Microbial maximal specific growth rate as a square-root function of biomass yield and two kinetic parameters

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ABSTRACT

Understanding how growth rates changes under different perturbations is fundamental to many aspect of microbial physiology. In this work, we experimentally showed that maximal specific growth rate is a square-root function of the biomass yield, the substrate turnover number, and the maximum synthesis rate of the substrate transporter under that condition. We used *Escherichia coli* cultures in lactose minimal medium as a model system by introducing genetic modifications, *in vitro* evolution, and ethanol stress to the cell. Deletion of *crr* affected all three parameters in different directions while deletion of *ptsG* decreased only the biomass yield. Ethanol stress negatively impacted all three parameters, while anaerobicity decreased biomass yield and transporter synthesis rate. In addition, laboratory evolution increased the growth rate in lactose mostly through enhancing the expression rate of the *lac* operon. Despite all these changes, the growth rate of the perturbed strain was successfully related to the three parameters by the square-root equation. Thus, this square-root relationship provides insight into how growth rate is altered by different physiological parameters.

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1. Introduction

Microbial growth rate is a fundamental parameter which determines the strain's fitness in competitive evolution and usefulness in many industrial settings. At the macroscopic level, growth rate is typically modeled in the form of Monod kinetics, which characterizes the effect of nutrient limitation. At the microscopic level, the whole cell model pioneered by the Cornell group (Ataai and Shuler, 1985; Domach et al., 1984; Shu and Shuler, 1989) is used to link intracellular mechanisms to the growth rate. Such models require detailed characterization of all the key steps in the cell. Perturbations such as environmental stress and genetic mutations can alter the growth rate by affecting various intracellular events, which ultimately change the growth rate of the cells. Heretofore, there is no good method to dissect the intracellular perturbations and understand how the growth rate changes in a quantitative way.

Using a set of general kinetic equations as a starting point, we have recently derived a relationship that relates the upper bound of the specific growth rate (μ) in terms of measurable physiological quantities (Wong et al., 2009). The boundary is the square-root product of the biomass yield (Y_B), the turnover number (k_{cat}) and the maximum protein synthesis rate (R_m) of the nutrient turnover enzyme

$$\mu \leq \sqrt{Y_B R_m k_{cat}} \qquad (1)$$

This relationship can be formulated for each of the nutrient needed for growth. If the particular uptake system (including its protein synthesis) functions at its maximal activity under the specified condition and is not degraded during growth, then the cell grows at the boundary

$$\mu_m = \sqrt{Y_B R_m k_{cat}} \qquad (2)$$

where μ_m is known as the unrestricted growth rate. When the culture is under nutrient limitation, the growth rate decreases in a way that is characterized by the Monod kinetics. In this case, the cell would grow at a rate below this boundary. The unrestricted growth typically occurs when no nutrient limitation or product inhibition occurs during batch growth under the condition of interest. Note that this equation relates the three biochemical parameters to growth rate. Further work will be required to determine how perturbations affect each biochemical parameter.

The basis of Eq. (2) comes from macroscopic mass balance that is typically used to model growth and substrate consumption in a culture (Bailey and Ollis, 1986; Shuler and Kargi, 2001; Stephanopoulos et al., 1998). By considering the substrate consumption rate and its efficiency (yield) for cell mass formation, Eq. (1) necessarily arises without any artificial constraints. This equation dissects the unrestricted growth into three components: a biomass yield (Y_B) and two kinetic parameters (R_m and k_{cat}), which are measurable and well-defined biochemical properties. Note that this equation does not require substrate uptake to be the limiting step. If downstream intracellular metabolism is the limiting step, then either a metabolite will accumulate or be secreted out, which decreases the growth yield. In addition, the

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relationship can be formulated for each of the nutrient needed for growth. The validity of this equation has been discussed in detail previously (Wong et al., 2009).

In this paper, we validate the square-root equation using *Escherichia coli* lactose metabolism as a model system. We used genetic knockout (*crr* and *ptsG*), laboratory evolution, ethanol stress, and anaerobicity to perturb the cell and measured the three parameters and the growth rate. We successfully demonstrated the square-root relationship between *E. coli* unrestricted growth rate and the three measured parameters.

2. Materials and methods

2.1. Bacterial strains and plasmids

All experiments involving lactose were performed using *E. coli* MG1655. MG1655Δ*crr* and MG1655Δ*ptsG* were obtained from P1 transduction using the Keio Collection (Baba et al., 2006) as the donor.

2.2. Culture conditions and measurement of μ_m

E. coli MG1655 and various other mutants were grown in M9 minimal media supplemented with 0.1% of carbon source (lactose), 1 mM MgSO$_4$, 1 μg/ml vitamin B1, 100 μM CaCl$_2$, and incubated in a 37 °C water bath shaking at 250 rpm. With the initial OD of 0.05, the cultures were allowed to double once before various measurements were taken to ensure that the cells had grown out of the stationary phase.

At each time point, samples were collected for OD measurement, β-galactosidase assay, or lactose determination. The sampling interval was determined by the growth rate. For wild-type strains or mutants with a growth rate similar to the wild-type, the typical sampling interval was between 15 and 30 min. For slower growing strains, the typical interval was between 30 and 90 min. At least five time points were taken per culture. The samples were grown to a final OD of 0.3. The exponential growth region was used to determine the unrestricted growth rate, μ_m.

Ethanol stress experiments were performed using MG1655. The cultures were grown aerobically in M9 minimal media with lactose as the sole carbon source and with different amounts of ethanol (% w/v).

2.3. Lactose, optical density, and β-galactosidase measurements

For lactose determination, 700 μl of culture was filtered immediately with a syringe filter (0.22 μm) to prevent further consumption of lactose by the cells. OD was measured by using a spectrophotometer at 600 nm (Beckman DU520). β-galactosidase enzyme activity was measured by adding 75 μl of cell culture into 675 μl of Z-buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM β-mercaptoethanol), 6 μl of chloroform and 12 μl of 0.1% SDS. The samples were vortexed until the solution became clear. Then 150 μl of 4 mg/ml *o*-nitrophenyl β-D-galacto-side (ONPG) was added to the mixture and the absorbance at 405 nm was measured every 10–60 s for 5–10 min using a spectrophotometer (Beckman DU640). β-galactosidase activity was determined by the slope of the absorbance kinetics. In the lactose concentration measurement, filtered supernatant was injected into HPLC with auto-sampler (Agilent 1100) equipped with a reflective index detector and an Aminex HPX87 column (0.5 mM H$_2$SO$_4$, 0.6 ml/min). Concentration was determined was extrapolated from standard curves.

2.4. Determination of biomass yield, Y_B

The biomass yield was determined by the linear regression coefficient of OD against the concentration of extracellular lactose. OD is converted to dry cell weight by the conversion factor, 1 OD = 0.33 g$_{dcw}$/L. The data used were within the period of unrestricted growth.

2.5. Determination of the lac operon R_m

The R_m is defined as the maximum synthesis rate of the substrate turnover enzyme under the specified condition: $R_m = (1/B)(dy_1/dt)$. Therefore, R_m was measured under fully induced condition in the unrestricted culture condition (with or without ethanol stress). To measure R_m for the lactose transporter, we used β-galactosidase as a reporter protein, since they were transcribed in the same operon. The β-galactosidase activity was measured as a function of time during mid-log growth when lactose was above 1 mM. The rate of β-galactosidase increase was determined and divided by the cell mass at that time. This measurement was made several times and the average was recorded.

2.6. Determination of k_{cat}

k_{cat} is defined as the maximum substrate uptake (when the substrate concentration is much greater than the k_m) per molecule of the protein under the condition of interest. To measure the k_{cat} for LacY, an independent culture about 100 ml at OD∼0.3 was harvested, concentrated 50-fold with fresh M9 minimal media without a carbon source (with or without ethanol stress). The 3 μl of this concentrated culture was used for β-galactosidase activity measurements. The 10 μl of 20% lactose was added to the culture and samples were taken and filtered every 1 min for 5 min. Lactose concentration was determined by HPLC. The k_{cat}^{app} was determined by dividing the rate of change in lactose concentration by the LacZ activity.

2.7. In vitro evolution experiments

The starting strain for the evolution is MG1655. To evolve *E. coli* in lactose, 20 separate cultures of 5 ml M9 minimal media supplemented with 0.5% of lactose, 1 mM MgSO$_4$, 1 μg/ml vitamin B1, and 100 μM CaCl$_2$ were grown at 37 °C. After a day of growth, each culture was diluted 100 fold into fresh media. Every 3–7 days, cells were frozen at −80 °C. The experiment continued for 70 days. Selected cultures were used to isolate single colonies and test for growth rate, yield, k_{cat} and R_m.

2.8. Anaerobic experiments

Cultures were grown in media contained in a sealed, airtight bottle, where the air inside the bottle was replaced with argon. The growth experiment was performed under normal conditions in a water bath shaker. Samples were removed with needles and syringes. OD, biomass yield and β-galactosidase activity measurements were performed as described earlier. For the turnover number measurements, all the cell culture transfers and periodic sampling through filtration were done inside an anaerobic chamber.

2.9. Normalization

To control for experimental bias introduced by the use of reporter enzyme and the inaccuracy of the conversion factors, we chose a reference state as a normalization basis. The reference state chosen is the wild-type MG1655 strain in unrestricted balanced growth. All the measurements were normalized by the data taken for this strain using the same method and the same conversion factors. Eq. (2) then becomes

$$\frac{\mu_m}{\mu_{m,ref}} = \sqrt{\frac{Y_B}{Y_{B,ref}} \frac{R_m}{R_{m,ref}} \frac{k_{cat}}{k_{cat,ref}}} \qquad (3)$$

3. Results

3.1. Deletion of ptsG (IICB^Glc)

The *ptsG* gene encodes the glucose transporter IICB^{Glc}, the membrane protein responsible for glucose uptake through the phosphotransferase system (PTS). To test the validity of the square-root equation, we deleted the glucose transporter, *ptsG*, and grew the strain in lactose minimal medium. The activity of β-galactosidase (LacZ) was measured as a reporter for lactose permease (LacY), since they are expressed from the same operon. k_{cat} and R_m were measured independently. The biomass yield was defined based on lactose consumption. Table 1 shows these data along with the data from the wild-type strain. Interestingly, deletion of *ptsG* decreased the biomass yield as compared to the wild type (Fig. 1), while k_{cat} and R_m remained unchanged. When the data were used in Eq. (3), they successfully calculated the decrease in growth rate (Table 1 and Fig. 2).

The decrease in the biomass yield was caused by the excretion of glucose in the medium (Fig. 3) after lactose is hydrolyzed. Since the *ptsG* mutant cannot re-uptake glucose, the biomass yield on lactose decreased. Apparently, lactose uptake was not the limiting step. The cell can take up and hydrolyze lactose, but cannot phosphorylate glucose intracellularly fast enough. Therefore, the glucose was excreted and the biomass yield decreased. In contrast, the wild-type cells can re-uptake glucose through IICB^{Glc}, and thus no glucose was detected in the medium. Since IICB^{Glc} does not have a known regulatory role in lactose metabolism, therefore it did not change either k_{cat} nor R_m.

3.2. Deletion of crr (IIA^Glc)

The *crr* gene codes for the PTS enzyme IIA^{Glc}. The Δ*crr* strain cannot grow using glucose as the sole carbon source. This strain was cultured in lactose minimal medium and the growth rate, Y_B, k_{cat} and R_m were measured. Deletion of *crr* changed all the physiological parameter measured (Table 1). Remarkably, the measured growth rate was consistent with that determined by
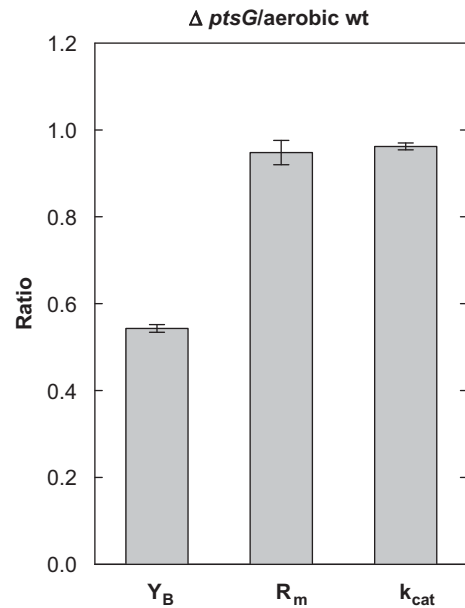
Fig. 1. Changes to Y_B, R_m, and k_{cat} in lactose growth for *E. coli* with *ptsG* deletion. The fold-changes in Y_B, R_m, and k_{cat} for Δ*ptsG* are normalized to the wild-type MG1655 strain in aerobic lactose minimal medium. *ptsG* deletion mainly disrupt the biomass yield while leaving R_m, and k_{cat} relatively unchanged.
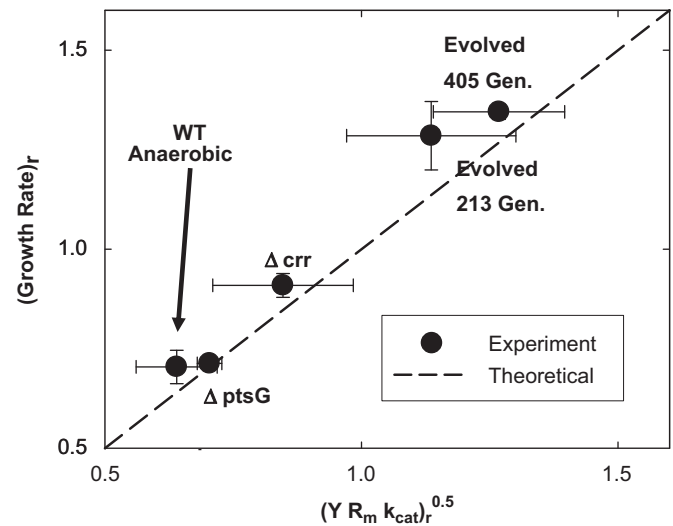

Fig. 2. The combined effect of Y_B, R_m, and k_{cat} determined the growth rate according to Eq. (3) from various perturbations. The growth rate determined by Eq. (3) $(Y_B R_m k_{cat})_r^{0.5}$ agreed well with the measured value (Growth Rate)$_r$. Dash line represents the theoretical square-root relationship. The subscript r denotes ratio, which is normalized to wild type in aerobic condition.

Table 1
Measured parameters and growth rate determined from the square-root relationship.

	Yield (g/g)	R_m (act./h/OD)	k_{cat} (mol/min/act.)	μ (meas.) (h^{-1})	μ (pred.) (h^{-1})
Δcrr	0.14 (0.01)	0.14 (0.01)	139.7 (19.7)	0.5 (0.02)	0.47 (0.06)
ΔptsG	0.12 (0.002)	0.3 (0.01)	51.7 (0.4)	0.39 (0)	0.39 (0.01)
213	0.24 (0.007)	0.48 (0.03)	41.8 (5.5)	0.71 (0.05)	0.63 (0.1)
405	0.24 (0)	0.53 (0.02)	47.4 (4.3)	0.74 (0.01)	0.7 (0.09)
WT w/o O₂	0.1 (0.01)	0.28 (0.01)	55.2 (5.4)	0.39 (0.02)	0.35 (0.02)
WT w O₂	0.22 (0.02)	0.31 (0)	53.8 (3.2)	0.55 (0.03)	–

Numbers in parenthesis are the standard deviation ($n = 3$). 213 and 405 represent the number of generation of lactose evolved strains. act. denotes β-galactosidase activity.
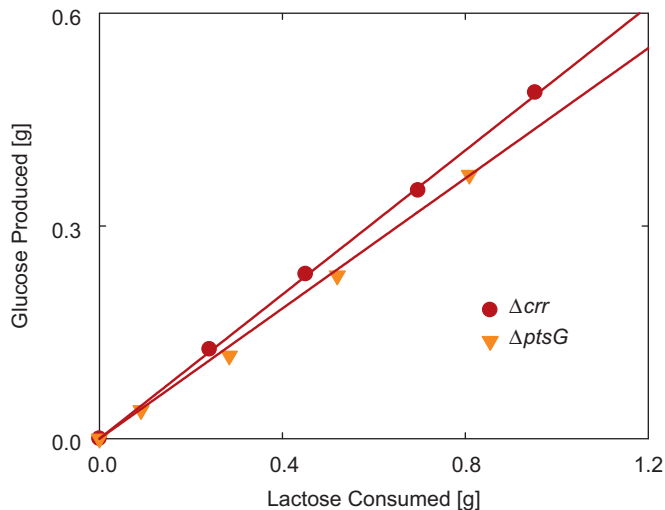
Fig. 3. Glucose production as a function of lactose consumed in Δcrr and $\Delta ptsG$ strains when grown in minimal media supplemented with lactose as the sole carbon source. When the glucose transporter process is disrupted, extracellular glucose accumulates after lactose is hydrolyzed by *lacZ* and ultimately causes the biomass yield to decrease.
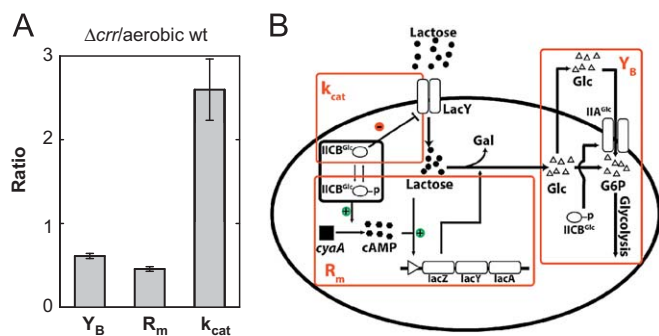

Fig. 4. Changes to key physiological parameters in lactose growth for *E. coli* with *crr* deletion and the lactose regulatory network: (A) *crr* deletion affects all three parameters that are ultimately compensatory in their effect on growth rate (Fig. 2). (B) A schematic diagram of the lactose metabolism and how genes in glucose transport system (*crr* and *ptsG*) affect Y_B, R_m, and k_{cat} of the lactose metabolic enzymes in a non-diauxic situation. Lactose is imported by LacY and metabolized into galactose (Gal) and glucose by LacZ. Some glucose is secreted and reuptaked by the glucose transporter systems IIA^{Glc} (*crr*) and IICB^{Glc} (*ptsG*). Deletion to both of these genes will lead to reduction in Y_B. IIA^{glc} can inhibit the transport of lactose by LacY, thus affecting the k_{cat}. The phosphorylated form of IIA^{glc} is also an activator of adenylate cyclase (*cyaA*) which produces cAMP and activates the *lac* operon. Therefore, IIA^{glc} affects the R_m as well. G6P is glucose-6-phosphate.

Eq. (3) (Table 1 and Fig. 2), providing another validation of this equation.

The unphosphorylated form of IIA^{Glc} binds to the lactose permease, LacY, and inhibits lactose uptake, a process known as inducer exclusion (Nelson et al., 1983). Deletion of *crr* caused a 2.6-fold increase in k_{cat} for lactose uptake because of the lack of inducer exclusion (Fig. 4a), which is consistent with previous results (Hogema et al., 1999). In contrast, R_m was reduced by 50% in this strain (Fig. 4a). The phosphorylated IIA^{Glc} is an inducer of adenylate cyclase, which synthesizes cAMP, a positive regulator of the *lac* operon through binding with the cAMP receptor protein. Thus, in the Δcrr strain, the *lac* operon is not fully induced because of the reduced cAMP level. The biomass yield (Y_B) was also reduced by 40% (Fig. 4a), due to the excretion of glucose (Fig. 3) which cannot be phosphorylated fast enough by the intracellular glucokinase. The combined effects of Y_B, R_m and k_{cat} successfully
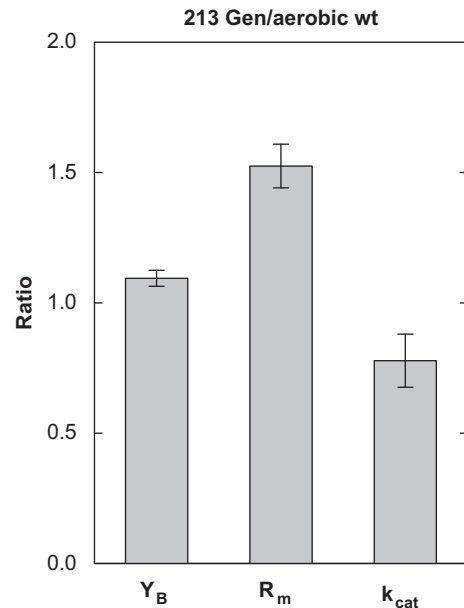
Fig. 5. Fast-growing *E. coli* evolved by serial transfer in lactose medium after 213 generations. The evolved strain at 213 generation showed altered Y_B, R_m, and k_{cat}, with R_m changes being the most significant.
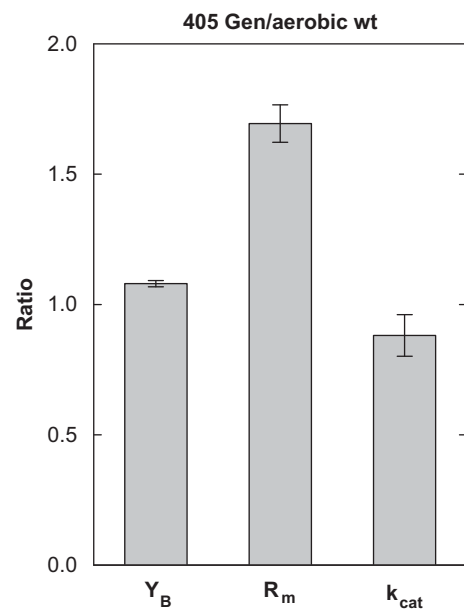
Fig. 6. Fast-growing *E. coli* evolved by serial transfer in lactose medium after 405 generations. Further improvement in growth rate in 405 generations is caused by a higher level of evolved R_m.

determined the perturbation on the growth rate (Fig. 2) based on Eq. (3). A schematic of the regulatory relationship between Y_B, R_m and k_{cat} and lactose regulatory network is depicted in Fig. 4b.

3.3. In vitro evolution

To test the square-root equation further, we isolated fast-growing *E. coli* strains by repeatedly transferring the *E. coli* culture to fresh lactose medium. It is known that repeated transfer of a culture may increase the growth rate under that condition. After about 200 generations, we began to observe a significant increase in the growth rate. At the 213 generation, R_m and Y_B were increased but k_{cat} was

slightly decreased (Fig. 5). At the 405 generation, the strain showed a further enhancement in R_m, but both Y_B and k_{cat} remained at the previous levels (Fig. 6). Remarkably, the growth rates were again determined by Eq. (3) (Fig. 2).

The result presented here is consistent with the in *vitro* evolution experiment reported by Dekel and Alon (2005), although the environmental condition is slightly different. Both this work and the previous work demonstrated that in *vitro* evolution in lactose increases the *lac* operon expression. However, here we demonstrated further that *lac* operon expression is the major reason for the growth rate improvement. Improving the biomass yield, however, may be difficult because the yield may be close to optimal or requires changes to many genes simultaneously.

3.4. Ethanol stress

We further tested the square-root equation by challenging the cells with ethanol. We exposed *E. coli* cultures grown in lactose minimal media to different concentrations of ethanol. Ethanol has been shown to alter membrane structures (Dombek and Ingram, 1984; Ingram, 1976), and thus may contribute to the decrease in the substrate uptake turnover number (k_{cat}). Moreover, the disruption of the membrane structure may cause intracellular metabolites to leak out of the cells, thus decreasing the biomass yield. The membrane damage may also negatively impact the energetic of the cell and result in the slight decrease in R_m.

As expected, we found that the growth rate decreased with increased ethanol concentration (Fig. 7A). Interestingly, Y_B, R_m and k_{cat} were all decreased as ethanol concentration increased, although each to a different extent (Fig. 7B). However, Eq. (3) is able to successfully calculate the growth rate (Fig. 7C).
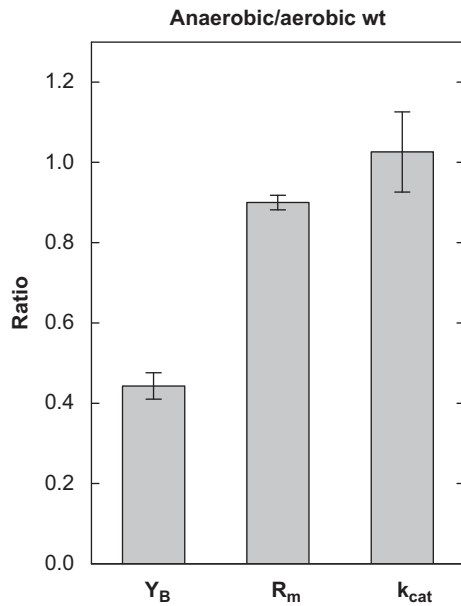
3.5. Anaerobic growth

Finally, we compared anaerobic growth with aerobic growth using the wild-type strain. Under anaerobic conditions, the cell excretes fermentation products and thus the biomass yield is expected to decrease. Consistent with this reasoning, we found that Y_B indeed decreased significantly under anaerobic conditions (Fig. 8). R_m also decreased slightly presumably due to the decreased protein synthesis rate in slower growth conditions. In contrast, k_{cat} remained unchanged as expected. Again, these three parameters were able to determine the growth rate changes according to Eq. (3) (Table 1 and Fig. 2), providing another validation of the square-root relationship.

4. Discussion

Growth rate and biomass yield are two distinct physiological variables, but the relationship between these two quantities has never been explicitly examined. We recently derived a square-root relationship from a set of mass balance equations (Wong et al., 2009). This square-root equation serves as the upper bound of the growth rate. However, if the substrate of interest is not limiting, which occurs under the unrestricted growth condition, the specific growth rate follows the upper bound in that condition. Using different stress and perturbations, we are able to relate the changes in growth rate to biomass yield and k_{cat} and R_m through this simple square-root equation. Some perturbations affects only one parameter (such as $\Delta ptsG$), while others affect all parameters. Therefore, square-root equation provides a way to dissect the causes of growth rate changes, in addition to gaining understanding of the relationship between growth rate and biomass
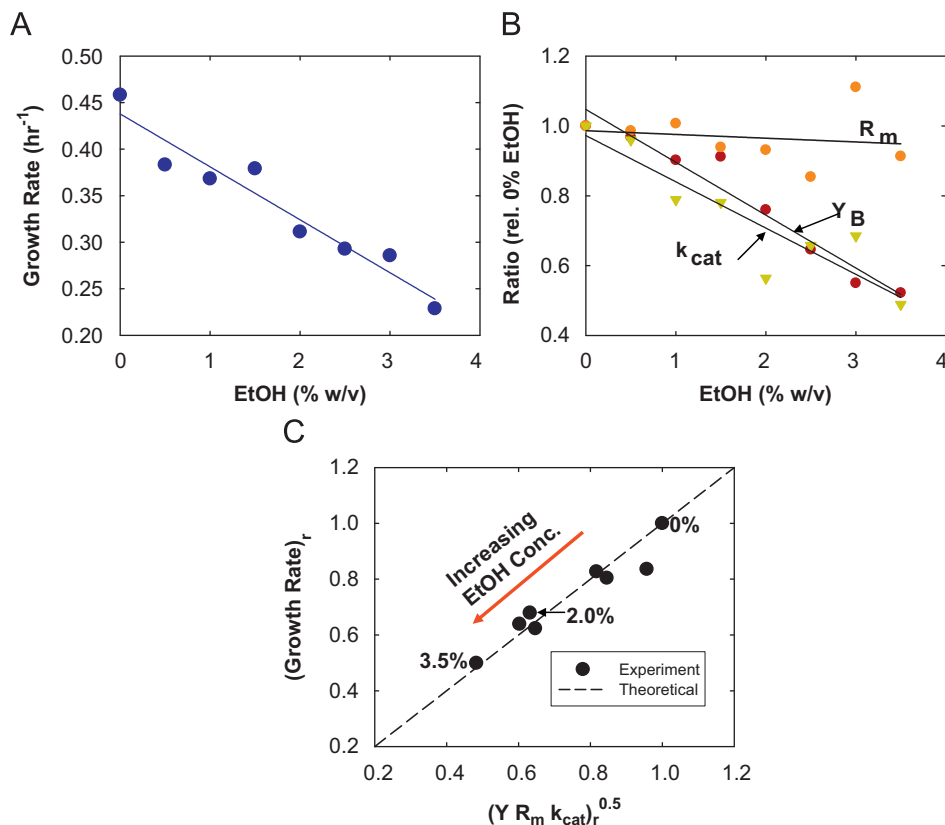
Fig. 7. Ethanol tolerance of the wild-type strain in lactose minimal medium: (A) the growth rate decreased with increasing ethanol concentration, (B) the effect of ethanol on Y_B, R_m, and k_{cat} and (C) when Y_B, R_m, and k_{cat} were considered, the growth rates were calculated quantitatively by the square-root relationship in Eq. (3) (marked by the 45° line).

Fig. 8. Changes to Y_B, R_m, and k_{cat} in lactose growth for *E. coli* in anaerobic condition. Under limited oxygen condition, the biomass decreased and this is the major cause for the observed growth defect (Fig. 2). The growth rate is again determined by Eq. (3) (Fig. 2).

medium and could not be re-taken. This situation resulted in a decrease in the yield, and was accounted for in the square-root equation.

yield. Although it is common to assume implicitly that an increase in growth yield will result in a proportional increase in growth rate, the square-root equation shown here demonstrates the intricate relationship between the two physiological parameters.

The square-root equation is a direct consequence of mass balance (Wong et al., 2009) without any artificial assumption. The only requirement is that the transporter protein is not degraded during unrestricted growth, which is believed to be true for all known microorganisms. It may appear that the relation is valid only when the substrate uptake is the limiting step. However, this is not the case as demonstrated by the results of $\Delta ptsG$ and Δcrr strains. In these strains, the uptake of lactose was faster than the downstream metabolism. Thus glucose was excreted to the

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