Experiment	**From Biothermodynamics to bacterial Lifestyles**
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Reading	Chapters 4.4 and Appendix 1 in BBOM 9th. Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 9th Edition, Prentice Hall, 1999. ISBN: 0-13-085264-3
Objectives	In this exercise the student will learn … • how basic laws of **equilibrium thermodynamics** are applied to biological processes • how one can better understand **bacterial lifestyles** if one understands bio-thermodynamics • how one can predict the effects of changing **environmental conditions** on bacterially mediated processes • how one can model **bacterial interactions** using Thermodyn©
Background	Biothermodynamics is the application of the laws of **equilibrium thermodynamics** to biochemical processes. Applying these laws under well defined **boundary conditions** allows one to gain insight into the **energetics** of microbial life styles. We assume, that microorganisms make use of any naturally occuring chemical process which proceeds **exergonically**. The evolutionary history contains many examples which illustrate that microbes have acquired a large number of rather unusual metabolisms, e.g. the use of inorganic compounds as electron donors, respiration with sulfate, ferric iron, halogenated hydrocarbons etc., and being able to extract energy from extremely energy-poor substrates like oxalate. With a thermodynamic model approach we can examine and define the conditions under which these reactions can **yield energy** and beyond which point they are no longer supporting microbial life. In the course of the exercise we will create concepts and define **limitations** and conditions for a number of reactions which are known to be mediated by microbes. Thermodynamics alone will not tell whether the microbes which could make use of an exergonic reaction will actually be where the reaction could proceed, and it will not tell how fast a reaction will occur. We will examine a few reactions which we derived in experiment 1 (Microbial Diversity in the Rumen) with regard to • the likelyhood with which they can take place, • the environmental conditions under which they can take place and • the interactions with the host needed to make them happen in the rumen. The reactions are treated as if they would obey ideal thermodynamic laws, that is they take place • at a standard temperature of 298.15 K (25 °C), • under ideally diluted conditions, such that activities of reactants equal concentrations. **Problem 1:** Under which boundary conditions is the degradation of glucose by *Ruminococcus flavefaciens* in axenic batch culture energetically feasible ? In exercise 3a (experiment 1) glucose fermentation by *R.flavefaciens* to acetate, formate and succinate was described by the stoichiometrically balanced equation $100\ C_6H_{12}O_6 + 48\ HCO_3^- \rightarrow 107\ CH_3COO^- + 62\ HCOO^- + 93\ ^-OOC(CH_2)_2COO^- + 59\ H_2 + 307\ H^+ + 34\ H_2O$ The reaction is thermodynamically feasible as long as the Gibbs free energy of the reaction is < 0. This depends on the actual activities of the substrates and products and on the standard free energy of the conversion reaction.

We will recall the background first.
From Physical Chemistry we remember that the reaction

$$aA^{n-} + bB^{m-} \rightarrow cC^{p-} + dD^{q-} + (p+q-n-m)H^{+}$$

will proceed in the direction as written, if $\Delta Gr < 0$. The free energy (ΔGr) is defined as

$$\Delta Gr = \Delta Gr^{o} + R \cdot T \cdot \ln Q$$

with Q being the ratio of the algebraic product of the activities (concentrations) of the reaction products, divided by the algebraic product of the activities of the reaction substrates; stoichiometric factors become exponents.

$$Q = \frac{\left[C^{p-}\right]^{c} \cdot \left[D^{q-}\right]^{d} \cdot \left[H^{+}\right]^{(p+q-n-m)}}{\left[A^{n-}\right]^{a} \cdot \left[B^{m-}\right]^{b}}$$

R is the gas constant $= 8.31451 \cdot 10^{-3}$ [$kJ \cdot mol^{-1} \cdot K^{-1}$] (concentration basis)
T is the thermodynamic temperature in [Kelvin]

ΔGr^{o} is calculated from the free energies of formation according to

$$\Delta Gr^{o} = \sum_{j} v_j Gf^{o}_{P_j} - \sum_{i} v_i Gf^{o}_{S_i}$$

(for the meaning of terms see Abbreviations below)

The temperature correction follows from

$$\Delta Gr^{o}_{T_{act}} = \Delta Gr^{o}_{T_{ref}} \cdot \frac{T_{act}}{T_{ref}} + \Delta Hr^{o}_{T_{ref}} \cdot \frac{T_{ref} - T_{act}}{T_{ref}}$$

Abbreviations :

Gf^{o}	standard free energy of formation [kJ/mol]
ΔGr^{o}	change of Gibbs free energy of reaction at standard conditions $= -R \cdot T \cdot \ln K^{o}$
ΔGr	change of Gibbs free energy of reaction under actual conditions
Hf^{o}	standard enthalpy of formation
ΔHr^{o}	enthalpy change of reaction at standard conditions
K^{o}	thermodynamic equilibrium coefficient
Q	ratio of actual activity products of reactants
R	gas constant $= 8.31451 \cdot 10^{-3}$ [$kJ \cdot mol^{-1} \cdot K^{-1}$] (concentration basis)
T	thermodynamic temperature in [Kelvin]
T_{ref}, T_{act}	reference and actual temperature, respectively
P_j, S_i	product and substrate of species j and i, respectively
v	stoichiometric factor

For our example

$$Q = \frac{\left[CH_3COO^{-}\right]^{107} \cdot \left[HCOO^{-}\right]^{62} \cdot \left[{}^{-}OOC(CH_2)_2COO^{-}\right]^{93} \cdot \left[H_2\right]^{59} \cdot \left[H^{+}\right]^{307} \cdot 1}{\left[C_6H_{12}O_6\right]^{100} \cdot \left[HCO_3^{-}\right]^{48}}$$

- activity of water in aqueous solutions is by convention 1;
- Proton concentration follows from pH; $\left[H^{+}\right] = 10^{-pH}$
- $C_6H_{12}O_6$ is α - D - glucose
- The concentrations (activities) of the other reactants are defined as boundary conditions.

The energies of formation (Gf^o) are :

Compound	Formula	Gf^0 [kJ/mole]
α - D - Glucose	$CH_2OH(CHOH)_4CHO$	- 917.2
Formate	$HCOO^-$	- 351.0
Acetate	H_3CCOO^-	- 369.4
Succinate	$^-OOC(CH_2)_2COO^-$	- 690.2
Bicarbonate	HCO_3^-	- 586.9
Hydrogen	H_2	+ 17.55
Proton	H^+	0
Water	H_2O	- 237.2
Methane	CH_4	- 34.4

Boundary conditions

Choose the conditions for the beginning and the end of the reaction in the batch culture as follows: (all concentrations in mole/l)

Reactant	beginning	end of experiment
Glucose	0,020	0,0002
Bicarbonate	0,030	0,020
Acetate	10^{-7}	0,020
Formate	10^{-7}	0,060
Succinate	$0.5*10^{-6}$	0,020
pH *	6.9	6.3
Hydrogen (dissolved)	variable 10^{-2} to 10^{-10}	variable 10^{-2} to 10^{-10}
Temperature	25°C [298.15K]	25°C [298.15K]

* remember: $[H^+] = 10^{-pH}$

Calculating ΔGr for the actual conditions at the beginning of the batch culture experiment using Thermodyn©

The Excel spreadsheet program Thermodyn© allows one to calculate free reaction energies for a number of microbially mediated chemical ractions. Thermodyn© is ment to be used as a learning tool to make applying thermodynamic laws in microbiology more understandable to the student. Comparing free reaction energies which are calculated for real conditions (activities, concentrations, pH, temperatures) make thermodynamics in many cases a more useful concept to understand processes in nature than if one has to rely on values calculated for standard state conditions solely. In addition, the graphs will aid in quickly getting an idea on how changes will influence the outcome of a reaction.

HOW TO PROCEED WHEN USING THERMODYN©

1. Define process of interest: e.g. Mixed acid glucose fermentation by *Ruminococcus flavefaciens*
2. Write process as a stoichiometrically balanced equation: 1 $C_6H_{12}O_6$ + 0,48 HCO_3^- → 1,07 CH_3COO^- + 0,62 $HCOO^-$ + 0,93 $^-OOC(CH_2)_2COO^-$ + 0,59 H_2 + 3,07 H^+ + 0,34 H_2O
   (The stoichiometric factors are reduced to 1 glucose. Please note that all numbers are written with commas; this is necessary if the preferences in defining the cells of your spread sheet are set the same way)
3. Define boundary conditions, variable and range of applicability: For beginning of batch culture experiment see under "Boundary conditions".

4. Enter reaction number, stoichiometric coefficient, formula, state, and activity into the corresponding spreadsheet columns (s stands for substrate, p for product): 1 is the reaction under beginning conditions, 2 under end conditions. Reactants may be entered as text (an in the 1st table) or as chemical formulas (an in the 2nd table)

Beginning of batch culture experiment

Reaction No.	(S,P)	Stoich. Coeff.	Enter formula	State	Special remarks	Activity	Variable
1	s	1	a-D-Glucose	aq		2,00E-02	
1	s	0,48	bicarbonate	aq		3,00E-02	
1	p	1,07	acetate	aq		1,00E-07	
1	p	0,62	formate	aq		2,00E-07	
1	p	0,93	succinate	aq		5,00E-07	
1	p	0,59	H2	aq			v
1	p	3,07	Proton	aq		1,26E-07	
1	p	0,34	water	l		1,00E+00	

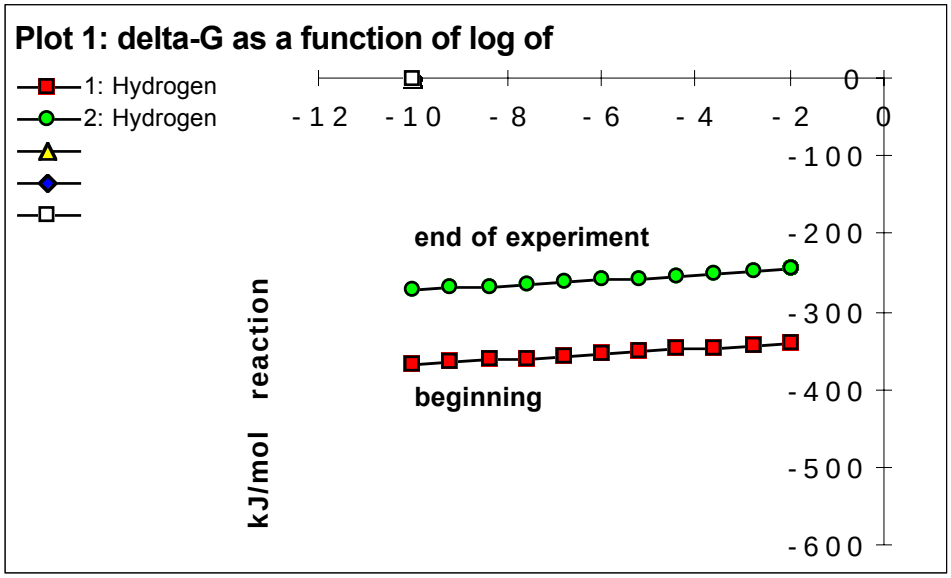
End of batch culture experiment

Reaction No.	(S,P)	Stoich. Coeff.	Enter formula	State	Special remarks	Activity	Variable
2	s	1	CH2OH(CHOH)4CHO	aq		2,00E-04	
2	s	0,48	HCO3-	aq		2,00E-02	
2	p	1,07	CH3COO-	aq		2,00E-02	
2	p	0,62	HCOO-	aq		6,00E-03	
2	p	0,93	(CH2)2(COO-)2	aq		2,00E-02	
2	p	0,59	H2	aq			v
2	p	3,07	H+	aq		5,01E-07	
2	p	0,34	H2O	l		1,00E+00	

5. Define temperature, boundary for variable and graphing options

Plot 1	
Temp. (K)	298,15
Min. variable:	1,00E-10
Max. variable:	1,00E-02
Log plot?:	x
Show react.1	x
Show react.2	x
Show react.3	
Show react.4	
Show react.5	

6. Run calculation and adjust scaling of graph coordinates if necessary.



red squares: conditions at the beginning of the batch culture experiment
green cicles: conditions at the end of the batch culture experiment

7. Interprete graphs and vary conditions.

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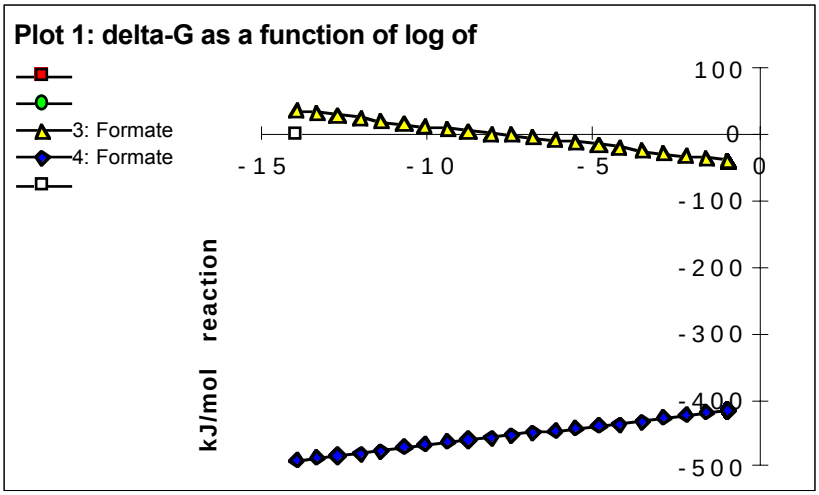
Problem 2: Proceed stepwise as outlined above to reconstruct the stoichiometric equation and the reaction conditions from the data given in the table below.

Comparison between glucose fermentation by *Ruminococcus flavefaciens* (reaction 4) and methane formation from formate by *Methanobrevibacter ruminantium* (reaction 3)

Reaction No.	(S,P)	Stoich. Coeff.	Enter formula[1]	State	Special remarks	Activity	Variable
4	s	1,61	CH2OH(CHOH)4CHO	aq		2,00E-04	
4	s	0,77	HCO3-	aq		2,00E-02	
4	p	1,73	CH3COO-	aq		2,00E-02	
4	p	1	HCOO-	aq			v
4	p	1,5	(CH2)2(COO-)2	aq		2,00E-02	
4	p	0,95	H2	aq		1,00E-07	
4	p	4,95	H+	aq		5,01E-07	
4	p	0,55	H2O	l		1,00E+00	
3	s	1	Formate	aq			v
3	s	0,25	Water	l		1,00E+00	
3	s	0,25	Proton	aq		5,01E-07	
3	p	0,25	Methane	aq		1,00E-04	
3	p	0,75	Bicarbonate	aq		2,00E-02	

[1] reactants may be entered in words or as chemical formula

The result of the comparison is shown in the figure below as a function of the formate concentration.



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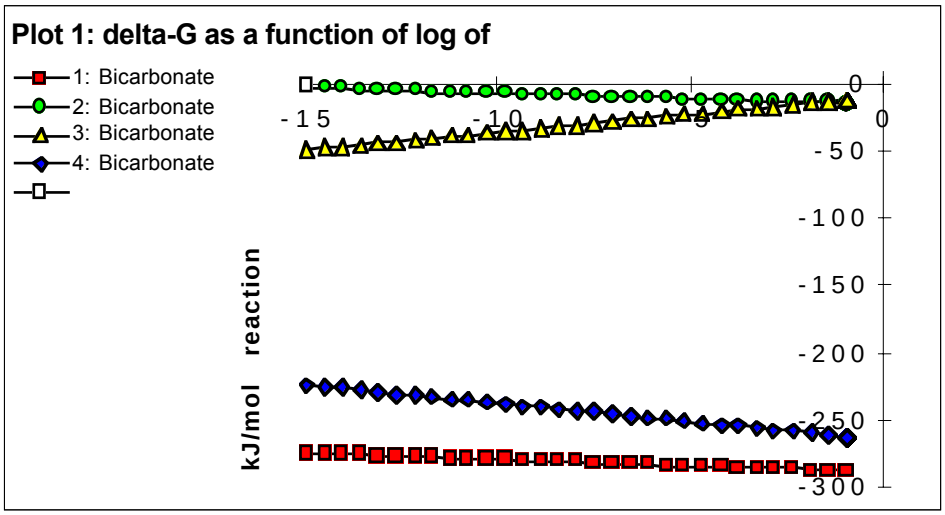
Problem 3: Glucose fermentation by *R. flavefaciens* (reaction 4) and methane formation from formate (reaction 3) and from hydrogen (reaction 2) by *M. ruminantium* presented as a function of variable bicarbonate concentration, and the overall reaction (1) if formate and hydrogen are efficiently removed by the methanogen.

Reaction No.	(S,P)	Stoich. Coeff.	Enter formula	State	Special remarks	Activity	Variable	Compound	Formula
4	s	1	CH2OH(CHOH)4CHO	aq		2,00E-04		a-D-Glucose	CH2OH(CHOH)4CHO
4	s	0,48	HCO3-	aq			v	Bicarbonate	HCO3-
4	p	1,07	CH3COO-	aq		2,00E-02		Acetate	CH3COO-
4	p	0,62	HCOO-	aq		2,00E-04		Formate	HCOO-
4	p	0,93	(CH2)2(COO-)2	aq		2,00E-02		Succinate	(CH2)2(COO-)2
4	p	0,59	H2	aq		1,00E-05		Hydrogen	H2
4	p	3,07	H+	aq		5,01E-07		Proton	H+
4	p	0,34	H2O	l		1,00E+00		Water	H2O
3	s	0,62	HCOO-	aq		2,00E-04		Formate	HCOO-
3	s	0,155	H2O	l		1,00E+00		Water	H2O
3	s	0,155	H+	aq		5,01E-07		Proton	H+
3	p	0,155	CH4	aq		1,00E-04		Methane	CH4
3	p	0,465	HCO3-	aq			v	Bicarbonate	HCO3-
2	s	0,59	H2	aq		1,00E-05		Hydrogen	H2
2	s	0,1475	HCO3-	aq			v	Bicarbonate	HCO3-
2	s	0,1475	H+	aq		5,01E-07		Proton	H+
2	p	0,1475	CH4	aq		1,00E-04		Methane	CH4
2	p	0,4425	H2O	l		1,00E+00		Water	H2O
1	s	1	CH2OH(CHOH)4CHO	aq		2,00E-04		a-D-Glucose	CH2OH(CHOH)4CHO
1	s	0,1625	HCO3-	aq			v	Bicarbonate	HCO3-
1	p	1,07	CH3COO-	aq		2,00E-02		Acetate	CH3COO-
1	p	0,93	(CH2)2(COO-)2	aq		2,00E-02		Succinate	(CH2)2(COO-)2
1	p	0,3025	CH4	aq		1,00E-04		Methane	CH4
1	p	2,7675	H+	aq		5,01E-07		Proton	H+
1	p	0,6275	H2O	l		1,00E+00		Water	H2O



Literature

- K.W. Hanselmann 1991. Microbial energetics applied to waste repositories. Experientia 47: 645-687 Birkhaüser Verlag, CH-4010 Basel/Switzerland.
- Thauer, R.K., Jungermann, K., and Decker, K. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bact. Rev. 41:100-180.
- Kurt Hanselmann 1994. Microbial activities and their eco-chemical influence. In: Chemical and biological regulation of aquatic systems, J.Buffle & RR.De Vitre (eds.), CRC Press, Lewis Publishers, Boca Raton

www. Links

The thermodynamic modelling program Thermodyn© used in this exercise can be downloaded from the microeco website http://www.microeco.unizh.ch
Please ask for the necessary access number and password during the course.
The program is based on Excel, please make sure that you have Microsoft Excel installed on your computer.
Thermodyn© works with Excel loaded on MacIntosh or on PC-Computers

Practical work

Details of the layout of the spread sheet and operating procedures are described under "INSTRUCTIONS" on the program. In short:

1) Define process of interest.
2) Write process as a stoichiometrically balanced equation.
3) Define boundary conditions, variable and range of applicability.
4) Enter equation number, state, boundary conditions and other required values into the corresponding spreadsheet columns.
5) Run calculation and adjust scaling of graph coordinates if necessary.
6) Interprete graphs and vary conditions.

Precautions

Please ask the advisor if you are not familiar with Excel. Do not worry about damaging the program and do not attempt to repair it; it is easier and probably faster to download a new copy from the internet.

Experiences gained

You will learn how to **formulate biochemical processes** as **stoichiometric equations**, define the **conditions** under which the processes might take place and analyze the outcome of **simulation runs**.

Timing

90 min

Reporting	• Describe and discuss the conclusions from the thermodynamic analysis of the interaction between *Ruminococcus flavefaciens* and *Methanobrevibacter ruminantium.* • Describe and discuss how the host affects the thermodynamics of the *R.flavefaciens* fermentation reactions.
Further Problems	Problems 1 to 3 see text above **4. *Ruminococcus flavefaciens* in axenic culture** **a)** In problem 1 which was presented above, we determined the free energy of the reaction varying the H_2 concentration. Define ΔGr for the same reaction at a constant H_2 concentration varying one of the other reactants. **b)** How would a pH change in the rumen affect the energetic performance of *R.flavefaciens*? **5. *Ruminococcus flavefaciens* and *Methanobrevibacter ruminantium* in axenic co-culture** **a)** Which methanogenic reaction is thermodynamically more favourable for *Methanobrevibacter ruminantium* the hydrogenotrophic one or the formatotrophic one? **b)** *In vitro*, the two organsims live in a syntrophic co-culture. Which are, thermodynamically speaking, the most successful conditions for the syntrophic interaction ? **c)** What kind of insights into the complex rumen ecosystem can we derive from the theoretical analyses of individual processes ?
Observations and Comments	