202
8	0.1504	0.0320	0.9739	1.0060	0.0225	0.6787	0.2952	0.9964
9	0.2000	0.0355	0.9278	0.9633	0.0243	0.6498	0.2759	0.9499
10	0.2720	0.0286	0.9705	0.9991	0.0267	0.6880	0.2824	0.9972
11	0.3240	0.0339	0.9506	0.9845	0.0316	0.6889	0.2616	0.9822

* Data of Lee et al., [22].

† First five points are from Figure 6. Last six points are from Figure 5 [22].

• Yeast extract and levan (as side product) are included in calculations.

Table IV. Point and 95% confidence interval estimates of the maintenance coefficient, m'_c , and the true biomass available electron yield, $n_{\max}$, for the data of Table III.

Equation and Method	Form	Maintenance Parameter		True Biomass Yield		Covariates Included	J
		Point Estimate	95% confidence interval	Point estimate	95% confidence interval		
Eq. (27), L.R. ^a	I	2.54	1.74, 3.33	0.0415	0.0303, 0.0659		
	II	2.81	1.42, 4.20	0.0458	0.0341, 0.0697		
Eq. (28), L.R.	I	2.07	1.54, 2.60	0.0413	0.0331, 0.0549		
	II	1.96	0.68, 3.24	0.0400	0.0314, 0.0522		
Eq. (29), L.R.	I	2.07	1.52, 2.62	0.0416	0.0331, 0.0560		
	II	1.95	0.64, 3.25	0.0401	0.0313, 0.0559		
Eq. (27), (28) (29)	I	1.97	1.41, 2.52	0.0369	0.0305, 0.0468	z_2, z_3	2.97
		1.96	1.25, 2.67	0.0415	0.0323, 0.0566	z_2	4.37
Method III		2.30	1.61, 2.99	0.0402	0.0303, 0.0582	z_3	5.28
	II	2.52	0.94, 4.10	0.0392	0.0308, 0.0539	z_2, z_3	0.0776
		2.05	-0.015, 4.12	0.0406	0.0294, 0.0656	z_2	0.127
		2.93	0.99, 4.86	0.0451	0.0333, 0.0699	z_3	0.111

^a L.R. refers to linear regression

For Form I, the eigenvalues are 2.09 and 5.74 for z_2 and z_3 , respectively.

For Form II, the eigenvalues are 1.09 and 0.182 for z_2 and z_3 , respectively.

Table V. Comparison of free energy maintenance parameters, m_{th} and m_{th}' , true growth yields, η_{th} and η_{th}' , and $\eta_{th}'^{max}$ product yields, ξ_{th} , between aerobic and anaerobic microbial growth processes.*

Form of Equations	$(m_{th} \text{ or } m_{th}') (n_{th}^{max} \text{ or } \eta_{th}^{max}) (\xi_{th}^{max})$	Organism	Substrate	Product	Source of Data
I	0.00439	Rhodospseudomonas	Glucose	Polyscharrides	Nishizawa et al. (34)
II	0.00465	sphaeroides			
I	0.00697	Rhizobium	Glucose	Polyscharrides	de Hollander et al. (35)
II	0.00404	Trifolii			
I	0.00360	Saccharomyces	Glucose	Ethanol	Dekkers et al. (13)
II	-0.00940	Cerevisiae			
		CBS 426			
I	0.00380	Saccharomyces	Glucose	Ethanol	von Meyenburg (36,37)
II	0.00370	Cerevisiae			
		CBS A26			
I	-0.00055	Saccharomyces	Glucose	Ethanol	Dekkers et al. (13)
II	-0.00402	Cerevisiae			
		CBS A26			
I	0.170	Zymomonas	Sucrose	Ethanol	Lee et al. (22)
II	0.218	Mobilis			

* Prime denotes anaerobic processes.

CHAPTER IV

ETHANOL PRODUCTION BY ZYMOMONAS MOBILIS ON GLUCOSE IN ANAEROBIC BATCH CULTURE

INTRODUCTION

Anaerobic growth of Zymomonas mobilis, ATCC 10988, on glucose in batch culture has been performed. Samples collected during the fermentation process were analyzed to determine biomass, ethanol production and glucose consumption. Also, carbon-dioxide production has been measured.

These measured variables have been used to check consistency of experimental data by utilizing previously presented [1-5, Chapter III], carbon and available electron balances.

Maintenance coefficient, m_e , and true growth yield, $\eta_{\max}$, in anaerobic processes have been defined in Chapter III. Previously introduced methods [Chapter II, III, 13] have been used to estimate m_e and $\eta_{\max}$ for the data collected during anaerobic growth of Z. mobilis on glucose in batch cultures.

It has been demonstrated that, having higher maintenance requirement and lower true growth yield are important properties for this organism. Also, it was found that these are desired properties when ethanol production is the main purpose of the process.

THEORY

Stoichiometric equation of microbial growth and product formation can be written as $\text{CH}_m\text{O}_l + a\text{NH}_3 + b\text{O}_2 = y_c \text{CH}_p\text{O}_n\text{N}_q + z \text{CH}_r\text{O}_s\text{N}_t + c\text{H}_2\text{O} + d\text{CO}_2$ (1) where CH_mO_l , $\text{CH}_p\text{O}_n\text{N}_q$ and $\text{CH}_r\text{O}_s\text{N}_t$ represent the elemental compositions of the organic substrate, biomass and extracellular product, respectively (for anaerobic processes, $b = 0$).

The reductance degree, γ , has been defined as the equivalents of available electrons per g atom carbon or the equivalents of oxygen required to oxidize the organic substance of interest to CO_2 , H_2O and NH_3 . Also, Minkevich and Eroshin [1] have shown that the fraction of carbon in biomass, σ_b , the reductance degree, γ_b , and energy content per equivalent of available electrons in biomass, Q_o , are relatively constant and equal to 0.462, 4.291 and 27 kcal/eq., respectively.

Utilizing equation (1), carbon and available electron balances can be written as [1-4]

$$y_c + z + d = 1 \quad \text{carbon balance} \quad (2)$$

and

$$\varepsilon + \eta + \xi_p = 1 \quad \text{available electron balance} \quad (3)$$

where, y_c , z and d are the fraction of substrate carbon converted to biomass, product and carbon dioxide, respectively, and ε , η and ξ_p are the fractions of available electrons in the substrate, which are transferred to oxygen, incorporated into biomass and utilized in product formation. Obviously, for anaerobic case, ε will be equal to zero and Equation (3) will be

$$\eta + \xi_p = 1 \quad (4)$$

Equations (2) and (4) have been used to check the consistency of data collected during the anaerobic fermentation of Zymomonas mobilis on glucose.

During fermentation, measured variables are biomass concentration X , g/l, specific carbon dioxide evolution rate Q_{CO_2} , moles CO_2 /g biomass (hr), specific substrate consumption rate, g substrate/g biomass (hr) and specific product formation rate, g product/g biomass (hr). In batch cultures both instantaneous and integrated balances are possible. For instantaneous balances, following relationships have been derived [5] to obtain y_c , z , d , η and ξ_p

$$y_c = \frac{\sigma_b \mu}{\sigma_s Q_s} \quad (5)$$

$$z = \frac{\sigma_p}{\sigma_s} \frac{Q_p}{Q_s} \quad (6)$$

$$d = \frac{12 \cdot Q_{CO_2}}{\sigma_s Q_s} \quad (7)$$

$$\eta = \frac{\sigma_b Y_b}{\sigma_s Y_s} \frac{\mu}{Q_s} \quad (8)$$

$$\xi_p = \frac{\sigma_p Y_p}{\sigma_s Y_s} \frac{Q_p}{Q_s} \quad (9)$$

For integrated balances the following equations are used.

$$y_c = \frac{\sigma_b}{\sigma_s} \frac{X - X_o}{(S_o - S)} \quad (10)$$

$$z = \frac{\sigma_p}{\sigma_s} \frac{P - P_o}{S_o - S} \quad (11)$$

$$d = \frac{12 \int_0^\Theta Q_{CO_2} \cdot X \cdot d\Theta}{(S_o - S) \sigma_s} \quad (12)$$

$$\eta = \frac{\sigma_b Y_b}{\sigma_s Y_s} \frac{(X - X_o)}{(S_o - S)} \quad (13)$$

$$\xi_p = \frac{\sigma_p Y_p}{\sigma_s Y_s} \frac{(P - P_o)}{(S_o - S)} \quad (14)$$

where X , S and P are the concentration of biomass, substrate and product at any time of interest, Θ , respectively, and X_o , S_o and P_o are the initial biomass, substrate and product concentrations, respectively.

The maintenance coefficient, m'_e , and true biomass available electron yield, $\eta'_{\max}$, may be estimated using the following equations which have been derived in Chapter III:

$$\frac{\mu}{\eta} = \frac{\mu}{\eta'_{\max}} + m'_e \quad (15)$$

$$\frac{\mu(\eta + \xi_p)}{\eta} = \frac{\mu}{\eta'_{\max}} + m'_e \quad (16)$$

$$\frac{\mu(y_c + d + z)}{\eta} = \frac{\mu}{\eta'_{\max}} + m'_e \quad (17)$$

Equations (15), (16) and (17) have been used to obtain estimates of m'_e and $\eta'_{\max}$. Also, Method III, which is explained in Chapter II, has been used with these equations to obtain better, combined, estimates of the parameters m'_e and $\eta'_{\max}$.

MATERIALS AND METHODS

Fermentor

A Bench-Scale Fermentor, Model 19, manufactured by the New Brunswick Scientific Co. was used. A 7 liter stirred container with four vertical baffles was used for cell cultivation. Different rates of agitation were provided by impellers with flat stirring paddles.

Temperature was maintained at 30°C with the aid of a temperature controller. For the first run, pH of the medium was maintained at 5 by a New Brunswick Scientific Model pH-22 controller. Foaming was controlled by using antifoam, a spray produced by Dow Corning.

Organism

Zymomonas mobilis, ATTC-10388, was used for all runs.

Medium Composition

Medium composition is listed below.

<u>Component</u>	<u>g/liter distilled water</u>
D-Glucose	100.00
Urea, $(\text{NH}_2)_2\text{CO}$	0.50
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1.00
* KH_2PO_4	2.00
Yeast extract (Difco)	10.00

*For run II, instead of 2 gm/l KH_2PO_4 , 6.5432 gm/l KH_2PO_4 and 4.51126 gm/l K_2HPO_4 were used as buffer to maintain pH at 7.0.

Fermentation

Innoculum preparation: Zymomonas mobilis was recovered from agar slants (20 gm/l glucose, 5 g/l yeast extract and 2% agar at 30 C). The cells were cultivated for 24 hrs. in 100 ml of broth with the same composition as the fermentor broth. Twenty ml of this culture was used to inoculate 200 ml of medium which was cultured for 12 hrs. This 200 ml of culture was used as inoculum.

Batch growth of Zymomonas mobilis on Glucose: Five liters of culture medium in the fermentor vessel were autoclaved at 20 psia for 15 minutes. After the temperature had returned to 30°C, it was connected to the fermenter and inoculated with 200 ml. of inoculum. During the fermentation process, gas flow rate, gauge pressure, pH and % CO₂ in exit gas were recorded at least every half hour. For biomass, glucose and ethanol analysis samples were taken by means of a sismamotor peristaltic pump. The first 60 ml were discarded and 25 ml samples were collected and 10 ml of these samples were analyzed.

Agitation was maintained at 400 RPM for Run I; it was set to 800 RPM during Run II.

Analytical Methods

Cell Dry Weight Analysis: The 10 ml sample collected each time was filtered on a previously weighed millipore membrane filter (Type RA 1.2 µm). The filtrate was saved for further glucose and ethanol analysis. The residue was washed with distilled water after the filtrate had been removed. The biomass was dried at 105°C for 24 hrs. and weighed again. Also values of biomass were corrected for drying loss of filter papers.

Glucose and Ethanol Concentration: The filtrates saved from all dry weight analysis were used for this analysis. The analysis was performed with HPLC, Varian 520 (Column: HPLC column for organic acid analysis, Aminex Ion Exclusion HPX-87H 300 x 7.8 mm; Detector: Refractive index detector; Oven temperature: 45°C; Solvent flow rate: 0.9 ml/min; 2% propanol was used as internal standard).

Carbon Dioxide in the Gas Phase: Beckman Model 864 CO₂ infrared analyser was used. Minimum flow rate requirement for this analyzer was 1 l/min. To satisfy this and strict anaerobic condition pure nitrogen gas has been used. Filtered nitrogen gas passed through a rotameter and entered the fermentor through a sprayer. The lower scale of CO₂ analyzer was calibrated using dry air while the upper scale was calibrated using a standard gas containing 2.3% CO₂. Both sample and standard gases were passed through drierite before entering the pressure regulator. The CO₂ scale was from 0% CO₂ to 2.5% CO₂. In the late period of the fermentation, nitrogen gas flow into fermentor was increased to keep CO₂ concentration in this range. Using a valve just before the drierite, excess gas was vented. By using the rotameter of the CO₂ analyzer, the flow rate into the analyzer was kept at 1 l/min throughout the process.

Volume of Broth: Volume of broth at each point was calculated simply by subtracting, subsequently, the volume of samples from original volume of broth. Also, for Run I, where pH controller was used, corrections were made for acid and base additions. No corrections were made for changes due to evaporation of water, nutrient consumption and formation of ethanol and biomass.

Gas Flow Rate: A rotameter was used to measure the flow rate of gas entering the fermentor. Gauge pressure of entering gas was also recorded and gas flow rate was corrected for pressure using a chart provided with the rotameter.

The molar air flow rate per unit volume of broth, Q_m , was calculated as follows:

$$Q_m = \frac{F}{V} \cdot \frac{1 \text{ mole gas}}{22.4 \text{ l}} \cdot \frac{273^\circ\text{K}}{303^\circ\text{K}} \cdot \frac{60 \text{ min}}{\text{hr.}} \quad (18)$$

where F was the gas flow rate, l/min, and V was the volume of broth at the time of interest. The molar flow rate was assumed to be the same for the inlet and exit streams to the fermentor.

Rate of Carbon Dioxide Evolution: Since the inlet gas stream is pure nitrogen, rate of carbon dioxide evolution is

$$Q_{\text{CO}_2} \cdot X = \frac{\% \text{ CO}_2 \text{ exit}}{100} \cdot Q_m \quad (19)$$

The specific rate of carbon dioxide evolution Q_{CO_2} was calculated by dividing $Q_{\text{CO}_2} \cdot X$ by the biomass concentration, X.

Specific growth rate: Specific growth rate is defined as

$$\mu = \frac{1}{X} \frac{dX}{d\theta} \quad (20)$$

To calculate μ , biomass concentration versus time plot was drawn. A smooth curve was passed through these experimental points. Smoothed values were read and used to obtain derivatives at the time in question by using a subroutine program (DGT3). By dividing the slope by the value of X at the same point in time, an estimate of the specific growth rate, μ , was obtained.

Specific Rate of Glucose Consumption: The specific rate of glucose consumption is

$$Q_S = - \frac{1}{X} \frac{dS}{d\theta} \quad (21)$$

Similarly, smoothed values were obtained from the substrate concentration versus time curve and used to estimate slopes at each point in question by using subroutine DGT3. The slope is again divided by the value of biomass concentration, X , at the same point in time.

Rate of Ethanol Production: Specific rate of ethanol production is defined as

$$Q_P = \frac{1}{X} \cdot \frac{dP}{d\theta} \quad (22)$$

It was calculated with the same procedure as that performed for glucose.

DISCUSSION AND RESULTS

Anaerobic growth of Zymomonas mobilis, ATCC 10988, on glucose in batch culture has been studied. This organism has received increasing attention recently because of its more ethanol tolerance and faster ethanol producing capability [6, 7, 8, 9]. Zymomonas mobilis has absolute requirement for pantothenic acid [10, 11]. It also produces levan when it grown on sucrose [12]. It follows Entner-Doudoroff pathway and produces 1 ATP per mole glucose used [10].

Measured variables during anaerobic growth of Z. mobilis on glucose in batch culture for Run #1 and Run #2 are presented in Tables I and II, respectively. As explained earlier in this chapter, during Run #1 pH was controlled by a pH controller. Some instrumental problems later prevented good pH control; therefore, for the second run phosphate buffer has been used and pH was controlled by this way. Another difficulty was encountered during biomass measurements. Because of very small size of the organisms, filter paper clogged very quickly and for that reason a very small sample size was filtered (5 to 10 ml). This affected the accuracy of biomass measurements. The CO₂ analyzer had a low range for CO₂ measurements (0 to 2.5% CO₂). Therefore, the inlet gas flow rate had to be increased during later stages of the fermentation process to reduce the CO₂ concentration. This dilution procedure affected the accuracy of the CO₂ measurements.

Calculated variables of Run #1 and Run #2 are presented on Table III and IV, respectively. Another common problem of analyzing the data was the necessity of obtaining derivatives of biomass, substrate and product, respectively, with respect to time. To take derivatives a subroutine program (DGT3) has been used. It was necessary to smooth the

experimental data before using this subroutine program by drawing a smooth curve through the experimental points and reading the corresponding smoothed values from the curve. Some scatterings of values of μ , Q_s and Q_p may be attributed to this smoothing procedure. The consistency of experimental data collected during Run #1 and Run #2 is examined and shown in Tables V and VI, respectively. As can be seen in Table V and VI, available electron and carbon balances are much lower than unity, during the early stages of fermentation for both Run #1 and Run #2. The carbon balance shows especially low values. This may be due to the CO_2 measurements. Theoretically Z. mobilis produces about 1.8 moles ethanol and 1.8 moles CO_2 from 1 mole of glucose. Therefore d/z ratio should be approximately 1/2. But results show that this ratio is much lower than 1/2 during the early stages of the process. Later on it reaches this value and during the late stages of the batch process it passes this value. Slow transport of CO_2 from the fermentation broth may be one reason for the low d/z ratio in Run #1. CO_2 may dissolve into the broth at higher rates than it is transported out during the early stages of the fermentation process. During the middle stages of fermentation process, as it is seen, better consistency is observed and the d/z ratio is much closer to 1/2. During the late stages of the fermentation process the inlet gas flow rate was increased; this probably enhances the mixing and mass transfer, and more CO_2 is released to the gas phase. This may probably explain having higher values for the d/z ratio during this stage of the fermentation.

Table VII shows the integrated available electron and carbon balances for Run #1 and #2. Reasonably good estimates were obtained from integrated balances.

During very late stages of fermentation process a small amount of lactic acid production was observed (from HPLC analysis). But, because of very low and very scattered values, it wasn't included in the calculations. Also, because of the reasonably good data consistency from the integrated balances, the yeast extract consumption was not included in the calculations.

Results of parameter estimation with Run #1, Run #2 and Run #1 and #2 combined are presented in Tables VIII, IX and X, respectively. Equations (15), (16), and (17), form II, are used with least squares parameter estimation (linear regression) to estimate the maintenance coefficient, m'_e , and the true growth yield, $\eta'_{\max}$. Also, Method III (Chapter II and 13) were used to obtain better estimates of parameters by utilizing all the available data obtained. Method III with all the covariates included gives the maximum likelihood estimate [13].

Estimates obtained from Run #1 show that maintenance requirement for this organism is much higher than conventional yeasts; that is,

$$3.8468 \leq m'_e \leq 4.0109$$

Results of Run #2 gave considerably lower values for m'_e ,

$$1.2423 \leq m'_e \leq 2.3387$$

The same trend has been observed for $\eta'_{\max}$ results,

$$\text{Run \#1; } 0.1233 \leq \eta'_{\max} \leq 0.2492$$

$$\text{Run \#2; } 0.0590 \leq \eta'_{\max} \leq 0.1423$$

Since the consistency of the data is much better for Run #2 than for Run #1, one would expect the parameter estimates to be better for Run #2, also. These results may be compared to the results obtained from the analysis of the data of Lee et al. [12] which are presented in Chapter III. The

results presented above for m'_e are contained within the interval from the data of Lee, et al. [12]

$$0.937 \leq m'_e \leq 4.10$$

However, for the true growth yield, $\eta'_{\max}$, the interval from the data of Lee, et al. [12]

$$0.0305 \leq \eta'_{\max} \leq 0.0539$$

is smaller than the intervals presented above. When only one mole of ATP is produced per mole of glucose consumed,

$$\eta'_{\max} \leq 0.165$$

where the upper limit corresponds to

$$Y_{\text{ATP}}^{\max} = 28.8 \text{ g cells/g mole ATP.}$$

As stated in Chapter III, the value $Y_{\text{ATP}}^{\max} = 8.3$, which is equivalent to $\eta'_{\max} = 0.054$, was found by Forrest [14] for this organism. The pH problem encountered during Run #1 may be one reason for having considerably different results from Run #1 and Run #2. Considering that one of the best ethanol producing strains (ATCC 10988) has been used in this study, our results show that this organism has a high maintenance coefficient. The high maintenance requirement and low growth yield are unique to this organism and they are desirable properties which are related to the ability of this organism to produce ethanol rapidly.

CONCLUSIONS

Previously described methods of parameter estimations have been used to analyze the data collected during the anaerobic growth of Zymomonas mobilis, ATCC 10988, on glucose in batch culture. A high maintenance requirement and low growth yield require this organism to produce large quantities of ethanol.

Since data collected from continuous fermentation and fed-batch fermentation are more reliable for parameter estimates, further experiments are needed to confirm the results.

NOMENCLATURE

a	Moles of ammonia per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
b	Moles of oxygen per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
c	Moles of water per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
d	Moles of carbondioxide per quantity of organic substrate containing one g atom carbon, g mole/g atom carbon.
m'_e	Rate of organic substrate consumption for maintenance, g equiv. of available electrons per g equiv. of available electrons in biomass (hr). (Prime denotes anaerobic processes.)
F	Gas flow rate, liters/min.
P	Product concentration, g/liter.
P_o	Initial product concentration, g/liter.
Q_{CO_2}	Rate of evolution of carbondioxide, g moles/g dry wt (hr).
Q_m	Molar air flow rate per unit volume of broth, g mole/l (hr).
Q_o	Heat evolution in fermentation per equivalent of oxygen uptake, kcal/g equiv.
Q_p	Rate of product formation, g/g dry wt (hr).
Q_s	Rate of organic substrate consumption, g/g dry wt (hr).
S	Organic substrate concentration, g/liter.
S_o	Initial organic substrate concentration, g/liter.
X	Biomass concentration, g/liter.
X_o	Initial biomass concentration, g/liter.
V	Volume of broth, liters.

y_c	Biomass carbon yield (fraction of organic substrate carbon in biomass), dimensionless.
z	Fraction of organic substrate carbon in products, dimensionless.
γ_b	Reductance degree of biomass, equiv. of available electrons per g atom carbon.
γ_p	Reductance degree of products, equiv. of available electrons per g atom carbon.
γ_s	Reductance degree of organic substrate, equiv. of available electrons per g atom carbon.
ε	Fraction of available electrons in organic substrate which is transferred to oxygen, dimensionless.
η	Fraction of available electrons in organic substrate which is incorporated into biomass, dimensionless.
$\eta'_{\max}$	"True" biomass available electron yield, dimensionless. (Prime denotes anaerobic process).
μ	Specific growth rate, hr^{-1} .
ξ_p	Fraction of available electrons in organic substrate which is incorporated into products, dimensionless.
σ_b	Weight fraction of carbon in biomass, dimensionless.
σ_p	Weight fraction of carbon in products, dimensionless.
σ_s	Weight fraction of carbon in organic substrate, dimensionless.
θ	Time, hrs.

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Table I. Measured variables for batch fermentation of *Zymomonas mobilis* on glucose under anaerobic condition; Run #1.*o

# of data points	Volume, l	CO ₂ , %	X, g/l	S, g/l	P, g/l	F, l/min
1	4.4295	0.370	0.325	82.21	4.00	1.545
2	4.3540	0.450	0.324	80.51	5.38	1.545
3	4.2810	0.563	0.372	78.78	6.37	1.545
4	4.2055	0.800	0.427	78.47	7.05	1.545
5	4.1310	0.950	0.454	75.27	8.41	1.545
6	4.0545	1.363	0.484	71.98	8.41	1.545
7	3.9785	1.975	0.578	73.63	9.02	1.545
8	3.9030	1.875	0.613	70.07	10.53	3.090
9	3.8260	2.088	0.678	68.63	10.42	3.090
10	3.7440	2.200	0.765	64.20	12.52	3.631
11	3.6640	2.225	0.922	62.52	13.37	4.635
12	3.5265	2.325	1.019	64.57	13.73	4.558
13	3.3885	2.388	1.118	58.86	17.43	5.562
14	3.2490	2.025	1.196	51.29	18.86	7.231
15	3.0080	1.925	1.360	47.20	20.27	7.231
16	2.7715	1.900	1.519	45.99	23.52	7.210
17	2.6750	2.025	1.611	42.44	24.44	7.200
18	2.5900	1.775	1.666	42.20	27.09	7.215
19	2.5030	1.500	1.666	36.97	28.24	7.201
20	2.4200	1.738	1.672	33.34	28.50	4.435

*With pH control at 5, RPM = 400.0.

oSamples were taken within half-hours.

Table II. Measured variables for batch fermentation of *Zymomonas mobilis* on glucose under anaerobic condition; Run #2.*o

# of data points	Volume, l	CO ₂ , %	X, g/l	S, g/l	P, g/l	F, l/min
1	4.970	1.000	0.527	86.00	1.50	1.115
2	4.801	1.068	0.580	86.50	1.94	1.115
3	4.689	1.250	0.520	82.35	1.95	1.115
4	4.576	1.450	0.580	85.00	2.09	1.115
5	4.426	1.750	0.600	85.00	2.59	1.115
6	4.308	2.125	0.660	82.00	3.37	1.120
7	4.192	0.593	0.630	83.11	3.32	4.480
8	4.040	0.688	0.740	81.60	3.69	4.480
9	3.915	0.825	0.800	80.00	5.16	4.480
10	3.795	1.038	0.874	75.86	5.00	4.480
11	3.641	1.288	1.000	74.98	6.25	4.480
12	3.524	1.550	1.143	72.41	7.91	4.480
13	3.388	1.925	1.250	70.05	8.25	4.480
14	3.229	2.288	1.508	69.33	9.69	4.480
15	3.103	1.938	1.644	63.00	10.76	6.780
16	2.971	2.013	1.900	56.66	11.95	6.780
17	2.813	1.888	1.968	60.17	15.50	6.780
18	2.590	1.868	2.104	51.00	17.61	6.780
19	2.466	2.088	2.264	48.39	20.98	6.780
20	2.308	2.338	2.380	36.99	25.00	6.720
21	2.201	2.013	2.628	32.78	27.19	9.080
22	2.046	2.125	2.662	26.17	30.51	9.080
23	1.910	2.250	2.880	16.92	33.81	9.080
24	1.783	2.200	3.030	4.63	36.90	9.080
25	1.669	0.125	3.142	1.13	39.89	9.056

*With phosphate buffer at 7, RPM = 800.

oSamples were taken within half hours.

Table III. Calculated variables in batch fermentation of Zymomonas mobilis on glucose, under anaerobic condition; Run #1.

# of data points	μ , hr ⁻¹	Q_{CO_2} , $\frac{\text{g moles}}{\text{g-hr}}$	Q_s , $\frac{\text{g}}{\text{g-hr}}$	Q_p , $\frac{\text{g}}{\text{g-hr}}$
1	0.04615	0.00958	10.769	3.631
2	0.15124	0.01189	10.803	5.093
3	0.20968	0.01317	9.409	4.032
4	0.18969	0.01661	9.368	3.747
5	0.19163	0.01889	11.013	3.524
6	0.21281	0.02569	8.264	3.719
7	0.20415	0.03202	5.190	3.287
8	0.20718	0.05844	7.341	3.263
9	0.24336	0.06001	7.374	2.949
10	0.28366	0.06730	6.536	3.268
11	0.25163	0.07367	5.965	3.254
12	0.20510	0.07116	5.397	2.944
13	0.18336	0.08459	4.919	3.131
14	0.20652	0.09094	5.017	3.344
15	0.20000	0.08211	4.412	2.721
16	0.15866	0.07852	4.279	2.436
17	0.09373	0.08163	4.345	2.669
18	0.04142	0.07163	4.502	2.281
19	0.01681	0.06251	4.802	2.101
20	0.01196	0.04596	4.785	2.392

Table IV. Calculated variables in batch fermentation of *Zymomonas mobilis* on glucose, under anaerobic condition; Run #2.

# of data points	μ , hr ⁻¹	Q_{CO_2} moles g-hr	Q_s , $\frac{g}{g-hr}$	Q_p , $\frac{g}{g-hr}$
1	0.02277	0.01027	1.898	0.373
2	0.03448	0.01032	1.724	0.345
3	0.05769	0.01379	1.923	0.769
4	0.07414	0.01470	2.586	1.034
5	0.09167	0.01773	3.333	1.083
6	0.10606	0.02020	3.030	1.288
7	0.15873	0.02426	4.667	1.587
8	0.17568	0.02486	4.054	1.689
9	0.19250	0.02848	3.825	1.875
10	0.24028	0.03382	4.577	2.002
11	0.26600	0.03823	4.590	2.000
12	0.24497	0.04161	4.374	1.749
13	0.24800	0.04914	4.328	1.800
14	0.23873	0.05079	4.642	1.889
15	0.21289	0.06214	4.258	2.129
16	0.16842	0.05833	3.947	2.315
17	0.16260	0.05579	4.573	2.794
18	0.14734	0.05607	4.990	2.970
19	0.12809	0.06118	5.300	2.760
20	0.13445	0.06901	5.252	2.416
21	0.12557	0.07624	5.327	2.192
22	0.11269	0.08549	5.853	2.253
23	0.10417	0.08963	6.250	2.079
24	0.08317	0.08924	5.211	1.980
25	0.04965	0.00521	2.447	1.909

Table V. Examination of data consistency using instantaneous available electron and carbon balances for growth of Zymomonas mobilis on glucose, under anaerobic condition; Run #1.

# of data points	η	ξ_p	$\eta + \xi_p$	y_C	z	d	$y_C + z + d$
1	0.00531	0.65996	0.66527	0.00495	0.43997	0.02669	0.47162
2	0.01734	0.92282	0.94017	0.01617	0.61521	0.03308	0.66442
3	0.02761	0.83893	0.86654	0.02574	0.55929	0.04199	0.58503
4	0.02509	0.78300	0.80801	0.02339	0.52200	0.05319	0.59859
5	0.02156	0.62640	0.64795	0.02009	0.41760	0.05145	0.48915
6	0.03190	0.88087	0.91277	0.02974	0.58725	0.09329	0.71028
7	0.04874	1.23975	1.28848	0.04543	0.82650	0.18509	1.05703
8	0.03497	0.87000	0.90496	0.03259	0.58000	0.23883	0.85143
9	0.04089	0.78300	0.82389	0.03811	0.52200	0.24413	0.80424
10	0.05377	0.97875	1.03252	0.05013	0.65250	0.30892	1.01156
11	0.05226	1.06773	1.11999	0.04872	0.71182	0.37052	1.13105
12	0.04708	1.06773	1.11481	0.04389	0.71182	0.39556	1.15127
13	0.04618	1.24568	1.29186	0.04305	0.83045	0.51588	1.38939
14	0.05101	1.30500	1.35600	0.04754	0.87000	0.54381	1.46135
15	0.05617	1.20713	1.26330	0.05236	0.80475	0.55836	1.41079
16	0.04594	1.11427	1.16021	0.04282	0.74285	0.55048	1.33616
17	0.02673	1.20246	1.22919	0.02491	0.80164	0.56372	1.39028
18	0.01139	0.99180	1.00320	0.01063	0.66120	0.47734	1.14917
19	0.00433	0.85641	0.86075	0.00404	0.57094	0.39056	0.96554
20	0.00309	0.97875	0.98184	0.00289	0.65250	0.28819	0.94358

Table VI. Examination of data consistency using instantaneous available electron and carbon balances for growth of *Zymomonas mobilis* on glucose, under anaerobic conditions; Run #2.

# of data points	η	ξ_p	$\eta + \xi_p$	y_c	z	d	$y_c + z + d$
1	0.01487	0.39150	0.40637	0.01386	0.26100	0.16243	0.43729
2	0.02478	0.39150	0.41628	0.02310	0.26100	0.17949	0.46360
3	0.03717	0.78300	0.82017	0.03465	0.52200	0.21520	0.77185
4	0.03552	0.78300	0.81852	0.03311	0.52200	0.17053	0.72564
5	0.03407	0.63619	0.67026	0.03176	0.42412	0.15959	0.61548
6	0.04336	0.83194	0.87530	0.04043	0.55463	0.19999	0.79504
7	0.04214	0.66582	0.70796	0.03928	0.44388	0.15594	0.63910
8	0.05369	0.81562	0.86932	0.05005	0.54375	0.18399	0.77779
9	0.06235	0.95956	1.02192	0.05812	0.63971	0.22337	0.92120
10	0.06505	0.85641	0.92146	0.06064	0.57094	0.22169	0.85326
11	0.07180	0.85294	0.92475	0.06694	0.56863	0.24988	0.88544
12	0.06938	0.78300	0.85239	0.06468	0.52200	0.28533	0.87201
13	0.07099	0.81412	0.88512	0.06618	0.54274	0.34065	0.94958
14	0.06372	0.79698	0.85070	0.05940	0.53132	0.32826	0.91898
15	0.06194	0.97875	1.04070	0.05775	0.65250	0.43786	1.14811
16	0.05286	1.14840	1.20127	0.04928	0.76560	0.44335	1.25823
17	0.04405	1.19625	1.24030	0.04107	0.79750	0.36597	1.20454
18	0.03658	1.16518	1.20176	0.03410	0.77679	0.33709	1.14798
19	0.02994	1.01953	1.04947	0.02791	0.67969	0.34628	1.05388
20	0.03172	0.90045	0.93217	0.02957	0.60030	0.39420	1.02407
21	0.02921	0.80537	0.83458	0.02722	0.53691	0.42936	0.99350
22	0.02386	0.75385	0.77771	0.02224	0.50257	0.43824	0.96305
23	0.02065	0.65141	0.67206	0.01925	0.43427	0.43023	0.88376
24	0.01977	0.74383	0.76360	0.01843	0.49588	0.51371	1.02803
25	0.02513	1.52731	1.55244	0.02343	1.01821	0.06386	1.10549

Table VII. Examination of data consistency using integrated available electron and carbon balances for anaerobic growth of Zymomonas mobilis on glucose in batch cultures.

Source of data	η	ξ_p	$\eta + \xi_p$	y_c	z	d	$y_c + z + d$
Run #1	0.03415	0.98135	1.01550	0.03184	0.65423	0.35550	1.04156
Run #2	0.03795	0.88027	0.91821	0.03538	0.58684	0.36877	0.99099

Table VIII. Point and 95% confidence interval estimates of the maintenance parameter, m'_e , and the biomass yield, $\eta'_{\max}$, for Run #1.

Equation and Method	Form*	Maintenance Parameter		True Biomass Yield		Covariates Included	J
		point estimates	95% confidence intervals	point estimates	95% confidence intervals		
Eq. (15), L.R. ^a	I	3.7412	3.1673, 4.3151	0.0792	0.0374, ∞		
	II	4.7454	2.7573, 5.7335	0.2467	0.0670, ∞		
Eq. (16), L.R.	I	3.9749	3.7668, 4.1829	0.1275	0.0772, 0.3662		
	II	4.4623	3.4846, 5.4401	0.2432	0.10579, ∞		
Eq. (17), L.R.	I	3.8802	3.7738, 3.9866	0.1719	0.1185, 0.3121		
	II	4.1436	3.6076, 4.6796	0.2723	1.5140, 1.3469		
Eq's. (15)(16)(17) Method III [†]		0.9572	0.4215, 1.4929	0.8246	0.4308, 9.5680	z_2, z_3	0.09053
	I	3.9289	3.8469, 4.0109	0.1649	0.1233, 0.2492	z_2	0.08096
		4.9753	0.3088, 9.6417	0.0959	0.0504, 1.0099	z_3	7.77540
Eq's. (15)(16)(17) Method III [†]		4.1849	3.8092, 4.5607	0.2825	0.1794, 0.6642	z_2, z_3	0.00744
	II	4.2508	3.7004, 4.8013	0.2615	0.1469, 1.1906	z_2	0.01520
		4.4246	3.5059, 5.3432	0.2640	0.1135, ∞	z_3	0.04294

^a L.R. refers to linear regression. Equation numbers are those of Chapter III.

* Form I: Equations (15), (16), (17) were divided by μ . Form II: Equations (15), (16), (17) as they are.

[†] Explained in Chapter II and (13).

For Form I, the eigenvalues are 8874.01 and 178.473 for z_2 and z_3 , respectively; for Form II the eigenvalues are 48.9215 and 2.9577 for z_2 and z_3 , respectively.

Table IX. Point and 95% confidence interval estimates of the maintenance parameter, m'_e , and the true biomass yield, $\eta'_{\max}$, for Run #2.

Equation and Method	Form*	Maintenance Parameter		True Biomass Yield		Covariates Included	J
		point estimates	95% confidence intervals	point estimates	95% confidence intervals		
Eq. (15), L.R. ^a	I	1.1484	0.7389, 1.5579	0.0603	0.0451, 0.0911		
	II	2.3629	1.5076, 3.2183	0.1490	0.0828, 0.7440		
Eq. (16), L.R.	I	0.3645	-0.1378, 0.8669	0.0492	0.0367, 0.0743		
	II	1.8309	0.9159, 2.7460	0.1219	0.0717, 0.4064		
Eq. (17), L.R.	I	0.3196	-0.2057, 0.8449	0.0462	0.0349, 0.0704		
	II	1.9658	0.8576, 2.1783	0.1269	0.0674, 1.0793		
Eq's. (15)(16)(17) Method III [†]		1.7905	1.2423, 2.3387	0.0835	0.0590, 0.1429	z_2, z_3	2.0715
	I	0.6345	0.1897, 1.0793	0.0527	0.0398, 0.0780	z_2	4.3490
		1.0796	0.4452, 1.7139	0.0589	0.0430, 0.0938	z_3	3.9029
Eq's. (15)(16)(17) Method III [†]		2.2864	1.6023, 2.9705	0.1462	0.0916, 0.3622	z_2, z_3	0.0229
	II	2.4559	1.7243, 3.1874	0.1564	0.0922, 0.5151	z_2	0.0262
		2.0073	1.0249, 2.9896	0.1295	0.0733, 0.5543	z_3	0.0465

^a L.R. refers to linear regression. Equation numbers are those in Chapter III.

* Form I: Equations (15), (16), (17) were divided by μ . Form II: Equations (15), (16), (17) as they are.

[†] Explained in Chapter II and (13).

For Form I, the eigenvalues are 612.834 and 1126.65 for z_2 and z_3 , respectively; for Form II, the eigenvalues are 9.91749 and 4.91059 for z_2 and z_3 respectively.

Table X. Point and 95% confidence interval estimates of the maintenance parameter, m_e' , and the true biomass yield, $\eta_{\max}'$, for Run #1 and #2, together.

Equation and Method	Form*	Maintenance Parameter		True Biomass Yield		Covariates Included	J
		point estimates	95% confidence intervals	point estimates	95% confidence intervals		
Eq. (15), L.R. ^a	I	3.3258	2.7608, 3.3258	0.2073	0.0636, ∞		
	II	3.1305	1.8938, 4.3673	0.1363	0.0685, 13.8222		
Eq. (16), L.R.	I	3.3715	2.7802, 3.9628	0.8643	0.0796, ∞		
	II	2.7089	1.6980, 3.7199	0.1228	0.0710, 0.4538		
Eq. (17), L.R.	I	3.2736	2.7135, 3.8337	0.8549	0.0835, ∞		
	II	2.7139	1.8221, 3.6057	0.1371	0.0798, 0.4864		
Eq's. (15)(16)(17) Method III [†]		3.1683	2.6523, 3.6843	0.2073	0.0675, ∞	z_2, z_3	13.7243
	I	3.3258	2.7743, 3.8773	0.3431	0.0728, ∞	z_2	16.3042
		3.1928	2.6704, 3.7152	0.3151	0.0765, ∞	z_3	14.0273
Eq's. (15)(16)(17) Method III [†]		2.8377	1.9133, 3.7621	0.1422	0.0807, 0.5957	z_2, z_3	0.036244
	II	2.7208	1.8005, 3.6411	0.1319	0.0772, 0.4495	z_2	0.036761
		2.9380	1.9609, 3.9152	0.1308	0.0774, 0.6389	z_3	0.04056

^a L.R. refers to linear regression. Equation numbers are those in Chapter III.

* Form I: Equations (15), (16), (17) were divided by μ . Form II: Equations (15), (16), (17) as they are.

[†] Explained in Chapter II and [13].

For Form I, the eigenvalues are 8057.5 and 4062.85 for z_2 and z_3 , respectively; for Form II, the eigenvalues are 53.5232 and 9.01544 for z_2 and z_3 , respectively.

APPENDIX

AN EMPIRICAL COMPARISON OF THE BOOTSTRAP
AND NORMAL THEORY METHODS FOR
CONSTRUCTING CONFIDENCE INTERVALS

The procedure used to estimate the confidence interval by the Bootstrap method is presented for the case where the maintenance parameter, m_e , and the true growth yield, $\eta_{\max}$, are the parameters of interest. The results are compared with those obtained from Normal Theory methods for several different error distributions.

To illustrate the procedure, consider Equations (16) and (17), Chapter II, for the case where there is no product formation.

$$\frac{\sigma_s \gamma_s}{\sigma_b \gamma_b} Q_S = m_e + \frac{\mu}{\eta_{\max}} \quad (1)$$

$$\frac{48Q_{O_2}}{\sigma_b \gamma_b} + \mu = m_e + \frac{\mu}{\eta_{\max}} \quad (2)$$

To facilitate presentation, Equations (1) and (2) are put into the following form,

$$y_{1i} = \alpha + \beta_{xi} + \varepsilon_{1i} \quad i = 1, 2, \dots, n \quad (3)$$

$$y_{2i} = \alpha + \beta_{xi} + \varepsilon_{2i} \quad i = 1, 2, \dots, n \quad (4)$$

where

$$y_{1i} = \frac{\sigma_s \gamma_s}{\sigma_b \gamma_b} Q_{Si}$$

$$y_{2i} = \frac{48Q_{O_2i}}{\sigma_b \gamma_b} + \mu_i$$

are system responses and also dependent variables, $x_i = \mu_i$ is independent variable and ε_{1i} and ε_{2i} are random errors with mean zero and bivariate normal.

$$\alpha = m_e$$

and

$$\beta = \frac{1}{\eta_{\max}}$$

are unknown parameters to be estimated.

From Equations (3) and (4), two least square estimates of α and β , $\hat{\underline{\alpha}}$ and $\hat{\underline{\beta}}$, can be calculated where $\hat{\underline{\alpha}}$ is a (2 x 1) vector of the form

$$\hat{\underline{\alpha}} = \begin{bmatrix} \hat{\alpha}_1 \\ \hat{\alpha}_2 \end{bmatrix}$$

and $\hat{\underline{\beta}}$ is (2 x 1) vector of the form

$$\hat{\underline{\beta}} = \begin{bmatrix} \hat{\beta}_1 \\ \hat{\beta}_2 \end{bmatrix}$$

Using these two least square estimates, $(\hat{\alpha}_1, \hat{\beta}_1)$ and $(\hat{\alpha}_2, \hat{\beta}_2)$, based on respectively Equation (3) and Equation (4), estimated errors can be obtained;

$$\hat{\epsilon}_{1i} = y_{1i} - \hat{\alpha}_1 - \hat{\beta}_1 x_i \quad i = 1, 2, \dots, n \quad (5)$$

$$\hat{\epsilon}_{2i} = y_{2i} - \hat{\alpha}_2 - \hat{\beta}_2 x_i \quad i = 1, 2, \dots, n \quad (6)$$

Randomly selecting n pairs of errors $(\epsilon^*_{1i}, \epsilon^*_{2i})$ from $(\hat{\epsilon}_{1i}, \hat{\epsilon}_{2i})$ $i = 1, 2, \dots, n$, imitated system responses y^*_{1i} and y^*_{2i} can be obtained, such that;

$$y^*_{1i} = \hat{\alpha}_1 + \hat{\beta}_1 x_i + \epsilon^*_{1i} \quad i = 1, 2, \dots, n \quad (7)$$

$$y^*_{2i} = \hat{\alpha}_2 + \hat{\beta}_2 x_i + \epsilon^*_{2i} \quad i = 1, 2, \dots, n \quad (8)$$

Least square estimates of α and β , (α^*_1, β^*_1) and (α^*_2, β^*_2) can be obtained using imitated responses from Equations (7) and (8), respectively.

We can imitate the experiment, independently, many times. Suppose after N repetitions, we obtain $(\alpha_1^{*(1)}, \beta_1^{*(1)})$, $(\alpha_1^{*(2)}, \beta_1^{*(2)})$, $\dots$, $(\alpha_1^{*(N)}, \beta_1^{*(N)})$ and, $(\alpha_2^{*(1)}, \beta_2^{*(1)})$, $(\alpha_2^{*(2)}, \beta_2^{*(2)})$, $\dots$, $(\alpha_2^{*(N)}, \beta_2^{*(N)})$. These least square estimates by using weighted least square estimate technique:

$$\hat{\underline{\alpha}}_{\text{pooled}} = [\underline{1} \quad \underline{A}^{-1} \quad \underline{1}]^{-1} \underline{1}' \underline{A}^{-1} \hat{\underline{\alpha}} \quad (9)$$

$$\hat{\underline{\beta}}_{\text{pooled}} = [\underline{1} \quad \underline{B}^{-1} \quad \underline{1}]^{-1} \underline{1}' \underline{B}^{-1} \hat{\underline{\beta}} \quad (10)$$

where,

A is sample variance-covariance matrix, 2 x 2, of

$$(\alpha_1^{*(1)}, \alpha_2^{*(1)}), \dots, (\alpha_1^{*(N)}, \alpha_2^{*(N)})$$

B is sample variance-covariance matrix, 2 x 2, of

$$(\beta_1^{*(1)}, \beta_2^{*(1)}), \dots, (\beta_1^{*(N)}, \beta_2^{*(N)})$$

1 is (2 x 1) vector of ones.

Second step is to construct 95% confidence intervals for α and β . For this purpose, pooled values of α and β we calculated for each repetition, as follows:

$$\alpha_{\text{pooled}}^{*(i)} = [\underline{1}' \hat{\underline{\Sigma}} \underline{1}]^{-1} \underline{1}' \hat{\underline{\Sigma}} \alpha^{*(i)} \quad i = 1, \dots, N \quad (11)$$

$$\beta_{\text{pooled}}^{*(i)} = [\underline{1}' \hat{\underline{\Sigma}} \underline{1}]^{-1} \underline{1}' \hat{\underline{\Sigma}} \beta^{*(i)} \quad i = 1, \dots, N \quad (12)$$

where,

$\hat{\underline{\Sigma}}$ is the estimate of variance-covariance matrix of errors, $\underline{\Sigma}$; the errors from Equations (5) and (6) may be used to construct $\hat{\underline{\Sigma}}$. Since the errors, ϵ_{1i}^* and ϵ_{2i}^* are pairs of the original errors, values of $\underline{\Sigma}$ estimated from ϵ_{1i}^* and ϵ_{2i}^* , should be similar to those estimated from $\hat{\epsilon}_{1i}$ and $\hat{\epsilon}_{2i}$.

Since the Bootstrap method is not an exact method, mathematically derived, to obtain confidence intervals a distribution curve should be constructed for this purpose.

From Equations (11) and (12),

$$\text{Var}(\alpha_{\text{pooled}}^*) = [\underline{1}' \hat{\underline{\Sigma}}^{-1} \underline{1}]^{-1} \underline{1}' \hat{\underline{\Sigma}}^{-1} \text{var}(\underline{\alpha}^*) \hat{\underline{\Sigma}}^{-1} \underline{1} [\underline{1}' \hat{\underline{\Sigma}}^{-1} \underline{1}]^{-1} \quad (13)$$

where,

$$\text{Var}(\underline{\alpha}^*) = d_1 \hat{\underline{\Sigma}}$$

$$d_1 = \left(\frac{1}{n} + \frac{(\bar{x})^2}{\sum_{i=1}^n (x_i - \bar{x})^2} \right)$$

Substituting and simplifying give,

$$\hat{\text{Var}} (\alpha^*_{\text{pooled}}) = d_1 \cdot \tau \quad (14)$$

where,

$$\tau = [1' \hat{\Sigma}^{-1} \underline{1}]^{-1}$$

Similarly

$$\hat{\text{Var}} (\beta^*_{\text{pooled}}) = d_2 \cdot \tau \quad (15)$$

where

$$d_2 = \frac{1}{\sum_{i=1}^n (x_i - \bar{x})^2}$$

Now, a distribution curve with zero mean can be constructed. Let,

$$C_i^\alpha = (\alpha^{*(i)} - \hat{\alpha}_{\text{pooled}}) / \sqrt{d_1 \tau} \quad i = 1, \dots, N \quad (16)$$

$$C_i^\beta = (\beta^{*(i)} - \hat{\beta}_{\text{pooled}}) / \sqrt{d_2 \tau} \quad i = 1, \dots, N \quad (17)$$

Renumbering the subscripts such that the values of C_i^α and C_i^β in increasing order; that is,

$$C_1^\alpha \leq C_2^\alpha \leq \dots \leq C_N^\alpha \quad \text{and,}$$

$$C_1^\beta \leq C_2^\beta \leq \dots \leq C_N^\beta$$

When N is large, 95% confidence intervals can be obtained; let

$$r = [\text{largest integer} \leq (0.025)N].$$

$$s = N - r$$

for α ,

$$\hat{\alpha}_{\text{pooled}} + C_{(r)}^\alpha \cdot \sqrt{d_1 \cdot \tau} \leq \alpha \leq \hat{\alpha}_{\text{pooled}} + C_{(s)}^\alpha \cdot \sqrt{d_1 \cdot \tau} \quad (18)$$

$$\hat{\beta}_{\text{pooled}} + C_{(r)}^\beta \cdot \sqrt{d_2 \cdot \tau} \leq \beta \leq \hat{\beta}_{\text{pooled}} + C_{(s)}^\beta \cdot \sqrt{d_2 \cdot \tau} \quad (19)$$

To compare the Bootstrap and Normal Theory Methods, for the specified variance-covariance matrix of the errors, α , β , x_i . . . ($i = 1, 2, . . . , n$) values and the error distribution type ϵ_{1i} and ϵ_{2i} , $i = 1, 2, . . . , n$, and y_{1i} and y_{2i} values are calculated. To this data set the Bootstrap and Normal Theory methods were applied to obtain average length of confidence intervals and coverages of α and β for six different error distribution types (Table 1).

Simulation results showed that Bootstrap method is a reliable method as well as other exact methods and can be used for different type of error distributions in which the exact methods are absent.

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Table 1. Average confidence interval lengths and coverages of generated data from Bootstrap [1] and Rao's [2], normal theory, methods. (98% and 95% confidence intervals were used for Bootstrap and Rao's methods, respectively).

Error distribution used for generation of dependent variables	BOOTSTRAP METHOD			RAO'S METHOD		
	Average length of C.I. of α	Coverage	Average length of C.I. of β	Average length of C.I. of α	Coverage	Average length of C.I. of β
Normal Distribution [†]	0.804027	92	3.804882	0.820519	95	4.181110
Normal Distribution* [†]	0.886874	93	4.155250	0.820519	95	4.181110
t Distribution ^o $D_f = 3$	0.017803	95	0.084350	0.018196	96	0.093472
Uniform Distribution ^o	0.019250	90	0.092500	0.021638	93	0.110531
Double Exponential Distribution ^o	0.019250	98	0.092500	0.020339	98	0.103769
Chi-Square Distribution ^o $D_f = 4$	0.019418	95	0.091919	0.020050	97	0.104201
Mixed Distribution ^o (Uniform and Normal)	0.019448	97	0.092157	0.019699	97	0.100721

[†] $\delta_{11} = 1.0$, $\delta_{12} = -0.05$, $\delta_{22} = 0.5$ were used
^o $\delta_{11} = 0.0005$, $\delta_{12} = -0.00002$, $\delta_{22} = 0.00008$ were used
* 99% C.I. was used for Bootstrap Method

ESTIMATION OF GROWTH YIELDS IN
AEROBIC AND ANAEROBIC CULTURES

by

MEHMET DURDU ONER

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AN ABSTRACT OF A MASTER'S THESIS

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In the microbial production of useful products, it is important to understand the allocation of substrate energy for maintenance, growth and product formation. Methods are presented to obtain point and 95% confidence interval estimates for the true growth yield parameter, true product yield parameter, and the maintenance parameter, in aerobic cultures. Three different statistical methods are presented which allow all data to be used simultaneously for those cases where more than the minimum number of measurements are made at each specific growth rate. These methods are applied to two sets of literature data. Point estimates are similar for the three methods, but interval estimates are considerably larger for the third method. The 95% confidence interval estimates of maintenance coefficient showed that, in aerobic cultures, maintenance requirements are small. Results obtained for true product yields are close to the theoretical maximum value. For Rizobium trifolii, the 95% confidence interval for true growth yield is $0.53 \leq \eta_{\max} \leq 0.68$.

Equations are developed and used to estimate the maintenance parameter and true growth yield in anaerobic cultures. Two sets of published data were analyzed to demonstrate the application of these equations. For the growth of Saccharomyces cerevisiae on glucose in chemostat, low values of maintenance requirement and growth yield were observed; [0,00866] and [0.0608, 0.118], respectively. In contrast to these results, a very high maintenance requirement was observed for Zymomonas mobilis; $0.937 \leq m'_e \leq 4.10$.

Zymomonas mobilis, ATCC 10988, was cultivated anaerobically on glucose in an instrumented fermentor. Samples collected during fermentation process were analyzed to determine biomass and ethanol production and glucose consumption. The CO₂ production was also recorded. Reasonably good consistency

was observed. Previously developed equations, for anaerobic cultures, were used to estimate the maintenance coefficient and true growth yield. Parallel to previous results, very high maintenance requirements were observed for this organism; for Run #1 and Run #2, $3.8469 \leq m'_e \leq 4.0109 \text{ hr}^{-1}$, $1.2423 \leq m'_e \leq 2.3387 \text{ hr}^{-1}$, respectively. For η'_{max} , $0.1233 \leq \eta'_{\text{max}} \leq 0.2492$ and $0.0690 \leq \eta'_{\text{max}} \leq 0.1429$ were found for Run #1 and #2, respectively. It was concluded that the high maintenance requirement and low growth yield are related to the ability of this organism to produce ethanol.