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Research Article

Temperature and Glycerol Formation: A Proposal to Explain the Causal Relationship Based on Glycolytic Enzyme Activities

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Abstract: Most yeast strains produce glycerol in larger quantities when cultivated at higher temperatures. This is probably the reason why red wines contain higher amounts of glycerol than white wines. In this work, we tried a kinetic and thermodynamic approach to suggest a mechanistic reason for this phenomenon. A glycolytic model consisting of the kinetics of the individual enzymes constituting it was the starting point. The temperature and ethanol effects on the apparent kinetics of individual enzymes were determined and were incorporated into the model. The Arrhenius equation energy of activation was determined for each enzyme and it was found that the enzymes in the upper part of the glycolytic pathway were more dependent on the temperature. The model improved with these changes could qualitatively simulate the ethanol and glycerol production curves and that more glycerol is produced at higher temperatures. Here

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we propose that the differences in the temperature dependence of the enzymes around the glycerol branch are the reason for glycerol accumulation at higher temperatures.

Key words: fermentation temperature, glycerol, glycolysis, *Saccharomyces cerevisiae*, yeast metabolism

Introduction

Temperature is one of the most important parameters for alcoholic fermentation since it can affect both the kinetics of the process in terms of duration and rate of fermentation and the final quality, i.e., production of secondary metabolites (Torija et al. 2003). Motivated by its biotechnological applications, the response of the mesophilic yeast *Saccharomyces cerevisiae* to suboptimal temperatures has been the focus of several studies. In particular, brewing and winemaking are two processes in which yeast is subjected to suboptimal temperatures (typically 12 to 15°C) to obtain specific desired flavor compounds (Cruz et al. 2012). As a typical example of temperature effect on fermentation, glycerol formation in red and white wines can be given. Glycerol is the most important by-product of alcoholic fermentation after ethanol and CO_2. Glycerol is produced from dihydroxyacetone phosphate (DHAP), a three-carbon intermediate of the glycolytic pathway, by a two-step process: Reduction of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G3P) by NADH-dependent glycerol 3-phosphate dehydrogenase (GPD), encoded by GPD1 and GPD2 followed by dephosphorylation of glycerol 3-phosphate to glycerol by a specific glycerol 3-phosphatase (GPP), encoded by GPP1 and GPP2 (Cronwright et al. 2002; Remize et al. 2001; Remize et al. 2003). The first step of glycerol formation, catalyzed by GPD, is rate-limiting step for glycerol production (Remize et al. 2001;

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Remize et al. 2003). As widely known, glycerol, as a by-product, is not wanted in industrial fermentations, since it affects the alcohol yield. On the other hand, in enology and to an extent in brewing, it affects the taste positively. It is well documented that by increasing the fermentation temperature the glycerol yield increases and for example, red wines, fermented at comparatively higher temperatures, contain more glycerol than white wines (Ough et al. 1972). For example, Du et al. (2012) showed that more glycerol was produced at 25°C than at 13°C. The same results were indicated by Gao et al. (2018) and they explained their findings as a result of the activity of glycerol-3-phosphate dehydrogenase being higher at 25 °C than at 13°C. In both studies, ethanol production was indicated to be influenced by temperature, in a way that it was slightly higher at 13°C than at 25°C. Similarly, Yalcin et al. (2008) investigated the effect of temperature on growth and glycerol formation kinetics of two indigenous wine strains of *Saccharomyces cerevisiae* from Turkey. Their results showed that the strains exhibited an increase in their specific glycerol production rates as the temperature was raised from 20ºC to 30ºC. The specific glycerol production rates declined at 35ºC, which was a sub-optimal temperature for the yeast growth.

However, the biochemical basis of this increase in glycerol yield phenomenon has not been explained yet. Other than this, glycerol is involved in various metabolic processes, thus its metabolism is under complex control. It may act as a redox valve to counterbalance the surplus of NADH produced during biomass formation under anaerobic conditions since ethanol production is a redox-neutral process (Michnick et al. 1997). Glycerol formation is also important for recycling inorganic phosphate used in glycolysis and glycerol 3-phosphate, the

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68 precursor of glycerol, is needed for the synthesis of glyceride lipids (glycerophospholipids and
69 triacylglycerols) (Nevoigt and Stahl 1997). Glycerol is the main osmoprotectant in most yeast
70 species, including *S. cerevisiae*; it acts as a compatible solute during hyperosmotic stress
71 (Hohmann 2002; Pahlman et al. 2001). Moreover, glycerol serves in oxidative, heat or cold stress
72 protection in different organisms. Here we try to isolate and address only the change of its
73 synthesis rate with temperature, keeping other conditions constant.

74 It is known that yeast cells go through different levels of regulation, such as
75 transcriptional, translational and metabolic regulations upon environmental changes like
76 temperature and ethanol concentration (Postmus et al. 2008). First of all, temperature itself has
77 an immoderate effect on the kinetic properties of enzymes. Secondly, concentration and/or
78 catalytic capacity of enzymes can be changed by temperature. Such hierarchical regulation could
79 be affected at the levels of transcription, mRNA degradation, protein synthesis or degradation,
80 and post-translational modification. Finally, the temperature may exert a metabolic regulation.
81 An altered metabolite environment for an enzyme, such as an altered substrate, product or
82 effector concentrations can lead to differing *in vivo* reaction rates (Postmus et al. 2008).

83 Alcoholic fermentation follows the same enzymatic pathway with glycolysis for the first
84 10 steps, and glycolytic enzymes are among the targets on which temperature and ethanol exert
85 their effect. Regarding temperature effect, however, there are only a few data available on *in*
86 *vitro* enzyme activity measurements at different temperatures for yeast glycolytic enzymes (Cruz
87 et al. 2012). Furthermore, temperature dependencies of glycolytic enzymes are hypothesized
88 differently on contradictory results in the literature. Performing the activity assays at 12 and

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89 30ºC, Tai et al. (2007) arrived at a conclusion that the temperature dependence of glycolytic
90 enzymes was very strong. According to their results, instead of transcriptional (vertical)
91 regulation, metabolic control, reflected by massive changes of intracellular concentrations of
92 glycolytic metabolites was dominant and it was the compensating factor for the suboptimal
93 kinetics of glycolytic enzymes to sustain the unchanged glycolytic flux observed. Cruz et al.
94 (2012), on the other hand, concluded that the glycolytic enzymes should have similar
95 temperature dependencies referring to the intracellular levels of glycolytic metabolites and
96 residual glucose concentration. In fact, encountering contradictory results in the literature is not
97 very surprising since we still lack a full mechanistic understanding of the effects of temperature
98 on biological processes across levels of the organization and the suite of adaptations that
99 organisms use to cope with these effects (Postmus et al. 2008). The different levels of control on
100 glycolytic flux related to temperature was investigated: In glucose-limited chemostat cultures,
101 the control was mainly through intracellular metabolite (Postmus et al. 2008) whereas, under
102 fermentative conditions, the effect of temperature on the catalytic rate and gene expression
103 contributed to the control of the flux (Postmus et al. 2012).
104 Another stress that yeast encounter in alcoholic fermentations is the alcohol itself. In a
105 typical wine fermentation, the stationary phase, during which most of the sugar (between 50 and
106 80%) is fermented, constitutes the majority of the fermentation period and non-growing yeast
107 cells are exposed to ethanol concentrations that gradually rise up to 12% (v/v) (Bisson 1993).
108 There are not many studies about the effect of ethanol on the activities of glycolytic enzymes.
109 Nagodawithana et al. (1977) investigated the effect of ethanol on hexokinase (HXK),

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phosphofructokinase (PFK), aldolase (ALD) and glycerol-3-phosphate dehydrogenase (GLYC3PDH) of *Saccharomyces uvarum*, formerly *Saccharomyces carlsbergensis*. HXK and GLYC3PDH were found to be inhibited non-competitively by ethanol and no inhibition was observed for PFK and ALD within the concentration range tested. There has still been just one study in which all glycolytic enzymes of baker's yeast were examined in terms of ethanol inhibition (Millar et al. 1982). They investigated the effect of ethanol on the purified enzymes in terms of both activity and denaturation. Both Nagodawithana et al. (1977) and Millar et al. (1982) suggested that inhibition of glycolytic enzymes by ethanol might play a role in the slowing down of the glycolytic rate. Nevertheless, it is known that the yeast cell ceases to grow long before the glycolytic pathway stops functioning; so that, one should keep in mind that the effects of alcohol are obviously more complex than just affecting the glycolytic pathway.

This study addresses our attempts to relate the effect of temperature on the rates of individual glycolytic reactions to overall fermentation behavior. To this end, kinetics of individual yeast enzymes involved in alcoholic fermentation was studied *in vitro* under various temperatures, and the effect of temperature was quantified using Arrhenius relation. Incorporating the effect of temperature along with that of ethanol to a previous model (Teusink et al. 2000), alcoholic fermentations at different temperatures were simulated and the outputs were compared with the experimental data. The improved application of the model simulates qualitatively the alcohol formation and offers an explanation for the glycerol formation alongside.

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Materials and Methods

Strain and Growth Conditions.

Brewers' yeast obtained from Efes Brewery in Kazan, Ankara, Turkey was used in this study. This yeast is company's own strain and it was included in the culture collection of our laboratory, with the code "EF412-H2". The yeast culture was kept at -80ºC in 20% glycerol until use. For enzyme assays, yeast was pre-grown in 20 mL yeast extract-peptone-dextrose (glucose 20 g/L) medium (YPD) in 100 mL cotton plugged Erlenmeyer flask. Pre-culture was inoculated from the glycerol stock and incubated at 30ºC and 200 rpm for about 12 hr until the optical density at 600 nm (OD_{600}) was around 1.0. This was used to inoculate the YPD (glucose 50 g/L) main culture. Twenty mL of pre-culture was added to 180 mL of the main culture medium in 500 mL Erlenmeyer flask and incubated at 30ºC and 140 rpm. For enzyme assays, cells were harvested after 15-16 hr, when OD_{600} reached around 9.0. The glucose concentration in the medium was 15–20 g/L at the time of harvest.

Extraction of Proteins for Enzyme Assays.

Yeast cells at OD_{600} of 9.0 were collected by centrifugation at 5000xg for 5 min. Cells were washed twice with 20 mM potassium phosphate buffer (pH 7.0) and suspended in the same buffer at a volume (mL) equal to the wet weight (mg) of cells. The suspension was pipetted slowly into liquid nitrogen. Droplets formed were kept at -80°C until further use.

Frozen cells were brought into powder form for the extraction of proteins via Mikro-D95 dismembrator (Sartorius, Göttingen, Germany) by shaking for 60 sec at 2000 rpm. The biomass in powder form was then suspended in cold extraction buffer (as specified in enzyme assay

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procedures). Cell debris was removed by centrifugation at 12500xg for 30 min at 4°C. The supernatant (crude extract) was used for enzyme assays, the protein concentration of extracts was determined by the Lowry method modified by Hartree (1972) with bovine serum albumin (BSA) as the standard.

Enzyme Assays for Activity Measurements.

All enzyme activities, except HXK, phosphoglucose isomerase (PGI), and alcohol dehydrogenase (ADH) (reverse direction-ethanol as substrate), were measured by monitoring the oxidation of NADH at 340 nm in a spectrophotometer with a thermostated cell comportment (Shimadzu UV-1202, Kyoto, Japan). The activities of HXK and PGI were measured by monitoring the reduction of $NADP^+$, and that of ADH (reverse direction-ethanol as substrate), was measured by monitoring the reduction of NAD^+. The extinction coefficient of NADH was taken as 6.22 mM/cm. Assays were done in 1.4 mL special glass cells (Hellma, Müllheim, Germany) having a light path of 1 cm.

A detailed description of each enzymatic assay can be assessed from Şahin (2009), as well as from the Appendix. For all enzymes, one unit of enzyme activity was defined as the μmole of substrate converted per min. Protein extracts were diluted with extraction buffer when necessary. All assays were performed with at least two concentrations of cell extract. Activities obtained by these experiments differed by less than 10%. Activities were reported as specific activities, which were defined as the unit of enzyme per mg of protein in the crude extract. Crude extracts contained 0.8-2 mg protein/mL.

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In studying the effect of temperature on activities of glycolytic enzymes, enzyme assays were carried out at 10, 15, 20, 25, 30 and 35°C. In order to minimize the effects of hierarchical and metabolic (metabolite environment) regulations on enzyme activities, yeast cells with same growth history (grown at 30°C) were harvested and the activity measurements of the crude extracts were carried out with the same reaction environment except for temperature. For the ethanol effect, enzyme assays were carried out by including ethanol at concentrations of 0, 5, 10, 15, or 20% (v/v) in final assay volume, and rates (or specific activities) were compared with the rates in the absence of ethanol, except for ADH assay using ethanol as substrate. All assays were carried out at 30°C.

The specific activities determined at 30°C were assumed to be the maximum velocity (V_{max}) values of the corresponding enzymes for the simulation purpose since the saturating conditions were used in the assays for most of the enzymes. Vmax values of hexose transport (HXT) and glycerol-3-phosphatase (GLYCPASE) are taken from literature as 163.7 mmol/L_{cyt}.min (one carrier model with low affinity) (Teusink et al. 2000) and 104 mmol/L_{cyt}.min (Cronwright et al. 2002), respectively. For the conversion of specific activity unit of U/mg protein into mmol/L_{cyt}.min, the cell cytosolic volume was taken to be 1.67 μL per mg dry yeast weight (about 3.75 μL cytosol per mg protein) (Cronwright et al. 2002; Teusink et al. 2000).

Short-Term Fermentation Kinetics.

Effects of temperature as well as ethanol on glucose consumption and ethanol and glycerol production were investigated in short-term under non-growing conditions (Teusink et al. 2000).

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Four temperatures (10, 15, 20, 30ºC) and three ethanol concentrations (5, 10, and 15 % v/v) were investigated in triplicate. Cells grown in YPD (glucose 5% w/v) medium until OD_{600} of 9.0 were collected by centrifugation at 5000xg for 5 min at 4°C. Glucose concentration in the medium was 1.5– 2.0% at the time of harvest. They were washed twice by 50 mM potassium phosphate buffer at pH 6.5 and re-suspended in 200 mM phosphate buffer (pH 6.5). Dry cell weight of this suspension was determined by filtering an aliquot on cellulose-acetate filters (0.45 μm pore size) and drying the filters in a microwave oven for 15 min. The cell concentration was adjusted to 22 mg dry weight/mL by adding phosphate buffer. Glucose solution (10% w/v), concentrated ethanol, and 25 mL screw-capped bottles containing 12.75 mL of cell suspension were placed in an incubator or in a water bath at the desired temperature for temperature equilibration before the experiment was commenced. Appropriate amounts of ethanol and water and 5 mL of glucose solution were added to the yeast suspension to a total volume of 25 mL, so that concentrations of the buffer, glucose, and cells were 100 mM, 2% (w/v), and 11 mg dry cell weight/mL, respectively. The culture bottles were incubated for 70 min and samples taken at regular time intervals were put into Eppendorf tubes on ice and centrifuged immediately at 12500xg for 1 min at 4ºC. Supernatants were kept at -20ºC until HPLC analyses. Glucose, glycerol and ethanol concentrations were determined by HPLC using an organic acid analysis column (Phenomenex, Torrance, CA, USA), and a differential refractometer (Schambeck RI2000, Germany). The column was kept at 60ºC and was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The signal from the detector was processed by CCDS data acquisition software (Dizge Analitik, Ankara, Turkey).

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Modeling.

A mathematical model developed for fermenting, non-growing yeast cell in a previous study (Teusink et al. 2000) was used after some modifications:

1. Isomerization reaction between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P) catalyzed by triose phosphate isomerase (TPI) was included as reversible Michaelis-Menten in the model as in the work of Rizzi et al. (1997).
2. The assessment of glycerol branch was modified: The two-step process of glycerol formation was modeled as Cronwright et al. (2002), in which GLYC3PDH activity was simulated by using a reversible two-substrate, two-product rate equation with non-competitive inhibition and GLYCPASE activity by using irreversible noncompetitive inhibition kinetics.
3. Three more ordinary differential equations (ODEs) describing the time dependencies of glucose, ethanol, and glycerol were included. In addition, a transport step for glycerol diffusion was also included in the model.
4. "Volume effect" factor was involved in the model to quantify the dilution of ethanol and glycerol due to excretion to the extracellular medium and the concentration of glucose as a result of its uptake into the cells.
5. Instead of the ODE for free variable phosphate in the original, ATP, ADP and AMP were treated as separate variables and their concentrations were kept constant. As determined by Teusink et al. (2000), the concentrations of ATP, ADP, and AMP were taken to be 2.52, 1.32, and 0.25 mM, respectively.

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6. When the model was run for realistic initial glucose concentrations (i.e., 18%, w/v) as in wine fermentations, an almost linear increase of ethanol with time with an abrupt stop at a given concentration was predicted. During batch fermentation, on the contrary, the rate of ethanol production is maximal for the early period in the process and declines progressively as ethanol accumulates in the surrounding broth (Dombek and Ingram 1987). The inhibition effect of increasing ethanol concentration was considered to help the model to simulate the parabolic trend of ethanol accumulation in the medium.

Computational Methods.

Mathematical Expressions for Temperature and Ethanol Effects on Enzyme Activities.

Temperature effect on reaction rates was expressed by Arrhenius relation for the kinetic rate constant (Equation 1).

$$k_{cat} = A.e^{(\frac{-E_a}{R.T})} \qquad (1)$$

Here k_{cat} is the rate constant, A is the pre-exponential constant (frequency factor, Arrhenius constant), T is the absolute temperature (K), E_a is the activation energy (J/mol), and R is the gas constant (8.314 J/K.mol).

Ethanol effect on each enzyme activity was expressed mathematically in the form given below:

$$Residual\ activity = a - b.e^{c.[ETOH]} \qquad (2)$$

where residual activity was defined as the relative activity compared to the activity observed without ethanol. The data obtained for the changes in the *in vitro* activities of enzymes with increasing ethanol concentrations were fitted by the non-linear least squares method by

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using curve fitting tool of MATLAB 6.5. Residual activity relations derived against ethanol concentration were used to calculate the inhibition factor for each enzyme in the model. Since the ethanol effect on the hexose transport step was not determined experimentally, studies in literature were referred (Leao and Vanuden 1982).

Simulations.

MATLAB 6.5 was used for programing where ODE23s was selected as the ODE solver. The values of the kinetic parameters of enzymes and transporters and the specific activities (V_{max}) used in the simulations are given in Supplemental Tables 1, 2, and 3, respectively.

Results

Effect of Temperature on Enzyme Activities.

The specific activities of the glycolytic enzymes and glycerol and ethanol branch enzymes were measured at five temperatures in order to quantify the effect of temperature on the reaction rates. The enzymes were extracted from yeast cells grown at a single temperature (30°C), so that the factors other than the direct effect of temperature on the capacity, i.e. level of transcription, protein turnover, and post-translational modifications were excluded. In addition, the temperature was assumed to have a negligible effect on the binding affinities of the enzymes to their substrates (Cruz et al. 2012). It is well known that, temperature influences the rates of enzyme reactions positively up to a point where protein denaturation starts and for mesophilic range. This effect on the rates of reactions can partially be explained by Arrhenius relation for k_{cat}.

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The specific activity data obtained in *in vitro* assays under different temperatures were used to draw Arrhenius plots. For all enzymes of glycolysis and the glycerol and ethanol branches investigated in this study, the plots were linear and the activation energy value (E_a) for each enzyme was calculated from the slopes of the lines (Supplemental Fig 1). Fig. 1 shows that the E_a values, thus the temperature dependency of the enzymes, were different. Broadly, the enzymes of the upper part of the glycolysis and the glycerol branch showed increased temperature dependency compared to the lower part and the ethanol branch.

Effect of Temperature on Fermentation Kinetics.

The effect of temperature on the alcoholic fermentation kinetics was followed in short-term fermentations. Temperature values (10, 15, 20 and 30^{o}C) were selected considering the yeast-based alcoholic processes such as wine fermentations, brewing, and alcohol production. The yeast pre-grown at 30°C was cultured at the selected temperatures and the concentrations of the main extracellular metabolites, namely glucose, ethanol, and glycerol, were followed. The glucose consumption, and ethanol and glycerol production kinetics at four temperatures are shown in Fig. 2 (represented by markers). The fermentation was slow at 10^{o}C, whereas the consumption and production rates were increased with temperature. The yields of ethanol and glycerol on glucose after 70 min of fermentation were compared (Fig. 3, black markers). The temperature had a slight effect on the ethanol yield, on the other hand, glycerol yield increased notably with temperature. The divergence of carbon to glycerol formation resulted in a slight decrease in the ethanol yield.

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296 **Modeling of Fermentation Kinetics at Different Temperatures.**

297 In order to incorporate the effect of temperature on activities of glycolytic enzymes and
298 its branches to glycerol and ethanol to the modified model of Teusink et al. (2000), an equation
299 for each enzyme relating the rate at a certain temperature to the reference temperature (30°C in
300 our case), was derived from Arrhenius relation by the use of activation energies.

301 $$V^T = V^*.e^{\frac{-E_a}{R}(\frac{1}{T}-\frac{1}{T^*})} \qquad (3)$$

302 where V^T is the enzymatic reaction rate (mmol/L_{cyt}.min) obtained at temperature T (K), V^* is the
303 rate obtained at temperature T^* (K), E_a (J/mol) is the activation energy of the respective enzyme,
304 and R is the gas constant (8.314 J/K.mol). The activation energy of the hexose transporter was
305 calculated as 53.19 J/mol from the data of Reinhardt et al. (1997). Their data were re-assessed
306 according to one component Michaelis-Menten kinetics and E_a values were re-calculated.
307 Temperature changes for glycogen, trehalose, and succinate branches were assumed to be the
308 same with that of the hexose transporter.

309 The mathematical expression of ethanol effect on each enzyme activity was derived by
310 plotting the residual activity data obtained *in vitro* against the corresponding ethanol
311 concentration (Supplemental Fig. 2). The values of the parameters in Equation 2 were obtained
312 by non-linear least squares method as given in Supplemental Table 4.

313 The glucose consumption and the ethanol and glycerol productions at different
314 temperatures as predicted by the modified model were represented by lines in Fig. 2. Comparison
315 of the simulations with the experimental data shows that the model was generally successful in
316 predicting the short-term fermentation behavior of yeast.

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Discussion

The temperature dependence of the glycolytic enzymes has been the subject of investigation by several researchers. Tai et al. (2007), Postmus et al. (2008) and Cruz et al. (2012) have all reported that the temperature dependencies for the glycolytic enzymes are comparable and compatible and in the vicinity of 50 kJ/mol. In this work, we also report temperature dependencies similar to the ones reported before but with an important difference. Our results also showed activation energies in the bracket of 30 to 70 kJ/mol; however, according to our results, the enzymes leading to the glycerol branch and those enzymes leading away from the glycerol branch had significantly different E_a values. Namely, HXK, PGI, PFK, ALD, TPI, and GLYC3PDH had E_a values between 40 and 66 kJ/mol; while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward and backward enzymes and phosphoglycerate kinase (PGK) and phosphoglycerate mutase (PGM) had E_a values all smaller than 32 kJ/mol.

These results indicate that the rates of the reactions in the upper part were more sensitive to the changes in temperature. As a result, at higher temperatures, the increase in capacity should be higher in the upper part compared to the lower part. The higher the temperature, the more DHAP may be directed towards glycerol branch due to the insufficient increase in the capacity of the enzymes downstream compared to the ones upstream, so that glycerol accumulates more in high-temperature fermentations. This may explain the higher glycerol content in red wines than that in white wines, the former of which is fermented at comparably higher temperatures (Scanes et al. 1998). Similarly, being fermented at relatively higher temperatures, ale contains more

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337 glycerol than lager beer (Zhao et al. 2015). We suggest that this effect may be the biochemical
338 reason for this phenomenon.

339 Our results with short-term fermentations at four temperature values showed that glycerol
340 production was affected notably. The glycerol yield at 30°C is almost five times larger than at
341 10°C. This trend, a common observation in alcoholic fermentations at different temperatures,
342 agrees with the activation energy levels calculated for the glycolytic enzymes in this study.

343 The Teusink kinetic model (Teusink et al. 2000) was supported by the temperature
344 dependence of the enzymes and was used to simulate the changes in the rates of glucose
345 utilization and ethanol and glycerol formation kinetics in batch fermentations at various
346 temperatures. The *in silico* results showed qualitative accordance with the general trend in
347 alcoholic fermentation, in which the rates of glycerol formation increase with temperature. In
348 addition to that, the simulations were successful in estimating the data obtained in short-term
349 fermentations in this study (Fig. 2). Accordingly, increase in the glycerol yield with temperature
350 could be approximated by the model (Fig. 3). These indicated that metabolic modeling based on
351 the rates of individual enzymatic reactions determined *in vitro* could be a promising way for the
352 estimation of the overall behavior in batch alcoholic fermentations, such as the ones conducted at
353 different temperatures.

354 ## Conclusion

355 In summary, here we propose and provide experimental evidence that the biochemical
356 explanation for the glycerol accumulation at higher fermentation temperatures lies in the
357 thermodynamics and the kinetics of the glycerol branch point enzymes. It is shown that the

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358 temperature dependence of the enzymes that lead to the glycerol branch point, and to glycerol
359 formation is higher than the dependence of the enzymes in the lower part of the glycolysis. This
360 hypothesis was also tested and supported using a glycolytic model (Teusink et al. 2000) based on
361 the kinetics of the individual enzymes from literature, which was improved here by including the
362 temperature and ethanol effects on the apparent rates. The kinetic model in this improved form
363 can also simulate the time profiles of the extracellular glucose and ethanol in batch
364 fermentations.

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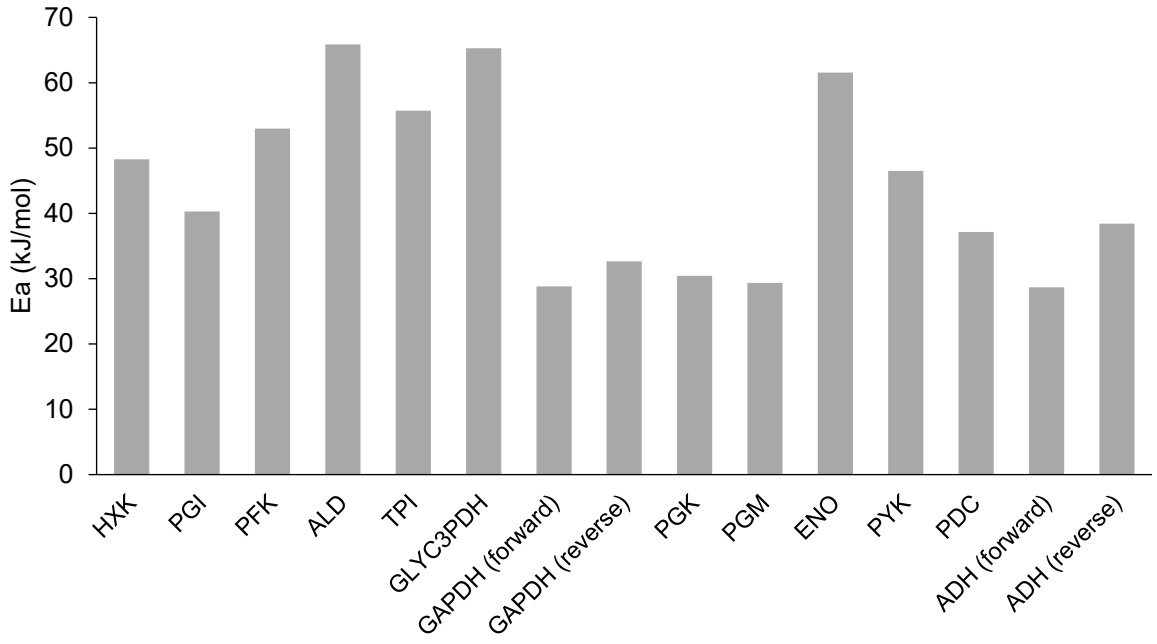

Figure 1 Activation energies of the enzymes of glycolysis and its branches to glycerol and ethanol. HXK: hexokinase, PGI: Phosphoglucose isomerase, PFK: Phosphofructokinase, ALD: aldolase, TPI: triose phosphate isomerase, GLYC3PDH: glycerol-3-phosphate dehydrogenase, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglycerate mutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase.

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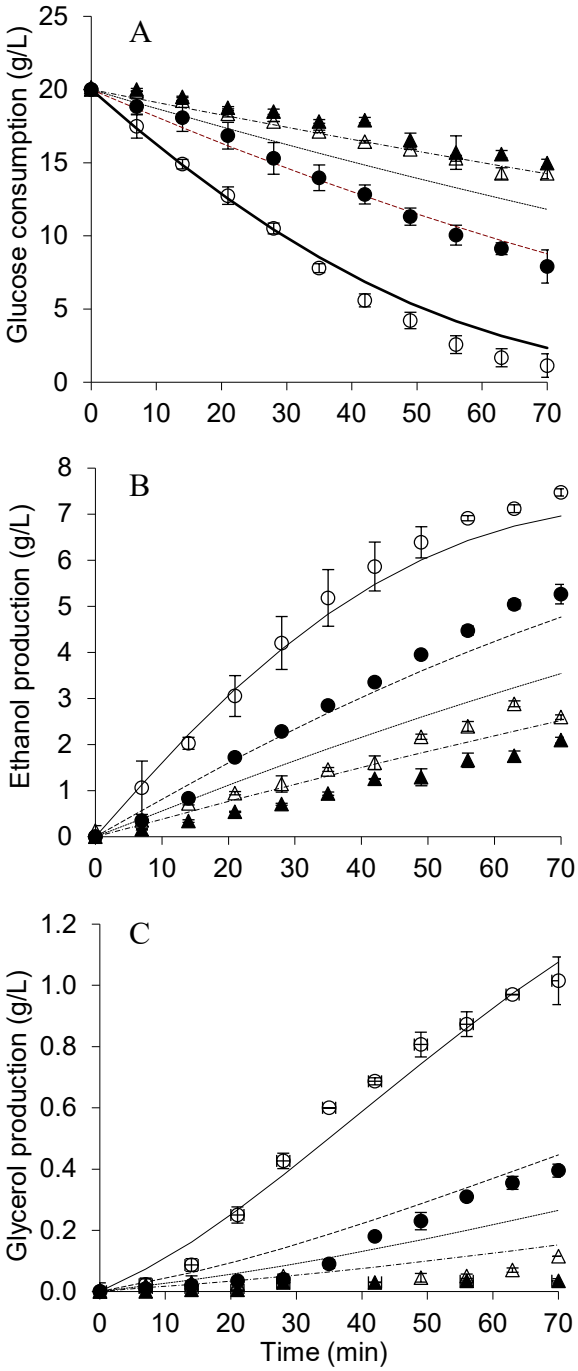

Figure 2 Experimental data (markers) and model simulations (lines) for the effect of fermentation temperature on glucose consumption (A) and ethanol (B) and glycerol (C) productions.
30°C: ○, ——;
20°C: ●, — — —;
15°C: Δ, – – – – –;
10°C: ▲, — · — ·.
Error bars represent standard deviations.

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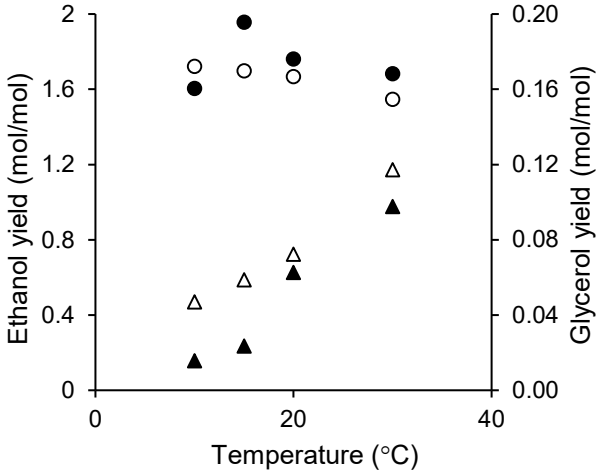

Figure 3 Effect of temperature on the molar yields of ethanol (●, ○) and glycerol (▲, Δ) based on the glucose consumed. Full markers: experimental; empty markers: simulation.

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Supplemental Table 1 Values of kinetic parameters used in the model.

Enzyme	**K_{eq}**	**K_a (mM)**	**K_b (mM)**	**K_p (mM)**	**K_q (mM)**	**K_i (mM)**	**K_{branch}**	**Other**
HXT (low affinity)	1[a]	55 (Glu_{out})[a]		55 (Glu_{in})[a]		0.91[e]		
HXK	3800	0.08 (Glu_{in})	0.15 (ATP)	30 (G6P)	0.23 (ADP)			
Glycogen branch							6	
Trehalose branch							2.4	
PGI	0.314	1.4 (G6P)		0.3 (F6P)				
PFK	**Table S2**							
ALD	0.069	0.3 (F16bP)		2.4 (DHAP)	2 (GA3P)	10 (GA3P)		
TPI[d]	0.045	0.38 (DHAP)		0.064 (GA3P)				
GLYC3PDH[b]	10000	0.2[c] (DHAP)	0.023 (NADH)	1.2 (Glyc3P)	0.93 (NAD)	*4.8 (F16bP)*[g] *0.73 (ATP)*[g] *2 (ADP)*[g]		
GLYC3PASE[b]		3.5 (Glyc3P)		1 (Pi)				1 (Pi)
GAPDH		0.21 (GA3P)	0.09 (NAD)	0.0098 (BPG)	0.06 (NADH)			
PGK (reverse)	3200	0.53 (G3P)	0.3 (ATP)	0.003 (BPG)	0.2 (ADP)			
PGM	0.19	1.2 (G3P)		0.08 (G2P)				
ENO	6.7	0.04 (G2P)		0.5 (PEP)				
PYK	6500	0.14 (PEP)	0.53 (ADP)	21 (PYR)	1.5 (ATP)			
PDC		4.33 (PYR)						1.9 (n)
Succinate branch							21.4	
ADH (reverse)	0.00001[f]	17 (ETOH)	0.17 (NAD)	0.11 (NADH)	1.11 (ACE)	90 (ETOH) 1.1 (ACE) 0.031 (NADH) 0.92 (NAD)		
ATPase							39.5	

All values are taken from Teusink et al. (2000) except:

[a]Teusink et al., 1998, [b] Cronwright et al., 2002, [c] Nader et al., 1979, [d] Rizzi et al., 1997, [e] "Interactive constant" Ki depends on the relative mobility of the unbound and bound carrier, [f] adjusted, original value is 0.000069, [g] F16bP, ATP and ADP are not used as effectors in the model.

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Supplemental Table 2 Values of parameters of PFK kinetics (Teusink et al., 2000).

	K_R (mM)	c	K (mM)	c_i	G_R	L_o
F6P	0.1	0				
ATP	0.71	3	0.65	100		
AMP			0.0995	0.0845		
F16bP			0.111	0.397		
F26bP			6.82×10^{-4}	0.0174		
Others					5.12	0.66

Supplemental Table 3 Specific activities determined *in vitro* at 30°C. V_{max} values of HXT and GLYCPASE are taken from literature.

Enzyme	Specific Activity (U/mg protein)	Specific Activity (mmol/L_{cyt}.min)
HXT[a]		163.7
HXK	1.7	452
PGI	1,78	473.3
PFK	0.69	184
ALD	1.26	334.7
TPI	25.87	6898.1
GAPDH (forward)	0.92	245.3
GAPDH (reverse)	6.3	1681.3
PGK	5.86	1561.3
PGM	9.99	2664
ENO	1.88	502.7
PYK	1.54	409.6
PDC	1.1	293.3
ADH (forward)	7.05	1880
ADH (reverse)	2.1	560
GLYC3PDH	0.16	41.6
GLYCPASE[b]		104

[a] One carrier model with low affinity from Teusink et al., 1998.

[b] Value taken from Cronwright et al., 2002.

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Supplemental Table 4 Values of parameters fitted for the effect of ethanol on enzyme activities.

Enzyme	a	b	c
HXK	1.0	33.29×10^{-6}	36.29×10^{-4}
PGI	1.16	15.97×10^{-2}	43.65×10^{-5}
PFK	1.06	57.63×10^{-3}	82.93×10^{-5}
ALD	1.0	32.03×10^{-5}	28.19×10^{-4}
TPI	1.01	10.78×10^{-3}	12.33×10^{-4}
GLYC3PDH	0	-1.0	-38.5×10^{-5}
GAPDH	0	-1.0	-53.65×10^{-5}
PGK	0	-1.0	-48.57×10^{-5}
PGM	1.33	33.23×10^{-2}	30.25×10^{-5}
ENO	1.3	30.1×10^{-2}	31.21×10^{-5}
PYK	1.02	19.27×10^{-3}	66.64×10^{-5}
PDC	1.997	1.0	99.2×10^{-6}
ADH (forward)	0	-1.0	-14.29×10^{-4}
ADH (reverse)	1.02	17.39×10^{-3}	10.52×10^{-4}

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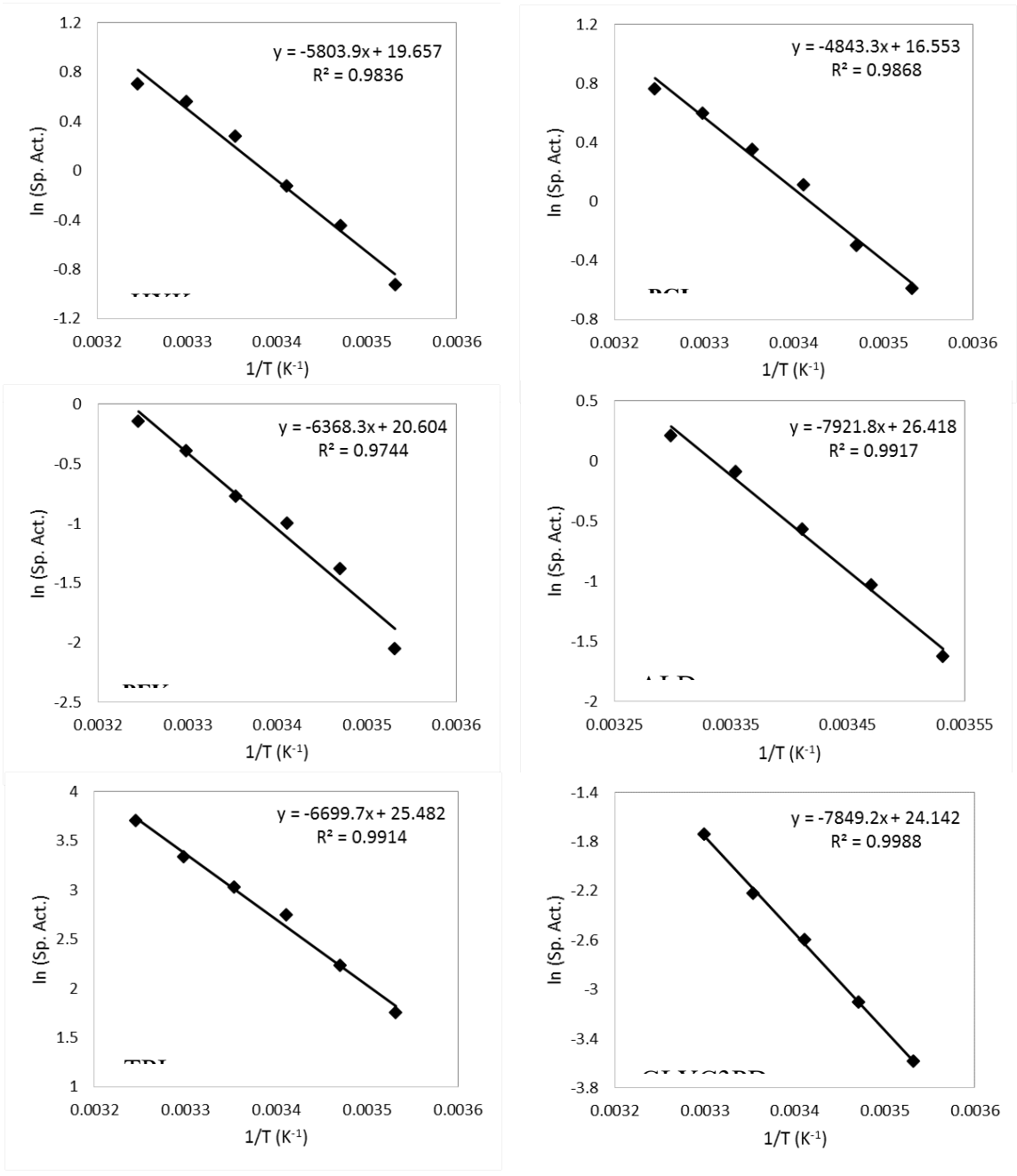
Supplemental Figure 1 Arrhenius plots of the enzymes of glycolysis and glycerol and ethanol branches. Plots were drawn using the specific activity data determined in vitro at 10°C, 15°C, 20°C, 25°C, 30°C and 35°C.



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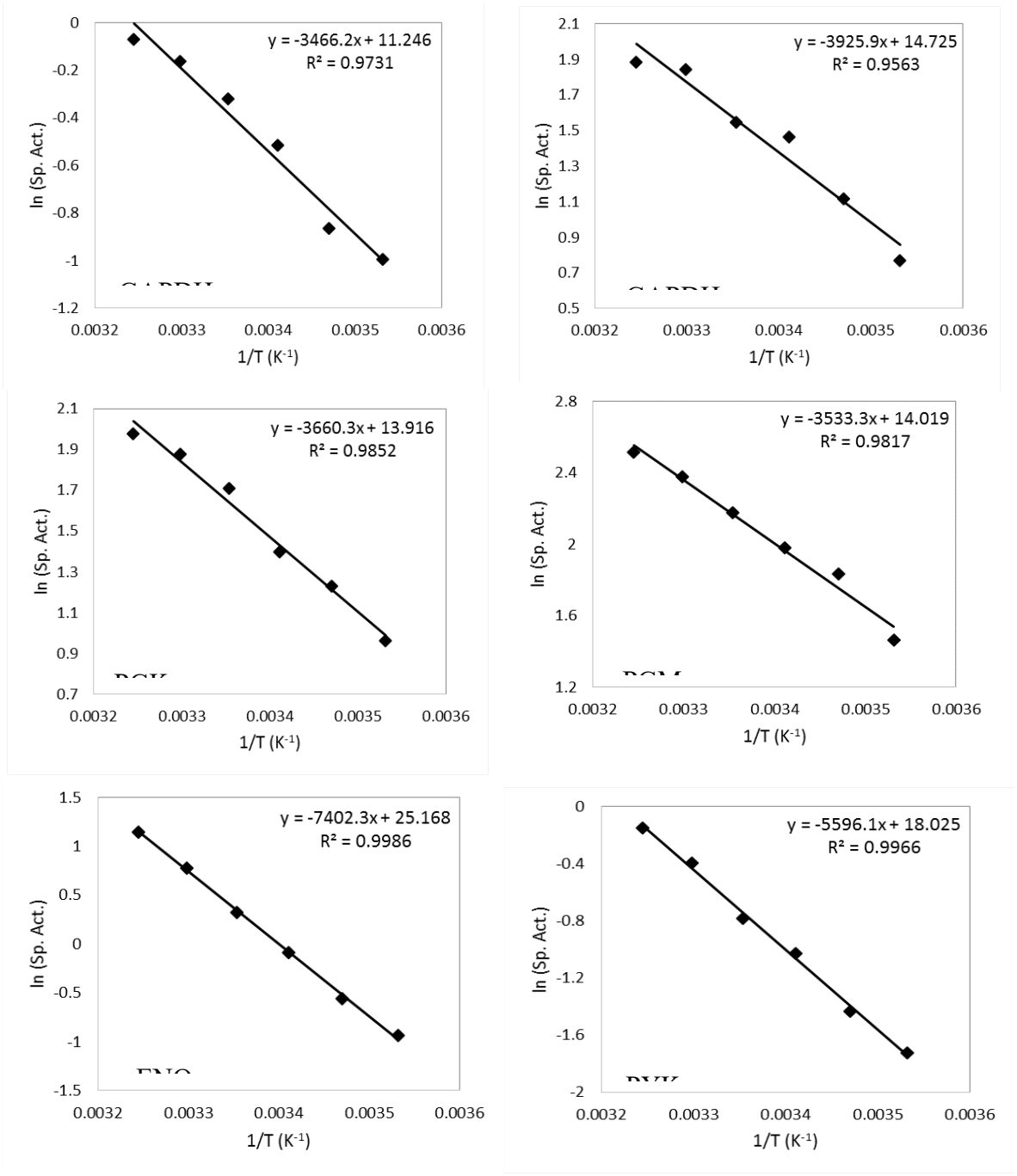

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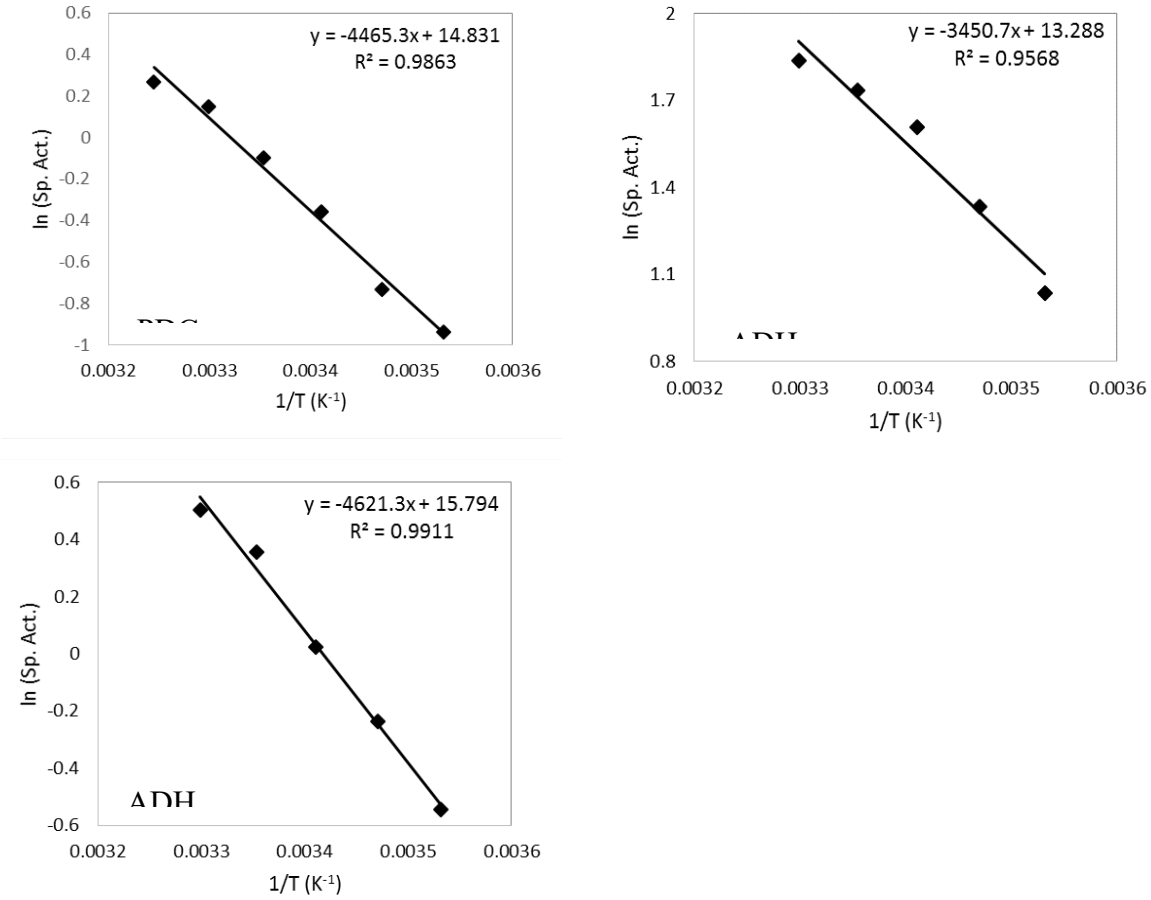

Supplemental Figure 1 Arrhenius plots of the enzymes of glycolysis and glycerol and ethanol branches. Plots were drawn using the specific activity data determined in vitro at 10ºC, 15ºC, 20ºC, 25ºC, 30ºC and 35ºC.

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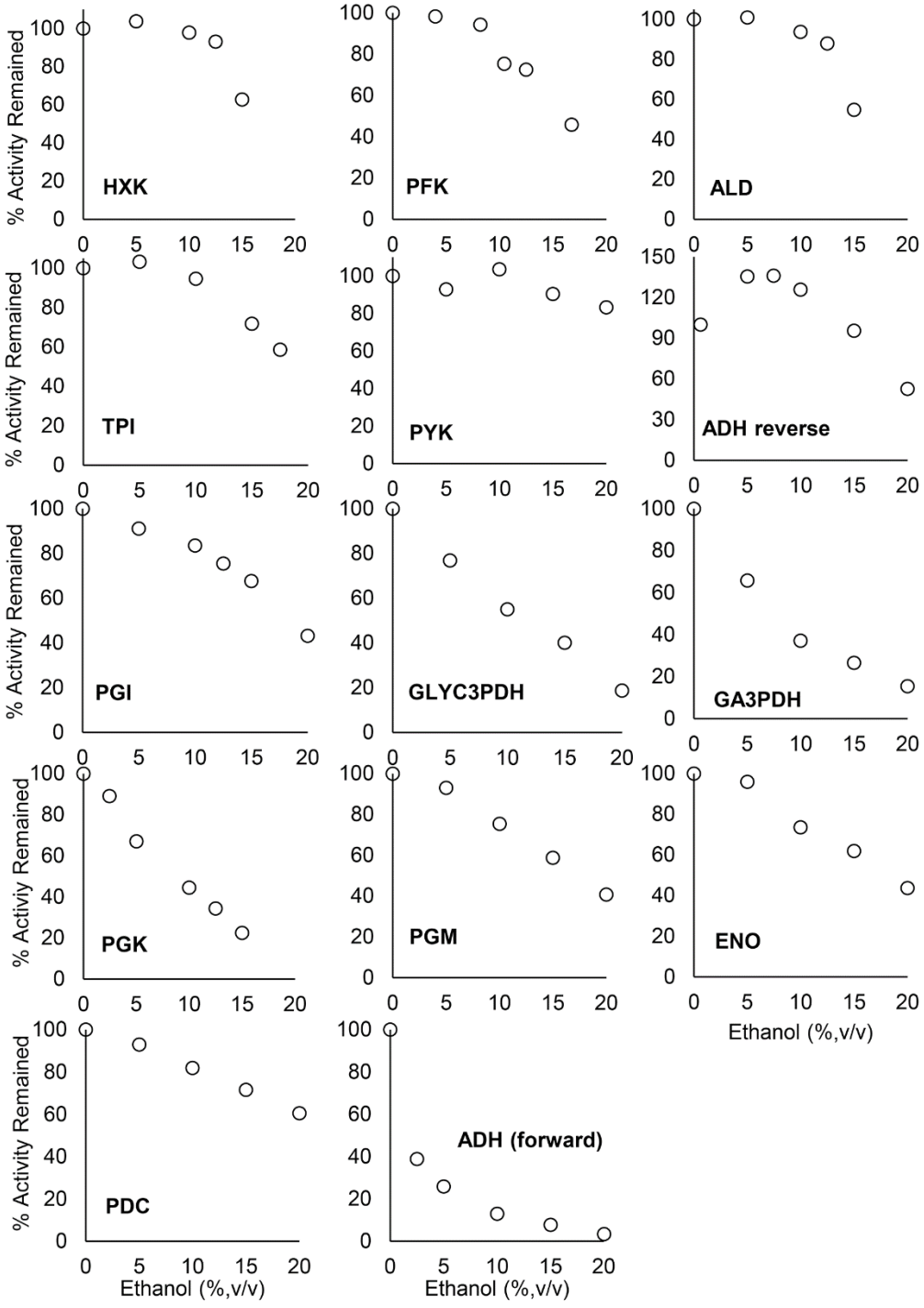

Supplemental Figure 2 Effect of ethanol on enzyme activities.

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APPENDIX

Enzyme Assays

Hexokinase

Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF).
Assay buffer: 50 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 0.2 mM $NADP^+$, 5 mM ATP, 2.8 U/mL G6PDH, and 10 mM glucose

Phosphoglucose isomerase

Phosphoglucose isomerase activity was measured in the reverse direction.
Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 0.2 mM $NADP^+$, 2.8 U/mL glucose6-phosphate dehydrogenase (G6PDH), and 2 mM fructose-6-phosphate (F6P)

Phosphofructokinase

Extraction buffer: 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$, 1 mM Dithioerythritol (DTE), and 1 mM PMSF.
Assay buffer: 70 mM PIPES buffer (pH 7.0) containing 5 mM $MgCl_2$
Reagents: 0.15 mM NADH, 1 mM ATP, 1 mM ADP, 0.1 mM fructose-2,6-bisphosphate (F26bP), 1.5 U/mL aldolase (ALD), 67.5 U/mL TPI, 2.5 U/mL GLYCPDH, and 5 mM F6P

Aldolase

Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 0.15 mM NADH, 50 U/mL TPI, 4.3 U/mL GLYCPDH, and 2 mM fructose-1,6-bisphosphate (F16bP)

Glyceraldehyde-3-phosphate dehydrogenase

For the forward direction:
Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$ and 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 2 mM NAD^+, 5 mM cysteine-HCl, 10 mM arsenate, 0.5 mM GA3PDH

For the reverse direction:

Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 0.15 mM NADH, 1 mM ATP, 0.9 mM EDTA, 0.2 mM DTE, 5 U/mL PGK (10 U/mL for ethanol effect), and 2 mM 3-phosph glycerate (3-PG)

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Phosphoglycerate kinase

Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 0.15 mM NADH, 1 mM ATP, 0.9 mM EDTA, 8 U/mL GAPDH, and 5 mM 3-PG

Phosphoglycerate mutase

Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 0.15 mM NADH, 1 mM ADP, 0.5 mM glycerate-2,3- bisphosphate, 0.9 mM EDTA, 14 U/mL LDH, 7 U/mL PYK, 0.95 U/mL enolase (ENO), and 2 mM 3-PG

Enolase

Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents: 0.15 mM NADH, 1 mM ADP, 0.9 mM EDTA, 14 U/mL lactate dehydrogenase (LDH), 7 U/mL PYK, and 0.2 mM 2-phospho glycerate (2-PG)

Pyruvate kinase

Extraction buffer: 100 mM PIPES buffer (pH 7.0) containing 10 mM KCl and 1 mM PMSF.
Assay buffer: 70 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 2 mM $MgCl_2$
Reagents: 0.2 mM NADH, 2 mM ADP, 1 mM F16bP, 10 U/mL LDH, and 2 mM phosphoenolpyruvate (PEP)

Triosephosphate isomerase

Extraction buffer: 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTE, 2 mM $MgCl_2$, and 1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 10 mM $MgCl_2$
Reagents: 0.15 mM NADH, 2.5 U/mL GLYC3PDH (5 U/mL for ethanol effect), and 0.8 mM DL- GAPDH (diluted with 10 mM K-PO_4 buffer at pH 7.0)

Glycerol-3-phosphate dehydrogenase

Extraction buffer: 10 mM triethanolamine (TEA) buffer (pH 7.5) containing 1 mM DTE, 1 mM EDTA, and 1 mM PMSF.
Assay buffer: 20 mM imidazole-HCl buffer (pH 7.0) containing 1 mM $MgCl_2$
Reagents: 0.1 mM NADH, 1 mM DTE, and 1.34 mM DHAP
Since TEA buffer interfered with Lowry method in protein determination, standard curve was prepared by inclusion of same amount of TEA that would come from crude. Therefore, specific activity of GLYC3PDH was calculated by protein amount calculated from this standard curve.

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Alcohol dehydrogenase

Extraction buffer: 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM DTE, and1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgSO_4$
Reagents:
Reverse direction: 2 mM NAD^+ and 100 mM ethanol
Forward direction: 0.15 mM NADH, and 5 mM acetaldehyde

Pyruvate decarboxylase

The effect of temperature on pyruvate decarboxylase (PDC) was investigated using a NADH linked assay like the other enzymes. For this assay extraction and assay buffers and reagents were as follows:
Extraction buffer: 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM DTE, and1 mM PMSF.
Assay buffer: 50 mM PIPES buffer (pH 7.0) containing 100 mM KCl and 5 mM $MgCl_2$
Reagents: 0.15 mM NADH, 0.2 mM thiamine pyrophosphate (TPP), 110 U/mL ADH, and 50 mM pyruvate (PYR)
The effect of ethanol concentration on PDC could not be investigated by NADH linked continuous assay since the most reasonable coupling enzyme for the assay, alcohol dehydrogenase was inhibited extremely by its product, ethanol. Therefore, a stop assay was developed, in which crude extract was incubated with the pyruvate and TPP, and ethanol, without the coupling enzyme alcohol dehydrogenase. Remaining pyruvate was measured enzymatically as described below.
The extraction and assay buffer used were the same as above. Pyruvate concentration was decreased to 10 mM in order to be measured accurately. Otherwise the percentage of the remaining pyruvate would be too high that sensitivity of the pyruvate assay would not be sufficient to detect the difference. With this concentration of pyruvate, activity was 30% lower than that of 50 mM. Similarly, relatively higher amount of crude extract (20-40 % of the assay volume) was used in the enzyme assay and incubation time was also longer (15 minutes). TPP concentration was also five times that of used in continuous assay. Assays were conducted in Eppendorf tubes containing 1 mL assay mixture in water bath at 30ºC. One hundred and fifty milliliter samples were taken from the assay at 5, 10 and 15 min. and mixed with the same volume of 500 mM EDTA to stop the enzymatic activity. Remaining pyruvate was determined enzymatically by measuring the oxidation of NADH while pyruvate is reduced to lactate by lactate dehydrogenase. Assay was conducted in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM NADH. After the sample was added absorbance at 340 nm was recorded and LDH (10 U/mL) was added to start the reaction. After the decrease in absorbance stopped the absorbance was recorded and subtracted from the initial absorbance. The difference was used to calculate the amount of pyruvate present initially, since the NADH and pyruvate were utilized stoichiometrically in equal amounts. Decrease in the pyruvate concentration was linear in the course of the assay (15 minutes), suggesting that enzyme was stable during the assay.