SUPPLEMENTARY MATERIAL

Kinetic modeling of *Zymomonas mobilis* Entner-Doudoroff pathway: insights into control and functionality.

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Table S1. Mathematical symbols and abbreviations for equations and kinetic models used in this study

Mathematical symbol/abbreviation	Definition
Mathematical symbols	
K_{eq}	Equilibrium constant
K_i	Inhibition constant
K_m	Affinity constant
v	Predicted enzyme activities
V_f	Maximal enzyme activities under saturating substrate and activator conditions, and in the absence of inhibitors
h	Hill coefficient
σ	Interaction factor quantifying the extent to which binding of a modifier molecule affects substrate and product binding to the enzyme
k	Rate constant
Abbreviations	
ACET	Acetaldehyde
ADH	Alcohol dehydrogenase
AK	Adenylate kinase
ATPcons	ATP consuming reactions
bPG	1,3-bisphosphoglycerate
ENO	Enolase
ETOHcy	Cytoplasmic ethanol
ETOHex	Extracellular ethanol
ETOHexp	Ethanol transport
GAP	Glyceraldehyde 3-phosphate
GAPD	Glyceraldehyde 3-phosphate dehydrogenase
GF	Glucose facilitator
GK	Glucokinase
GLUCcy	Cytoplasmic glucose
GLUCex	Extracellular glucose
GLUC6P	Glucose 6-phosphate
GPD	Glucose 6-phosphate dehydrogenase
KDPG	2-keto-3-deoxy-6-phosphogluconate
KDPGA	2-keto-3-deoxy-6-phosphogluconate aldolase
PDC	Pyruvate decarboxylase
PEP	Phosphoenolpyruvate
PGD	6-phosphogluconate dehydratase
PGK	3-phosphoglycerate kinase
PGL	6-phosphogluconolactonase
PGLACTON	6-phosphogluconolactone
PGLUCONATE	6-phosphogluconate
PGM	Phosphoglycerate mutase
P3G	3-phosphoglycerate
P2G	2-phosphoglycerate
PYK	Pyruvate kinase
PYR	Pyruvate

Table S2. Enzyme activities used in simulation of glucose consuption in cell free extracts reported by Algar & Scopes (1985).

Reaction	Activity μmol/l/s
1 GF – glucose facilitator	0
2 GK – glucokinase	300
3 GPD – glucose 6-phosphate dehydrogenase	420
4 PGL – 6-phosphogluconolactonase	311
5 PGD – 6-phosphogluconate dehydratase	539
6 KDPGA – 2-keto-3-deoxy-6-phosphogluconate aldolase	900
7 GAPD – glyceraldehyde-3-P dehydrogenase	900
8 PGK – 3-phosphoglycerate kinase	900
9 PGM – phosphoglycerate mutase	3600
10 ENO – enolase	450
11 PYK – pyruvate kinase	1350
12 PDC – pyruvate decarboxylase	840
13 ADH I – alcohol dehydrogenase I	6
13 ADH II – alcohol dehydrogenase II	642
14 ATPcons – ATP consuming reactions	200
15 AK – adenylate kinase	150
16 ETOHexp – ethanol transport	0

Table S3. ATP and glycolytic flux measurements used for co-response analysis.

These measurements are taken from Rutkis *et al.,* (2013). For each strain, measurements were made with no addition and 50 μM DCCD. The penultimate column contains the finite change approximation to the ATP:glycolytic flux co-response coefficient with respect to ATPase.

					ln		Observed	Calculated	Using
			q (g/g/h)	ATP (μM)	q (g/g/h)	ATP (μM)	∂ln(ATP)/∂ln(J)	∂ln(ATP)/∂ln(J)	Mid-point ln(J)
Zm6	◆	**Control**	4.36	1210	1.47	7.1			
		DCCD 50	3.23	1800	1.17	7.5	-1.333	-0.800	1.32
Zm6 -cytB	●	**Control**	4.9	1330	1.59	7.19			
		DCCD 50	3.47	1760	1.24	7.47	-0.800	-1.943	1.415
Zm6-cydB	▲	**Control**	4.12	1070	1.42	6.98			
		DCCD 50	2.82	1420	1.04	7.26	-0.737	-0.474	1.23

Enzyme Kinetics and origin of parameters used in the study.

1. Glucose Facilitator **GF**

Z. mobilis transports glucose across the cell membrane via a constitutive, stereospecific, carrier–mediated facilitated diffusion system. This low affinity glucose transport system has Km values for D-glucose uptake 5000-15000 μM (DiMarco & Romano, 1984). Since reported *Vf* values for glucose facilitator in membrane vesicles (around 1500 μmol/l*s, see DiMarco & Romano, 1985; Parker *et al.,* 1997) apparently do not support typically observed specific glucose uptakes rates in growing *Z. mobilis* (Rogers *et al.,* 1979; Rogers *et al.,* 1982; Algar & Scopes, 1985), we have assumed the initial *Vf* value to be 7000 μmol/l*s referring to the highest glucose uptake value (10 g/g/h) reported for growing *Z. mobilis* culture (Lee *et al.,* 1980). Since glucose intracellular concentration quickly reaches a plateau close, but not above, external concentration (DiMarco & Romano, 1985), we have assumed that for this reaction Keq = 1. *Z. mobilis* glucose facilitator follows hyperbolic kinetics (Weisser *et al.,* 1995), therefore Michaelis-Menten kinetics was used for this reaction.

2. Glucokinase **GK**

Glucokinase reaction is the first reaction in Entner-Doudoroff pathway and converts glucose into glucose 6–phosphate. The Km values for this dimeric enzyme, determined by *Scopes et al.* (1985), are 800 μM and 220 μM for ATP and glucose respectively, higher than reported earlier (Doelle, 1982). The kinetic parameters for the reverse reaction are scarce, so we assumed Km = 1000 μM for glucose-6-phosphate and ADP. The initial glucokinase *Vf* value (6000 μmol/l*s) used for parameter optimization, as for most of the reactions in this work, was taken from the 18th hour of batch fermentation, when fermentative activity reached its maximum (Osman *et al.,* 1987).

The equilibrium constant for glucokinase reaction we used in this study, Keq = 450, is in good agreement with Keq values reported earlier for hexokinase (Robbins *et al.,* 1957). Also it has been shown that ATP is a competitive inhibitor for glucose-6-phosphate dehydrogenase, with an estimated Ki=15000 μM (Scopes *et al.,* 1985). Reversible two-substrate two-product Michaelis-Menten kinetics were used to describe this reaction.

3. G-6-P dehydrogenase **GPD**

G-6-P dehydrogenase has been found to be inhibited by phosphoenolpyruvate (PEP) in the micromolar range with sigmoidal kinetics and Hill coefficients up to 2 (Scopes, 1997). Besides PEP allosteric inhibition, earlier reports suggest that ATP is competitive with NAD, Ki = 1400 μM (Scopes 1985). Since the equilibrium constant L of Monod-Wyman-Changeux equation for the free T-state/free R-state is not known, we have used the universal rate equation for systems biology derived by the Triple-J Group for Molecular Cell Physiology (Rohwer *et al.,* 2006):

$$\frac{V_f \alpha^h \beta^h}{\left(\frac{1+\mu^h}{1+\sigma^{4h}\mu^h}\right) + \left(\frac{1+\sigma^{2h}\mu^h}{1+\sigma^{4h}\mu^h}\right)[\alpha^h + \beta^h] + \alpha^h \beta^h}$$

Where α and β are the concentrations of substrates A and B scaled by their half-saturation constants $A_{0.5}$,$B_{0.5}$ ($\alpha = A / A_{0.5}$; $\beta = B / B_{0.5}$), h the Hill coefficient, μ the concentration of allosteric modifier M scaled by its half-saturation constant $M_{0.5}$, and σ is an interaction factor quantifying the extent to which binding of a modifier molecule affects substrate and product binding to the enzyme, thus leading to allosteric inhibition or activation. We have used a non-linear least-squares method with the Excel solver (Microsoft) to optimize the universal rate equation's kinetic parameters to fit the experimental data reported by Scopes (1997).

Initial Glucose-6-P dehydrogenase activity varies widely during the batch fermentation, so the *Vf* =3500 μmol/l/s for GPD, that we used as initial value for parameter optimization, was set accordingly to the activity reported at 18th hour of fermentation. Within the physiological pH range, equilibrium constant for GPD reaction is Keq~1.4 (Glaser & Brown, 1955; Wurster *et al.,* 1970), which clearly indicates that the reverse reaction is feasible under certain conditions. Nevertheless, there are no data available for reverse reaction substrate affinities, and therefore we have assumed Km = 1000 μM for glucose-6- phosphate and NADH.

The complete equation we have used for GPD is thus a modification of the universal rate equation for reverse reactions (Rohwer *et al.,* 2006).

4. 6-phosphogluconolactonase **PGL**

6-phosphogluconolactone, the product of the oxidation of glucose-6-phosphate, hydrolyses spontaneously; however the rate of spontaneous reaction is not sufficient for the need of high glycolytic flux in *Z. mobilis*. 6-phosphogluconolactonase catalyses hydrolysis of 6-phosphogluconolactone to open chain 6-phosphogluconate. Reported Km values for 6-phosphogluconolactone vary from 20 μM to 29 μM (Scopes, 1985), and as in previous studies (Altintas *et al.,* 2006), the average value of 25 μM was used for PGL reaction. Glucose-6-phosphate as a competitive inhibitor, competing with 6-phosphogluconolactone, has a Ki value of 300 μM (Scopes, 1985). The equilibrium constant for the PGL reaction was calculated from known Keq constants for GPD, and PGL reactions combined (Wurster *et al.,* 1970; Casazza *et al.,* 1986). According to our calculations Keq = 6400, indicating that this reaction occurs in thermodynamically favorable direction. Despite the large Keq value we have assumed, that reverse reaction is feasible with assumed initial Km = 1000 μM for 6-phosphogluconate.

According to Osman *et al.* (1987), the combined activity of 6-phosphogluconolactonase, 6-phosphogluconate dehydratase and KDPGA aldolase does not exceed 2000 μmol/l*s, that was set as the initial *Vf* for parameter optimization. However earlier studies report significantly higher 6-phosphogluconolactonase activity reaching *Vf* = 35000 μmol/l*s (Scopes, 1985), most likely indicating, that PGL activity lies within these values.

The complete equation used for PGL obeys Michaelis-Menten kinetics.

5. 6-phosphogluconate dehydratase **PGD**

PGD catalyses an essentially irreversible dehydration of 6-phosphogluconate, in the E-D pathway and is iron-containing enzyme, that rapidly loses its activity in oxidizing conditions. Km for 6-phosphogluconate was determined to be 40 +/- 10 μM, and 3-phosphoglycerate as a competitive inhibitor has an apparent Ki=2000 μM (Scopes, 1984).

Based on the same assumptions as for the PGL reaction, for parameter optimization we used *Vf* =2000 μmol/l*s as an initial value. Furthermore, earlier reports suggest more than ten fold higher value – *Vf* = 25000 μmol/l*s (Scopes, 1984) - likewise implying that actual PGD activity is partially cryptic.

PGD obeys simple one substrate and one product irreversible Michaelis-Menten kinetics.

6. 2-keto-3-deoxy-6-phosphogluconate aldolase **KDPGA**

KDPGA, a Class I aldolase from Entner-Doudoroff glycolytic pathway, is a trimeric enzyme that catalyzes reversible cleavage of 2-keto-3-deoxy-6-phosphogluconate into the three-carbon units, pyruvate and glyceraldehyde-3-phosphate (Scopes, 1984). According to Scopes, Km values for 2-keto-3-deoxy-6-phosphogluconate was found to be 250 ± 40 μM, which is substantially greater than the values quoted for other bacteria (Scopes, 1984). Notwithstanding relatively high equilibrium constant Keq=1300 μM in the physiological pH range (Goldberg *et al.,* 2004), indicating that KDPGA most likely operates in the cleavage direction, accumulation of pyruvate and glyceraldehyde-3-phosphate at low mM range can cause KDPGA to reverse. Km values for *Z. mobilis* KDPGA reverse reaction substrates have not been reported yet, therefore, we assumed Km=1000 μM for pyruvate and glyceraldehyde-3-phosphate.

Based on the same assumptions, as in case with PGL and PGD , *Vf* was set to 2000 μmol/l*s as initial value for parameter optimization, but according to Scopes (1984), KDPGA activity is significantly higher. Reversible Michaelis-Menten kinetics for one substrate and two products was used to describe this reaction.

7. Glyceraldehyde-3-P dehydrogenase **GAPD**

Z. mobilis Glyceraldehyde-3-P dehydrogenase is the least well characterized enzyme in E-D pathway, with no information about Km values for either reaction. Meanwhile it shows 51% amino acid similarity with yeast - *Saccharomyces cerevisiae*. Therefore, as the best possible assumption, we used same Km values for parameter optimization as in the model of yeast glycolysis (Teusink *et al.,* 2000) - Km= 210 μM; 90 μM; 10 μM and 60 μM, for glyceraldehyde-3-phosphate, NAD, bisphosphoglycerate and NADH respectively.

Since in our model we did not include orthophosphate in biochemical equations, we have assumed the equilibrium constant Keq = 0.04 for GAPD reaction according to intracellular orthophosphate concentration at physiologycal pH levels (De Graaf *et al.,* 1999) and Keq values reported by Goldberg et al. (2004).

For parameter estimation, *Vf* was set to 15000 μmol/l*s for the GAPD reaction

(Osman *et al.,* 1987), and according to Pawluk *et al.* (1986), glyceraldehyde-3-P dehydrogenase obeys Michaelis-Menten kinetics.

8. 3-phosphoglycerate kinase **PGK**

Phosphoglycerate kinase catalyzes one of the two ATP generating reactions in E-D pathway. Nevertheless, kinetic properties for this enzyme have not been studied widely. According to Pawluk *et al.* (1986), Km = 1500μM and 1100 μM for 3-phosphoglycerate and ATP respectively. More recent studies indicate similar Km values at $30^{O}C$: Km = 800μM for 3-phosphoglycerate and Km = 1000μM for ATP (Thomas & Scopes, 1998), which is in good agreement with values reported for other bacteria. Most probably, since PGK is difficult to assay in the forward direction, precise Km values for bisphosphoglycerate and ADP have not been established. Due to similarity between *Z. mobilis* PGK and PGK from *Saccharomyces cerevisiae,* we assumed Km values for the forward reaction the same as reported by Teusink *et al.* (2000): Km = 3 μM for bisphosphoglycreate and Km = 200 μM for ADP.

The equilibrium constant for the PGK reaction, Keq = 3000, is similar to Keq values reported earlier (Teusink *et al.,* 2000, Krietsch *et al.,* 1970).

PGK activity varies widely during the different fermentation phases, and based on the same assumptions as before, we selected PGK activity (9000 μmol/(l*s)) at the 18th hour of batch as the initial value for parameter optimization (Osman *et al.,* 1987).

Pawluk *et al.* (1986), observed minor negative co-operativity for ATP and 3-phosphoglycerate, but since reported effects were small, as in other studies, we have not included cooperative binding in the rate equation, but rather used reversible two-substrate, two-products Michaelis-Menten kinetics.

9. Phosphoglycerate mutase **PGM**

Phosphoglycerate mutase is dimeric enzyme, and as in *Sacharomyces cerevisiae,* it is 2,3 bisphosphoglycerate-dependent. The Km value for 3-phosphoglycerate was determined to be 1100 μM (Pawluk *et al.,* 1986), which is almost identical to that Teusink *et al.* (2000) measured in yeast. The Km value for 2-phosphoglycerate is not known, hence based on the same assumptions as with PGK, according to Teusink *et al.,* we assumed Km=80 μM for 2-phosphoglycerate. As an initial value for parameter

estimation, we set *Vf* =45000 μmol/(l*s) for PGM (Osman *et al.,* 1987).

We have assumed the equilibrium constant at physiological pH range similar to that of Teusink *et al.* (2000), Keq=0.2, also implying that 3-phosphoglycerate intracellular content must be kept almost an order of magnitude higher than 2-phosphoglycerate, to support glucose convertion to ethanol via the ED pathway.

We have not taken 2,3 bisphosphoglycerate activation into account in the corresponding equation, assuming that the enzyme is saturated with 2,3 bisphosphoglycerate and that the PGM reaction obeys reversible one substrate Michaelis-Menten kinetics.

10 Enolase **ENO**

Z mobilis enolase catalyses a simple uninhibited one-substrate reaction with Km = 80 μM for 2-phosphoglycerate, that, as in the case of PGK and PGM, is comparable with values reported for the yeast (Pawluk *et al.,* 1986; Teusink *et al.,* 2000). Since the reverse reaction is physiologically feasible: Keq~4 (Wold *et al.,* 1957), and the Km for PEP has not been determined for the *Z. mobilis* enolase reaction*,* as initial parameter we used the value reported for yeast by Teusink: Km = 500 μM. According to Osman *et al.* (1987), *Vf* = 20000 μmol/(l*s) for ENO reaction, and as before it was set as initial value for parameter estimation. We used reversible one substrate Michaelis-Menten kinetics to describe this reaction.

11 Pyruvate kinase **PYK**

Pyruvate kinase, catalyses the 2nd ATP generating reaction in the E-D pathway, and unlike many other bacterial pyruvate kinases, it is not object to allosteric control. Km values were estimated to be 80 μM and 170 μM for PEP and ADP respectively (Pawluk *et al.,* 1986). The equlibrium constant that we have used for the pyruvate kinase reaction PYK = 5000 is comparable to that used elsewhere (Teusink *et al.,* 2000), and it indicates that the reverse reaction is almost impossible under physiological conditions. However, as in other glucolytic models, we did not exclude it, and assumed Km values similar to yeast: Km=210 μM for pyruvate and Km=1500 μM for ATP (Teusink *et al.,* 2000). Initially, for parameter estimation, we set Vmax = 70000 μmol/(l*s) for the PYK reaction (Osman *et al.,* 1987). According to Pawluk *et al.* (1986), Michaelis-Menten kinetics characterizes the PYK reaction.

12 Pyruvate decarboxylase **PDC**

Pyruvate decarboxylase, a key enzyme in the ethanol formation, has an apparent Km value for pyruvate of 400 μM, which was determined in the presence of 20 mM Mg^{2+} and 1.5 mM thiamine pyrophosphate in sodium citrate buffer, pH 6.0 (Bringer-Meyer *et al.,* 1986). The initial value for the limiting rate for parameter estimation was set to 7000 μmol/(l*s) (Osman *et al.,* 1987). Unlike in yeast, *Z. mobilis* pyruvate decarboxylase does not exhibit cooperativity, hence we used Michaelis-Menten kinetics that leads to irreversible one substrate one product equation.

13 Alcohol dehydrogenases **ADH I** and **ADH II**

Z. mobilis alcohol dehydrogenases have been studied and well described (Wills *et al.,* 1981; Hoppner & Doelle, 1983; Scopes, 1983; Kinoshita *et al.,* 1985; Neale *et al.,* 1986). The zinc-containing isoenzyme ADHI in the anaerobic culture is less active than the iron-dependent ADHII, that accounts for more than 80% of total alcohol dehydrogenase activity (Kinoshita *et al.,* 1985). In this study, due to detailed characteristics of Km values, we used kinetic parameters for both alcohol dehydrogenases obtained by Kinoshita et al.. The Keq for the alcohol dehydrogenase reaction is highly pH sensitive, and, as in other studies, where glycolytic flux was analyzed at pH range 6.5 - 7.0 (Teusink *et al.,* 2000), we selected Keq=10000 in the direction of acetaldehyde reduction. Activities for the acetaldehyde reduction reaction and half-saturation constants for both enzymes are recalculated to intracellular volume and represented in Table. 2. ADHI and ADHII follow a sequential bi-bi mechanism.

14 ATP consumption **ATPcons**

ATP-consuming processes are required for glycolysis to proceed, since PGK and PYK reactions demand a continual ADP supply. Apart from energy consumption by protein synthesis during the different growth phases, membrane bound F_0F_1 type ATPase is considered to be responsible for a significant part of ATP turnover in *Z. mobilis* (Reyes & Scopes, 1991). Notwithstanding the complexity of total ATP hydrolyzing reactions in the cell, we used simple Michaelis–Menten kinetics for ATP

hydrolysis (Vinogradov, 2000) with an assumption that H^+-dependent F_0F_1 type ATPase operates at saturating Mg^{2+} concentrations (Vinogradov, 2000) and is responsible for vast majority of ATP conversion to ADP. Our, assumed Km=500 μM for ATP lies between the values reported by Reyes & Scopes - 100 μM and Lazdunski & Belaich – 1000 μM (Reyes & Scopes, 1991; Lazdunski & Belaich, 1972). The initial activity of the ATP consuming reaction was set to *Vf* = 6000 μmol/l/s, that is slightly lower, than values reoported earlier for *Z. mobilis* and *E. coli* (Reyes & Scopes, 1991; Vogel & Steinhart 1976).

15 Adenylate Kinase **AK**

According to Zikmanis et al. (2001), *Z. mobilis* possesses cytoplasmic and membrane-bound adenylate kinases, but it is not supported by data from sequenced genomes of various *Z. mobilis* strains (Seo *et al.,* 2005; Kouvelis *et al.,* 2009; Pappas *et al.,* 2011; Desiniotis *et al.,* 2012). Therefore we have used one reaction with initial activity 1100 μmol/l/s according to Zikmanis *et al.* (2001). Due to the lack of exact Km values for *Z. mobilis,* and high AK sequence similarity with *E. coli,* we have used parameters obtained by Saint Girons *et al*. (1987). Simple Michaelis–Menten kinetics for 2 substrates and 2 products was used to describe AK kinetics.

16 Ethanol transport **ETOHexp**

As previously (Altintas *et al.,* 2005), we have presumed that ethanol diffuses freely across the cell membrane, rapidly equilibrating between intracellular and extracellular concentrations. Previous studies indicated, that ethanol transport across the cell membrane would not be a rate-limiting step in the E-D pathway, and the obtained rate constants k (in to out), at $29^{O}C$, were $6.8 \pm 0.4\ s^{-1}$ (Schoberth *et al.,* 1996). Therefore, we assumed simple mass action kinetics for this reaction with k = 7 s^{-1}.

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