Improving ethanol production by studying the effect of pH using a modified metabolic model and a systemic approach

Sajjad Ghaffari Nasab[1] and Ehsan Motamedian[2]

[1]Tarbiat Modares University Faculty of Chemical Engineering
[2]Tarbiat Modares University

October 5, 2020

Abstract

pH is an important factor affecting the growth and production of microorganisms; especially, it is effective on the efficiency of ethanologenic microorganisms. It can change the ionization state of metabolites via the change in the charge of their functional groups that may lead to metabolic alteration. Here, we estimated the ionization state of metabolites and balanced the charge of reactions in genome-scale metabolic models of Saccharomyces cerevisiae, Escherichia coli, and Zymomonas mobilis at pH levels 5, 6, and 7. The robustness analysis was first implemented to anticipate the effect of proton exchange flux on growth rates for the constructed metabolic models at various pH. In accordance with previous experimental reports, the models predict that Z. mobilis is more sensitive to pH rather than S. cerevisiae and the yeast is more regulated by pH rather than E. coli. Then, a systemic approach was proposed to predict the pH effect on metabolic change and to find effective reactions on ethanol production in S. cerevisiae. The correlated reactions with ethanol production at predicted optimal pH in a range of proton exchange rates determined by robustness analysis were identified using the Pearson correlation coefficient. Then, fluxes of these reactions were applied to cluster the various pHs by principal component analysis and to identify the role of these reactions on metabolic differentiation because of pH change. Finally, 12 reactions were selected for up and down-regulation to improve ethanol production. Enzyme Regulators of the selected reactions were identified using the Brenda database and 11 selected regulators were screened and optimized via Plackett-Burman and 2-level full factorial designs, respectively. The proposed approach has enhanced yields of ethanol from 0.18 to 0.36 mol/mol carbon. Hence, not only a comprehensive approach for understanding the effect of pH on metabolism was proposed in this work, but also it successfully introduced key manipulations for ethanol overproduction.

1. Introduction

Almost all processes in microorganisms are dependent on pH, which is why intracellular pH in all cellular systems is a tightly regulated physiological parameter. pH can affect molecules of living organisms such as metabolites, protein folding and their functions, enzyme activity, and interactions of lipids (Orij, Brul, & Smits, 2011).*Saccharomyces cerevisiae* , *Escherichia coli,* and*Zymomonas mobilis* are the most widely used microorganism in bio-based productions such as ethanol (Joshi, Joshi, Bhattarai, & Sreerama, 2019; Monk et al., 2016; Valgepea et al., 2017; Yang et al., 2010). The desirable characteristics of these microorganisms needed for efficient production are to overcome many harsh conditions used in industrial ethanol production; for instance, low pH levels to reduce contamination risks (Benjaphokee et al., 2012; Kuroda et al., 2019). Hence, changes in pH levels may cause stressful conditions that affect cellular metabolism and ethanol production. These stress conditions change metabolite charges, redox balances and the gene expression profile by activating or repressing specific genes involved in different reaction pathways, such as the central metabolic pathway, transcription regulation, protein folding, and cell cycle (Dong, Hu, Fan, & Chen, 2017). The efficiency of the metabolism pathways to produce ethanol depends on the redox control. Alteration in intracellular pH can change NAD: NADH ratio, which must be redox balanced through an electron shunt.

This electron transfer has enabled the production of ethanol (Contador et al., 2015; Williams-Rhaesa et al., 2018). *Z. mobilis* and*S. cerevisiae* are ethanologenic microorganisms and are also sensitive to pH, while *E. coli* is a neutralophilic bacteria that maintain its cytoplasmic pH within a restricted range (Jones & Doelle, 1991; Krulwich, Sachs, & Padan, 2011; Shioi, Matsuura, & Imae, 1980; J. L. Slonczewski, Rosen, Alger, & Macnab, 1981; Joan L. Slonczewski, Fujisawa, Dopson, & Krulwich, 2009). Thus, it is vital to investigate different behavior and effect of charge balance due to pH change on the metabolism of these microorganisms.

In order to develop a productive and robust desirable bio-based producer, some researchers have recently used a constraint-based modeling approach to model pH variability in bio-systems, based on the fact that cell activity is restricted by controlling physiochemical constraints (Edwards, Covert, & Palsson, 2002; Reed, Vo, Schilling, & Palsson, 2003). There have been many clarifications in different ways, including the effects of pH on metabolic flux distributions (Çalik & Ileri, 2007), a shift in carbon flux by pH alteration (Jo, Lee, & Park, 2008), thermodynamic feasibility of metabolic pathways due to pH (Vojinović & Von Stockar, 2009). Metabolic models were used to predict the impact of pH upon variation in cell activity. Anderson et al. (Andersen, Lehmann, & Nielsen, 2009) used constant acid disassociation to model acid production at pH 1.5 ~ 6.5. Effects of pH on the capability of *R. eutropha* to produce poly[R-(-)-3hydroxybutyrate] (PHB) was investigated under pH 6, 7, and 8 values by Park et al. (Park, Kim, & Lee, 2011). In these studies, a metabolic network involving varied biochemical reactions was used to construct a metabolic model based on constraints using a pseudo-steady-state assumption with mass and charge balance on each metabolite and reaction (Swayambhu, Moscatello, Atilla-Gokcumen, & Pfeifer, 2020). Therefore, the proton exchange rate can indicate the charge balance of the network in genome-scale metabolic models. Examining the interaction of proton exchange flux and ethanol production on growth can lead to a better understanding of metabolism associated with the charge balancing and metabolite charge alteration as a result of pH variations. The impact of pH on growth and ethanol production has not yet been evaluated with genome-scale metabolic models of microorganisms.

In this research, using genome-scale models of *S. cerevisiae, E. coli,* and *Z. mobilis* that their charge balance has been modified at various pH values (5, 6, and 7), the impact of metabolite charge alteration because of the pH change on the proton exchange rate and ethanol production was investigated to evaluate the metabolic differences of these microorganisms under different pH levels. Furthermore, a systemic approach has been proposed to identify reactions affecting ethanol production rates at various pH levels. Moreover, comparing the flux distributions of *S. cerevisiae* at optimal pH=5 (Papapetridis et al., 2016) with other pH levels can indirectly lead to the identification of the optimum flux distributions for ethanol production. Hence, this approach offers strategies for improving ethanol production that was experimentally evaluated on *S. cerevisiae* by adding inhibitors or activators of enzymes as regulators to the medium (Ehsan Motamedian, Sarmadi, & Derakhshan, 2019). Finally, the design of experiment (DOE) was implemented to maximize each selected compound's concentration.

2. Materials and methods

2.1. Genome-scale metabolic models

In this study, the genome-scale models of iMM904 (Mo, Palsson, & Herrgård, 2009), iEM439 (E Motamedian, Saeidi, & Shojaosadati, 2016), and iJO1366 (Orth et al., 2011) for *S. cerevisiae, Z. mobilis,*and *E. coli* were used, respectively. The intracellular reversible reaction fluxes were constrained between -1000 to 1000 mmol/gDCW/h, while intracellular irreversible reaction fluxes had zero lower limits. For the simulation of growth on minimal glucose media by the three metabolic models, lower bounds of exchange reactions were set to zero except for glucose, NH_4, H_2O, SO_4, O_2, H^+, phosphate and inorganic ions exchange reactions. Growth on glucose was simulated by the maximum uptake rate of 10 mmol/gDCW/h. Flux balance analysis (FBA) was used to simulate the growth, and the biomass reaction was used as an objective function to be maximized in all *in silico*experiments. MATLAB (R2017b) was used for modeling using the COBRA Toolbox, and the GLPK (GNU Linear Programming Kit) package was used to solve linear programming problems. The reconstructed metabolic models were presented at pH=7 and so, the metabolic models were modified at other pH levels according to the method presented in the next section. In this research, pH levels

of 5, 6, and 7 were selected for simulation based on the reported intracellular pH range for *S. cerevisiae*(Valli et al., 2005), *Z. mobilis* (Kalnenieks, Pankova, & Shvinka, 1987), and *E. coli* (Martinez et al., 2012). The unrealistic intracellular pH level of 5 for *E. coli* only was also assumed for the intracellular medium of the three cells to study the effect of high acidity.

2.2. Modification of genome-scale metabolic models

One of the important effects of the variation in the intracellular pH is the change of the ionization state of metabolites and metabolites contain certain particular functional groups that can lose or obtain protons at various pH levels. To modify the metabolic networks at different pH values, Marvin view software 19.2, 2019, developed by ChemAxon (http://www.chemaxon.com) was used to estimate the charge of metabolites based on their pKa for intended pH value. The mol files of each metabolite, which are generally classified as data files in plain text format containing molecular details, atom, bonds, co-ordinates, and communication detail, were obtained from Chebi (https://www.ebi.ac.uk/chebi) and PubChem (https://pubchem.ncbi.nlm.nih.gov/) databases.

The change of metabolites charge can result in the disruption of the charge balance of reactions and so, the charge balance of the reactions was automatically checked at different pH levels according to the method presented in Figure 1. Initially, the proton was removed from each reaction in the case of presence. Then, charges of the metabolites at the right and left side of each reaction were separately summed, and the total charge of the right side was subtracted from the left side. If it was positive (negative), the proton with the coefficient of the subtracted value was added to the left (right) side of the reaction. The reaction was left unchanged if the subtracted value was zero. The charge balance of transport reactions was not changed because the proton is mechanistically added to the reactions (e.g., simple transport). The charge of the exchange reactions was not changed because they are pseudo reactions that exchange metabolites between the cell and the environment. The accuracy of the method was evaluated by comparing the modified model with the original model at pH=7, and the charge balance of some reactions was not changed due to the fact that these reactions exchange protons between subsystems to transfer materials (e.g., mitochondria to cytosol). *S. cerevisiae, Z. mobilis,* and *E. coli* contained 8, 2, and 6 kinds of these reactions, respectively. These reactions were balanced separately, which are shown in Table S1 and S2.

By changing metabolite charges at different pH levels, the charge balance of each reaction would be changed, which affects in proton exchange rates. To determine the sensitivity of growth to the proton exchange rate at different pH levels, developed models could be analyzed with robustness analysis which can assess the optimal value of the objective function by varying the flux of a reaction and indicating the sensitivity of the objective function to changes in a particular reaction. In this regard, the sensitivity of cell growth to proton exchange has been assessed via robustness analysis. Mat files of modified metabolic models are also provided in Supplementary File 3.

2.3. A systemic approach to identify key reactions for ethanol production

In this research, a systemic approach was applied for *S. cerevisiae* to find candidate reactions for up and down-regulation for the overproduction of ethanol. As *S. cerevisiae* is the main industrial ethanologenic microorganism and the production rate of ethanol is dependent on pH, we assumed that by recognizing the key reactions for ethanol production at optimal pH, not only can we maintain the cell at optimum pH, but also increasing ethanol production indirectly.

Initially, the optimal pH level for ethanol production was identified based on a double robustness analysis determining the sensitivity of growth to proton exchange and ethanol production fluxes. Then, maximum ethanol production and flux distribution at optimal growth in the selected range of proton exchange rate for the desired pH level were determined. The absolute Pearson correlation coefficient of 0.95 (p-value of 0.05) between the flux of each reaction and ethanol production rate was used to determine key reactions coupled with ethanol production for optimal growth at the desired pH. If the coefficient was positive (negative), the reaction was selected for up (down) regulation. The Pearson correlation coefficient (between the flux of each key reaction and growth rate) was also applied to determine growth associated reactions. For evaluation

of the role and importance of each key reaction on metabolic differentiation, fluxes of the key reactions at pH levels of 5, 6 and 7 were applied to cluster by PCA. Figure 2 indicates the flowchart of the systemic approach for finding the key reactions for ethanol overproduction and identification of the effect of pH on the metabolism.

2.4. Experimental materials and method

2.4.1 Statistical approaches for optimization

To evaluate the applicability of the systemic approach for the metabolic engineering of microorganisms, the regulatory defined medium was designed for *S. cerevisiae* to remove intracellular constraints through recognized candidates (Ehsan Motamedian et al., 2019). Activator and inhibitor compounds of enzymes were obtained from the BRENDA database for up and down regulations, respectively and the regulatory effect of these compounds has been confirmed empirically. Each regulator was added separately to the medium, and its impact on the production of ethanol was evaluated experimentally. Moreover, DOE was used to screen and maximize the concentration of each fruitful compound that was applied. Plackett-Burman design is beneficial in screening from a long list of compounds, because of fewer required runs. This approach suggests that the main impacts will be much higher than the interactions between two factors. Hence, this methodology can be used to identify the most significant independent variables for the optimization stage. The experimental data analysis was conducted using Design Expert® software version 7.0 (STAT-EASE Inc., Minneapolis, USA). As shown in Table S3, each independent variable was evaluated at two levels, a high (+) and a low (-) level. The concentration ranges of the chosen compounds were determined by experimental studies based on the literature review. The ranges should be neither short nor wide in order to represent the effect of changing factor's value appropriately. It is worth noting that for a proper comparison, the ethanol concentration assessments were conducted when the glucose concentration was depleted to zero.

In the present study, the significant factors identified in the Plackett-Burman experiment were employed in a full 2-level factorial design. The approach is ideally suited for considering the effects of interaction among the variables that affected the response based on the contribution percentage of the evaluated variables, and it generally works well for optimizing the process. The optimal conditions were predicted and assessed for maximum ethanol production obtained from 8 experiments using Design Expert$^{(r)}$ software version 7.0. The actual and coded values of factors are shown in Table S4. Variables that significantly affected the production of ethanol were determined using a confidence level above 95% or a p-value below 0.05. The data are statistically evaluated by analysis of variance (ANOVA).

2.4.2 Microorganism and culture media

The industrial yeast strain *S. cerevisiae* (obtained from Bidestan Alcohol Production Company, Iran) was used in this study. This strain is commonly used in the production of alcohol and can be used as active dry yeast. The cultures were maintained on YPD agar plates containing Dextrose (2%, w/v), peptone water (1%, w/v), yeast extract (0.5%, w/v), and agar (2%, w/v). The seed culture also was prepared by the YPD medium containing (20 g L^{-1} dextrose, 10 g L^{-1} yeast extract, 20 g L^{-1} peptone water) at 30degC and 180 rpm. The minimal medium including Dextrose, 20 g/l; KH_2PO_4, 13g/l; K_2HPO_4, 2.7g/l; $(NH_4)_2SO_4$, 5g/l; K_2SO_4, 2g/l; $(MgSO_4).7H_2O$, 2.5g/l; NaCl, 0.1g/l; $CaCl_2.2H_2O$, 0.35g/l; H_2SO_4, 1 ml; 4.5 ml of trace elements solution including $FeSO_4.7H_2O$, 2g/l; $MnSO_4.H_2O$, 0.3g/l; $CoCl_2.6H_2O$, 0.05g/l; H_3BO_3, 0.002g/l; $CuSO_4.5H_2O$, 0.6g/l; $ZnCl_2$, 1g/l; KI, 0.008g/l; $MoNa_2O_4.2H_2O$, 0.002g/l; Biotin, 0.04g/l was used for the cultivation. 47 ml of minimal medium was inoculated with 3 ml of seed culture at the mid-exponential phase at 30degC, 150 rpm, and the initial measured pH of the medium was adjusted to 5. The chosen compounds were added to the minimal medium in compliance with the Brenda database concentrations. Concentrated solutions of these compounds have been separately sterilized by autoclaving them at 121degC for 15 min.

2.4.3 Analytical methods

Biomass formation has been measured at specific time points by assessing the optical density (OD_{600} nm) using a spectrophotometer (Cary50Conc, UV–vis Spectrophotometer, Australia). For the determination

of ethanol and glucose concentrations, the main culture was harvested by centrifugation (6000xg, 12 min, 4degC), and the supernatant was analyzed via G.C. The Glucose concentrations were measured enzymatically using a colorimetric glucose oxidase kit (Pars Azmun, Iran).

The concentration of ethanol was determined by using gas chromatography GC-2550TG (Teif Gostar Faraz Company, Iran), Hydrogen gas as the carrier gas and the injection, column and flame ionization detection (FID) temperature was maintained at 120degC, 250degC and 250degC respectively. Ethanol (99/9%, Merck) was used as the internal standard.

3. Results

3.1. Evaluation of modified genome-scale metabolic models at different pH levels

Figure 3 demonstrates the effect of proton exchange flux on the growth rate of *Z. mobilis* , *S. cerevisiae,* and *E. coli* . As the steady-state condition is assumed by FBA, the amount of charge entering the cell has to be equivalent to the leaving amount; thus, protons are consumed or produced for the charge balance. It can be inferred from Figure 3 that *Z. mobilis* is more sensitive to pH rather than *S. cerevisiae* and the yeast is more regulated by pH rather than *E. coli* . *E. coli* is a neutralophilic bacterium that can maintain its cytoplasmic pH in a narrow range of ˜7.5–7.7 and can grow at extracellular pH values from ˜5.5–9.0 (Martinez et al., 2012). However, the influence of extracellular pH on intracellular value is significantly more for acidophilic microorganisms *Z. mobilis* and *S. cerevisiae* . By decreasing the external pH from 7.0 to 2.2, a progressive reduction of the internal pH from 7.1 to 5.1 was observed in exponentially grown cells (Valli et al., 2005). Experimental data (Kalnenieks et al., 1987) showed that the reduction of extracellular pH of *Z. mobilis* from 5.6 to 3.5 would change the intracellular pH from 6.4 to 5.75.

A comparison of the results shows that *Z. mobilis* is the most sensitive microorganism to proton exchange and Figure 3a indicates that this bacterium is only capable of producing protons. The charge balance of this microorganism is merely possible to the proton production rate of approximately 6 mmolgDCW^{-1}h^{-1}. This range is small in comparison with two other microbes and indicates the less flexibility of *Z. mobilis* metabolism to pH changes. Furthermore, the proton exchange range for optimal growth of *Z. mobilis* is in the limited amount of 0.5 to 1 mmolgDCW^{-1}h^{-1}. *Z. mobilis* is an ethanologenic bacterium that prefers the maintenance of intracellular pH rather than more growth. Motamedian et al.(Kalnenieks et al., 1987; E Motamedian et al., 2016) expressed that more proton exchange leads to further growth only when growth and energy are coupled. So, the low flexibility of *Z. mobilis* metabolism to pH changes and its tendency for the maintenance of intracellular pH can justify its energy-uncoupled growth.

Comparison of Figures 3b and 3c indicates that the metabolic sensitivity of *S. cerevisiae* is more than *E. coli* . Similar to *Z. mobilis* , *S. cerevisiae* also mostly produces protons and the optimal growth of *S. cerevisiae* is accompanied by proton production in the limited range of proton exchange rates, which is near to zero. It is evident that as the internal pH drops to acidic condiction, charges of metabolites would lead to positive numbers (Figure S1). Given that the steady-state assumption, it is expected that proton is produced in optimal growth condition, which can be seen in the robustness analysis of *S. cerevisiae* and *Z. mobilis* since glucose as the carbon source has no charge and ammonium with positive charge can be the determinant substrate for charges entering the cell. However, *E. coli* has the opposite behavior by consuming protons, as the internal pH drops and at pH=5, the optimal proton consumption rate for the growth of *E. coli* is equivalent to -10 mmolgDCW^{-1}h^{-1} the biomass charge will be more positive in acidic pH (Figure S2). This effect of the biomass charge can also be seen in pH=7 for *Z. mobilis* and*S. cerevisiae* , which biomass charge has a noticeable difference with other pH levels (Figure S2).

Figures S3-8 demonstrate another metabolic similarity of *S. cerevisiae* and *Z. mobilis* in which optimal growth rate is associated with maximum ethanol at all pH levels while the results for*E. coli* is not monotonic (Figure S9-11). In this regard, *S. cerevisiae* and *Z. mobilis* have more in common as they are known as industrial ethanologenic organisms with an ethanol yield of 0.41 and 0.5 g/g (Table S5). However, Maximal ethanol production under optimal growth for *E. coli* is obtained for pH=6 and this maximal value for pH=7 is more than that for pH=5. The experimental data also confirm that the optimal pH for ethanol production

by *E. coli* is 6.5. Besides, the lowest yield of ethanol production is reported for *E. coli* 0.09 g/g, while this bacterium has the highest growth yield, according to Table S5. Bekers et al.(Bekers, Heijnen, & Van Gulik, 2015) experimentally evaluated the amount of NAD: NADH ratio assuming a decrease in intracellular pH would increase this ratio, which means more NADH consumption and higher ethanol production. *Z. mobilis* and*S. cerevisiae* are the acidophilic microorganisms and capable of more ethanol production than *E. coli* , which is the neutrophilic bacteria. In fact, cells with the capability of higher ethanol production showed tightly regulated metabolism and responsiveness to pH changes due to the imbalance of NAD: NADH ratio. Consequently, *Z. mobilis* , which converts 95% of its carbon source into ethanol and CO_2, then *S. cerevisiae* and *E. coli* are more regulated by pH, respectively.

Despite the similarity of the metabolism of *S. cerevisiae* and*Z. mobilis* , *S. cerevisiae* is more flexible and its sensitivity to proton exchange rate reduces as the internal pH decreases (Figures 3a and 3b). The metabolic models predict that pH=5 is the most appropriate level for growth and ethanol production (Figures S6-8). Sensitivity to proton production rate at this pH level disappears and maximal ethanol production in the wide range of proton production rates under optimal growth rate is possible. Experimental data confirm the predicted optimal pH for *S. cerevisiae* and Narendranath et al. (Narendranath & Power, 2005) stated that the ideal pH for *S. cerevisiae* is at the optimum enzyme activity of that product, which is 5-5.5 for the production of ethanol and yeast growth. Furthermore, the growth yield (Table S5), growth rate (Figures 3a and 3b) and tolerance to environmental stresses of *S. cerevisiae* are significantly higher than *Z. mobilis* . These characteristics have made *S. cerevisiae* the main strain for ethanol production in the industry.

3.2. Applying the systemic approach for improving ethanol production of *S. cerevisiae*

To find candidate reactions for *S. cerevisiae* , the correlated reactions with ethanol production at pH=5 were identified using the Pearson correlation coefficient (between fluxes of each reaction and ethanol production) in the range of proton exchange rate between -2 and 23 mmolgDCW^{-1}h^{-1}. This range was determined based on robustness analysis (Figure 3b), considering that the metabolic model of pH=5 was robust for growth compared to the two other models and maximum ethanol is produced at optimal growth in this range (Figure S8). So, flux distributions at optimal growth in the selected range were determined and correlated reactions with ethanol production were determined (Supplementary File 2). These 252 reactions are key reactions that indicate the manner of using metabolism for maximal ethanol production during optimal growth. 67 reactions with positive coefficient are candidates for up-regulation and 185 with negative coefficient are predicted for down-regulation. The Pearson correlation coefficients between these reactions and growth indicate that the correlated reactions with ethanol production are non-growth associated and vice versa (Supplementary File 2). So, the correlation coefficients predict that up and down-regulation of candidate reactions for overproduction of ethanol results in growth reduction(Naghshbandi et al., 2019; Pagliardini et al., 2013).

For more screening of the key reactions and determining the important genes affected by the pH change, PCA was performed using fluxes of the key reactions at pH levels of 5, 6 and 7. Figure 4a illustrated a clear distinction between pH=5, and the other three pH models were achieved based on the first PC. Only the first two components have been considered as they demonstrate the high percentage of variation between pH=5 and other pH levels: 90.18% for PC1 and PC2 (Figure 4b). The decomposition of data by PCA indicates the contribution of each correlated metabolic reaction to the differentiation of models at various pH values. Among the 252 key reactions, 12 reactions including ILETA, ME2m, PDHm, ICDHyr, CSm, ACONTm, DESAT16, NDPK1, THRD_L, ICL, AGTi, and MDH were essential for the discrimination of pH models based on the PCA results presented in Figure 4c. These reactions were selected to evaluate the effects of regulators on their enzymes on ethanol production. Table S6 provides information for these 12 reactions in detail.

It can be seen that various approaches have been suggested by the systemic approach for ethanol overproduction. Figure 5 shows the comprehensive connection of each proposed reaction, which is suitable for up-regulation or down-regulation. For instance, through observing the model reactions, mitochondrial pyruvate is not capable of converting to acetaldehyde and must enter the cytosol for conversion to ethanol. Thus,

according to the PDHm reaction (Table S7), coenzyme A reacts with the mitochondrial pyruvate to produce acetyl coenzyme A. The product of this reaction is then converted to citrate by the CSm reaction and the produced citrate is responsible for providing isocitrate via ACONTm reaction. Cytosolic isocitrate is generated by mitochondrial isocitrate from the CITtcm and then converted to 2-oxoglutarate by the ICDHyr reaction. This metabolite is further produced to cytosolic pyruvate by the ALATA_L reaction, which leads to acetaldehyde by PYRDC. Eventually, acetaldehyde is reduced to ethanol by the ALCD2ir.

The predicted redirecting flux to the TCA cycle under acidic conditions is expected. Isocitrate dehydrogenase (ICDHyr), citrate synthase (CSm), Aconitate hydratase (ACONTm), which were ethanol-associated (or non-growth associated) reactions and Malic enzyme NADP mitochondrial (ME2m), with growth correlated coefficient (or non-ethanol associated), were the identified candidate reactions in TCA. The predictions of the systemic approach were in compliance with fluxes determined by experimental data, where Blank et al.(Blank & Sauer, 2004) showed that malic enzyme and mitochondria were more active under acidic conditions, as was clear from metabolic reactions located at crucial branch points in central metabolism. Furthermore, previous studies have supported the predicted effects of some reactions. These were the bottleneck reactions for both growth and ethanol production at pH=5. Isocitrate dehydrogenase (ICDHyr) reaction, which is encoded by IDP2, converts isocitrate to ∝-ketoglutarate, Scalcinati et al.(Scalcinati et al., 2012) showed that overexpression of IDP2 has resulted in an increased NADPH production to supply the required energy for biomass and yeast growth. The alanine glyoxalate aminotransferase (AGTi) reaction, which encoded by AGX1, converts glyoxalate and alanine to glycine and pyruvate. Chidi et al.(Chidi, Rossouw, & Bauer, 2016) experimentally confirmed that the deletion of AGX1 increased pyruvic acid, which led to improved ethanol production.

3.3. Identification of regulators for the selected reactions

In this section, new compounds have been added to the medium, not only as a nutrient but also as a regulator to maintain the flux distributions at the optimum pH level. To assess the model's power to detect unknown yeast bottleneck reactions, we have used the Brenda database to identify activators/inhibitors for the target reactions (Table S8). The available BRENDA activators and inhibitors have been applied separately to the medium for the assessment of each efficient regulator. The concentrations (extracted from BRENDA) and the effects of each regulator on ethanol yield are shown in Figure 6 and providing information on glucose depletion time and yeast growth were presented in Table S3, 8. In order to precisely evaluate the regulatory effect of each compound, ethanol yield production was assessed as moles of produced ethanol per one carbon mole of all added carbon sources in the medium. The control sample yield (without any regulator) was 0.18 mole ethanol/mole carbon equals to 5.5 g/l ethanol. We experimentally tested 15 regulators and 11 compounds were selected for more evaluation, including EDTA, glycerol, sodium acetate trihydrate, and cysteine as the activator and glycine, ammonium chloride, imidazole, zinc chloride, calcium carbonate, magnesium sulfate, and zinc sulfate as the inhibitor. The other four remaining compounds (including copper (II) chloride, copper (II)chloride dehydrate, phenanthroline monohydrate, silver nitrate) were inhibited growth and were excluded from optimization experiments.

3.4. Screening and optimization of the selected regulators

The 11 selected regulators were evaluated using a Plackett-Burman design for their effects on the yield of ethanol mol/mol carbon (Table S9). Table S9 demonstrates that the design matrix is chosen to screen significant variables for ethanol production and the corresponding response. The model appropriateness was assessed, and the variables indicating statistically significant impacts were screened via their p-values of ANOVA. Factors with confidence levels above 85% ($P < 0.15$) were considered to have significant effects and were therefore selected for further optimization studies. Imidazole was estimated to be the most significant factor, with a probability value of 0.0008, followed by glycine (0.04), and glycerol (0.13). The other insignificant variables were dismissed, and 2-level full factorial design then calculated the optimum values for the three variables. We evaluated the eleven best-anticipated regulatory interactions, including two regulators with a strong in vitro impact in *S. cerevisiae*with no prior evidence for their regulatory significance. The majority of these regulators had not been already proposed in *S. cerevisiae* .

To identify the right combination of regulators for optimal ethanol production, the concentration of the aforementioned variables has been optimized. In this study, a 2^3 two-level full factorial design was used for three independent variables at two levels each, resulting in 8 experiments. Table S4 presents the full experimental plan regarding their actual and coded values, and the corresponding results of experiments. ANOVA statistical results for the selected factorial model are given in Table 1. The Model F-value of 697.8 indicated that the model was significant, so there was only a 0.014% probability that a ' Model F-value ' could occur due to noise, according to the statistical results for the quadratic models provided in Table 1. The values of 'prob $>$ F' for a model below 0.05 ($<$ 0.0001) suggested that the model was statistically significant, with a confidence interval of 99.99%.

Significant terms are shown in Table 1 indicates that interaction terms of BC and AB are very significant, respectively. In Table S10, the optimal concentrations suggested by the statistical model are provided, and it was expected that the optimal yield of 0.36 mol/mol carbon was obtained, which was verified experimentally. Additionally, ethanol concentrations improved from approximately 5.5 g/l to 11.6 g/l. The findings show that the approach was able to adjust the flux distribution at optimum pH by adding certain regulatory compounds so that more ethanol was produced, and 2.1 fold higher than the measured yield for the control sample was achieved.

4. Discussion

Our results address critical questions in metabolism, such as changes in metabolic fluxes due to pH constraint, what shifts the metabolic pathways, and what induces cells to adjust the way they perform their metabolism, as illustrated by the shift in metabolism from pH 7 to 5. Our strategy proposes optimizing growth under the constraint of pH as the central principle shaping metabolism. The pH changes contribute to a redirection of metabolic fluxes (for example, shift to TCA cycle under acidic conditions) as the exchange rates of proton increase and cells aim to optimize their growth. This finding suggests that the developed charge-balanced models are capable of revealing the physiological properties of the cells and that the models are a more suitable platform for addressing the challenges of using these cells in bio-based production.

Our work also shows that a limit of pH in modeling could constrain cellular metabolism. This constraint is undoubtedly a universal and physical metabolism restriction that can be applied not only in eukaryotes (yeast) but also in prokaryotes (*E. coli* , *Z. mobilis*). Besides, our principle of metabolic flux prediction provides an advantage over conventional FBA-based techniques, since it systematically investigates metabolism in order to perform better and more effectively in metabolic engineering approaches. The findings draw on previous research in *S. cerevisiae* and other microorganisms, which is aligned with our results that substrate concentration changes are a significant contributor to overall flux distribution changes(Daran-Lapujade et al., 2004; Gerosa et al., 2015; Valgepea, Adamberg, Seiman, & Vilu, 2013). Meanwhile, despite the value of*S. cerevisiae* as an industrial microorganism, our observations into its regulatory metabolic flux at various pH levels may be of substantial benefit in systems metabolic engineering. Therefore, using genome-scale metabolic models to evaluate pH effect can provide comprehensive solutions to improve biofuels, biomedicine, and bio-based production, especially reduced products such as ethanol.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Author contributions

S.G. & E.M. designed and performed research and wrote the paper.

References

Andersen, M. R., Lehmann, L., & Nielsen, J. (2009). Systemic analysis of the response of Aspergillus niger to ambient pH. *Genome Biology* , *10* (5), R47.

Bekers, K. M., Heijnen, J. J., & Van Gulik, W. M. (2015). Determination of the in vivo NAD: NADH ratio in Saccharomyces cerevisiae under anaerobic conditions, using alcohol dehydrogenase as sensor reaction. *Yeast* , *32* (8), 541–557.

Benjaphokee, S., Hasegawa, D., Yokota, D., Asvarak, T., Auesukaree, C., Sugiyama, M., ... Harashima, S. (2012). Highly efficient bioethanol production by a Saccharomyces cerevisiae strain with multiple stress tolerance to high temperature, acid and ethanol. *New Biotechnology* , *29* (3), 379–386. https://doi.org/10.1016/j.nbt.2011.07.002

Blank, L. M., & Sauer, U. (2004). TCA cycle activity in Saccharomyces cerevisiae is a function of the environmentally determined specific growth and glucose uptake rates. *Microbiology* , *150* (4), 1085–1093. https://doi.org/10.1099/mic.0.26845-0

Çalik, P., & Ileri, N. (2007). pH influences intracellular reaction network of β-lactamase producing Bacillus licheniformis. *Chemical Engineering Science* , *62* (18–20), 5206–5211. https://doi.org/10.1016/j.ces.2007.01.081

Chidi, B. S., Rossouw, D., & Bauer, F. F. (2016). Identifying and assessing the impact of wine acid-related genes in yeast. *Current Genetics* , *62* (1), 149–164. https://doi.org/10.1007/s00294-015-0498-6

Contador, C. A., Shene, C., Olivera, A., Yoshikuni, Y., Buschmann, A., Andrews, B. A., & Asenjo, J. A. (2015). Analyzing redox balance in a synthetic yeast platform to improve utilization of brown macroalgae as feedstock. *Metabolic Engineering Communications* , *2* , 76–84. https://doi.org/10.1016/j.meteno.2015.06.004

Daran-Lapujade, P., Jansen, M. L. A., Daran, J. M., Van Gulik, W., De Winde, J. H., & Pronk, J. T. (2004). Role of Transcriptional Regulation in Controlling Fluxes in Central Carbon Metabolism of Saccharomyces cerevisiae: A chemostat culture study. *Journal of Biological Chemistry* , *279* (10), 9125–9138. https://doi.org/10.1074/jbc.M309578200

Dong, Y., Hu, J., Fan, L., & Chen, Q. (2017). RNA-Seq-based transcriptomic and metabolomic analysis reveal stress responses and programmed cell death induced by acetic acid in Saccharomyces cerevisiae. *Scientific Reports* , *7* , 42659.

Edwards, J. S., Covert, M., & Palsson, B. (2002). Metabolic modelling of microbes: The flux-balance approach. *Environmental Microbiology* , *4* (3), 133–140. https://doi.org/10.1046/j.1462-2920.2002.00282.x

Gerosa, L., Haverkorn Van Rijsewijk, B. R. B., Christodoulou, D., Kochanowski, K., Schmidt, T. S. B., Noor, E., & Sauer, U. (2015). Pseudo-transition Analysis Identifies the Key Regulators of Dynamic Metabolic Adaptations from Steady-State Data. *Cell Systems* ,*1* (4), 270–282. https://doi.org/10.1016/j.cels.2015.09.008

Jo, J. H., Lee, D. S., & Park, J. M. (2008). The effects of pH on carbon material and energy balances in hydrogen-producing Clostridium tyrobutyricum JM1. *Bioresource Technology* , *99* (17), 8485–8491. https://doi.org/10.1016/j.biortech.2008.03.060

Jones, C. W., & Doelle, H. W. (1991). Kinetic control of ethanol production by Zymomonas mobilis. *Applied Microbiology and Biotechnology* , *35* (1), 4–9. https://doi.org/10.1007/BF00180626

Joshi, B., Joshi, J., Bhattarai, T., & Sreerama, L. (2019). *Currently Used Microbes and Advantages of Using Genetically Modified Microbes for Ethanol* . *Bioethanol Production from Food Crops* . Elsevier Inc. https://doi.org/10.1016/B978-0-12-813766-6/00015-1

Kalnenieks, U. Z., Pankova, L. M., & Shvinka, Y. (1987). Proton motive force in the bacterium Zymomonas mobilis. *Biogeochemistry* ,*52* (5), 617–620.

Krulwich, T. A., Sachs, G., & Padan, E. (2011). Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews Microbiology* , *9* (5), 330–343. https://doi.org/10.1038/nrmicro2549

Kuroda, K., Hammer, S. K., Watanabe, Y., Montaño López, J., Fink, G. R., Stephanopoulos, G., ... Avalos, J. L. (2019). Critical Roles of the Pentose Phosphate Pathway and GLN3 in Isobutanol-Specific Tolerance in Yeast. *Cell Systems* , *9* (6), 534–547.e5. https://doi.org/10.1016/j.cels.2019.10.006

Martinez, K. A., Kitko, R. D., Mershon, J. P., Adcox, H. E., Malek, K. A., Berkmen, M. B., & Slonczewski, J. L. (2012). Cytoplasmic pH response to acid stress in individual cells of Escherichia coli and Bacillus subtilis observed by fluorescence ratio imaging microscopy.*Applied and Environmental Microbiology* , *78* (10), 3706–3714. https://doi.org/10.1128/AEM.00354-12

Mo, M. L., Palsson, B. Ø., & Herrgård, M. J. (2009). Connecting extracellular metabolomic measurements to intracellular flux states in yeast. *BMC Systems Biology* , *3* (1), 37.

Monk, J. M., Koza, A., Campodonico, M. A., Machado, D., Seoane, J. M., Palsson, B. O., ... Feist, A. M. (2016). Multi-omics Quantification of Species Variation of Escherichia coli Links Molecular Features with Strain Phenotypes. *Cell Systems* , *3* (3), 238–251.e12. https://doi.org/10.1016/j.cels.2016.08.013

Motamedian, E., Saeidi, M., & Shojaosadati, S. A. (2016). Reconstruction of a charge balanced genome-scale metabolic model to study the energy-uncoupled growth of Zymomonas mobilis ZM1.*Molecular BioSystems* , *12* (4), 1241–1249.

Motamedian, E., Sarmadi, M., & Derakhshan, E. (2019). Development of a regulatory defined medium using a system-oriented strategy to reduce the intracellular constraints. *Process Biochemistry* .

Naghshbandi, M. P., Tabatabaei, M., Aghbashlo, M., Gupta, V. K., Sulaiman, A., Karimi, K., ... Maleki, M. (2019). Progress toward improving ethanol production through decreased glycerol generation in Saccharomyces cerevisiae by metabolic and genetic engineering approaches. *Renewable and Sustainable Energy Reviews* ,*115* (August), 109353. https://doi.org/10.1016/j.rser.2019.109353

Narendranath, N. V, & Power, R. (2005). Relationship between pH and medium dissolved solids in terms of growth and metabolism of lactobacilli and Saccharomyces cerevisiae during ethanol production.*Appl. Environ. Microbiol.* , *71* (5), 2239–2243.

Orij, R., Brul, S., & Smits, G. J. (2011). Intracellular pH is a tightly controlled signal in yeast. *Biochimica et Biophysica Acta - General Subjects* , *1810* (10), 933–944. https://doi.org/10.1016/j.bbagen.2011.03.011

Orth, J. D., Conrad, T. M., Na, J., Lerman, J. A., Nam, H., Feist, A. M., & Palsson, B. Ø. (2011). A comprehensive genome-scale reconstruction of Escherichia coli metabolism—2011. *Molecular Systems Biology* , *7* (1).

Pagliardini, J., Hubmann, G., Alfenore, S., Nevoigt, E., Bideaux, C., & Guillouet, S. E. (2013). The metabolic costs of improving ethanol yield by reducing glycerol formation capacity under anaerobic conditions in Saccharomyces cerevisiae. *Microbial Cell Factories* , *12* (1), 1–14. https://doi.org/10.1186/1475-2859-12-29

Papapetridis, I., Van Dijk, M., Dobbe, A. P. A., Metz, B., Pronk, J. T., & Maris, A. J. A. (2016). Improving ethanol yield in acetate-reducing Saccharomyces cerevisiae by cofactor engineering of 6-phosphogluconate dehydrogenase and deletion of ALD6. *Microbial Cell Factories* ,*15* (1), 1–16. https://doi.org/10.1186/s12934-016-0465-z

Park, J. M., Kim, T. Y., & Lee, S. Y. (2011). Genome-scale reconstruction and in silico analysis of the Ralstonia eutropha H16 for polyhydroxyalkanoate synthesis, lithoautotrophic growth, and 2-methyl citric acid production. *BMC Systems Biology* , *5* (1), 101.

Reed, J. L., Vo, T. D., Schilling, C. H., & Palsson, B. O. (2003). An expanded genome-scale model of Escherichia coli K-12 (i JR904 GSM/GPR).*Genome Biology* , *4* (9), R54.

Scalcinati, G., Otero, J. M., Van Vleet, J. R. H., Jeffries, T. W., Olsson, L., & Nielsen, J. (2012). Evolutionary engineering of Saccharomyces cerevisiae for efficient aerobic xylose consumption. *FEMS Yeast Research* , *12* (5), 582–597. https://doi.org/10.1111/j.1567-1364.2012.00808.x

Shioi, J. I., Matsuura, S., & Imae, Y. (1980). Quantitative measurements of proton motive force and motility in Bacillus subtilis. *Journal of Bacteriology* , *144* (3), 891–897. https://doi.org/10.1128/jb.144.3.891-897.1980

Slonczewski, J. L., Fujisawa, M., Dopson, M., & Krulwich, T. A. (2009). *Cytoplasmic pH Measurement and Homeostasis in Bacteria and Archaea . Advances in Microbial Physiology* (Vol. 55). Elsevier. https://doi.org/10.1016/S0065-2911(09)05501-5

Slonczewski, J. L., Rosen, B. P., Alger, J. R., & Macnab, R. M. (1981). pH homeostasis in Escherichia coli: measurement by 31P nuclear magnetic resonance of methylphosphonate and phosphate. *Proceedings of the National Academy of Sciences of the United States of America* ,*78* (10 I), 6271–6275. https://doi.org/10.1073/pnas.78.10.6271

Swayambhu, G., Moscatello, N., Atilla-Gokcumen, G. E., & Pfeifer, B. A. (2020). Flux Balance Analysis for Media Optimization and Genetic Targets to Improve Heterologous Siderophore Production. *IScience* ,*23* (4), 101016. https://doi.org/10.1016/j.isci.2020.101016

Valgepea, K., Adamberg, K., Seiman, A., & Vilu, R. (2013). Escherichia coli achieves faster growth by increasing catalytic and translation rates of proteins. *Molecular BioSystems* , *9* (9), 2344–2358. https://doi.org/10.1039/c3mb70119k

Valgepea, K., de Souza Pinto Lemgruber, R., Meaghan, K., Palfreyman, R. W., Abdalla, T., Heijstra, B. D., ... Marcellin, E. (2017). Maintenance of ATP Homeostasis Triggers Metabolic Shifts in Gas-Fermenting Acetogens. *Cell Systems* , *4* (5), 505–515.e5. https://doi.org/10.1016/j.cels.2017.04.008

Valli, M., Sauer, M., Branduardi, P., Borth, N., Porro, D., & Mattanovich, D. (2005). Intracellular pH distribution in Saccharomyces cerevisiae cell populations, analyzed by flow cytometry. *Appl. Environ. Microbiol.* , *71* (3), 1515–1521.

Vojinović, V., & Von Stockar, U. (2009). Influence of uncertainties in pH, pMg, activity coefficients, metabolite concentrations, and other factors on the analysis of the thermodynamic feasibility of metabolic pathways. *Biotechnology and Bioengineering* , *103* (4), 780–795. https://doi.org/10.1002/bit.22309

Williams-Rhaesa, A. M., Rubinstein, G. M., Scott, I. M., Lipscomb, G. L., Poole, F. L., Kelly, R. M., & Adams, M. W. W. (2018). Engineering redox-balanced ethanol production in the cellulolytic and extremely thermophilic bacterium, Caldicellulosiruptor bescii. *Metabolic Engineering Communications* , *7* . https://doi.org/10.1016/j.mec.2018.e00073

Yang, S., Land, M. L., Klingeman, D. M., Pelletier, D. A., Lu, T. Y. S., Martin, S. L., ... Brown, S. D. (2010). Paradigm for industrial strain improvement identifies sodium acetate tolerance loci in Zymomonas mobilis and Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America* , *107* (23), 10395–10400. https://doi.org/10.1073/pnas.0914506107

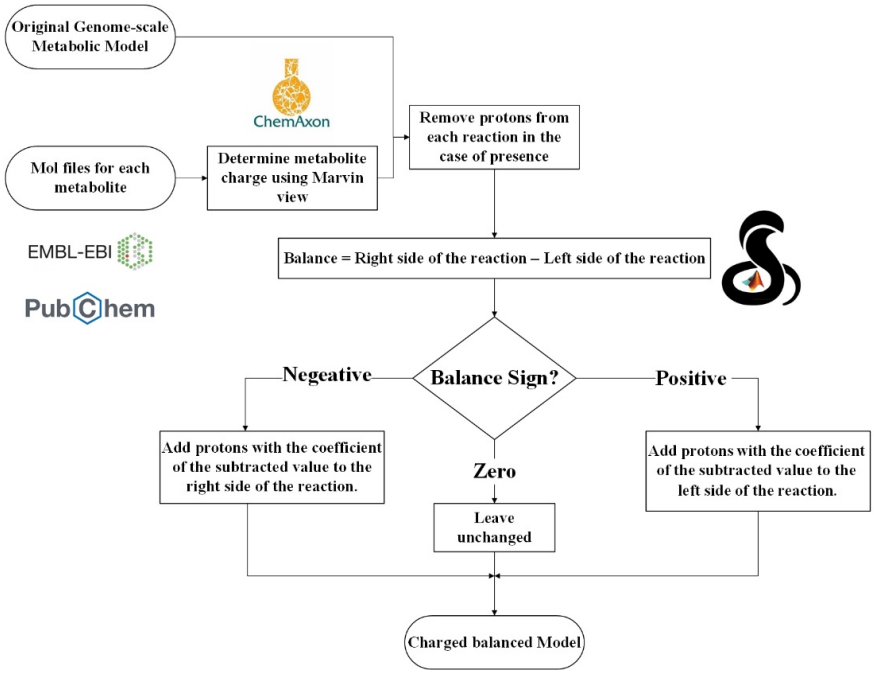


Figure 1. Flowchart of the charge balance method of a metabolic model at a specific or intended pH.

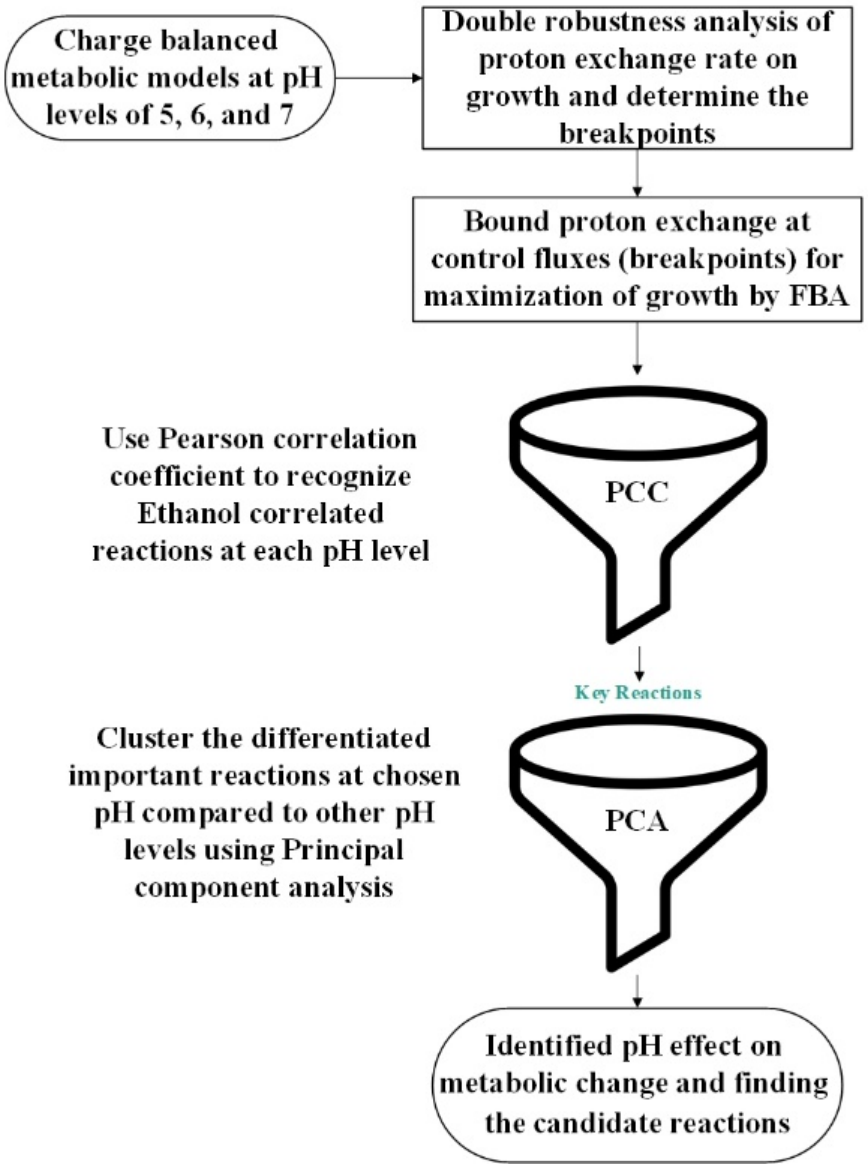
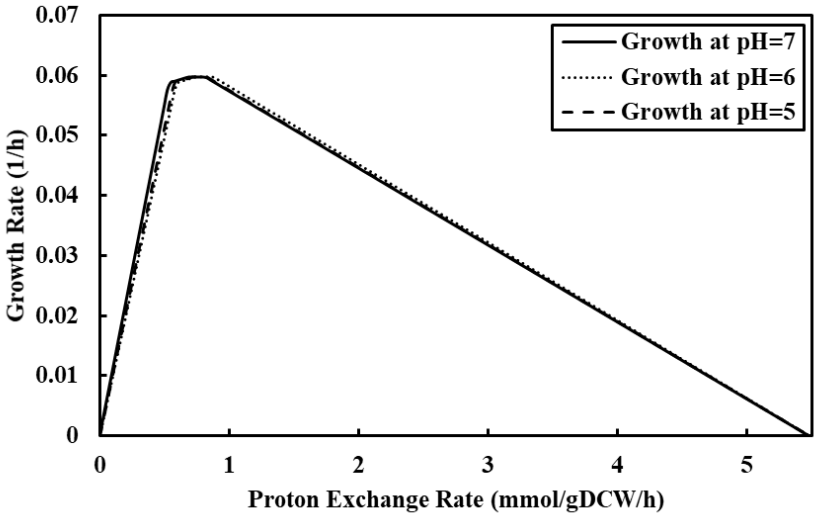
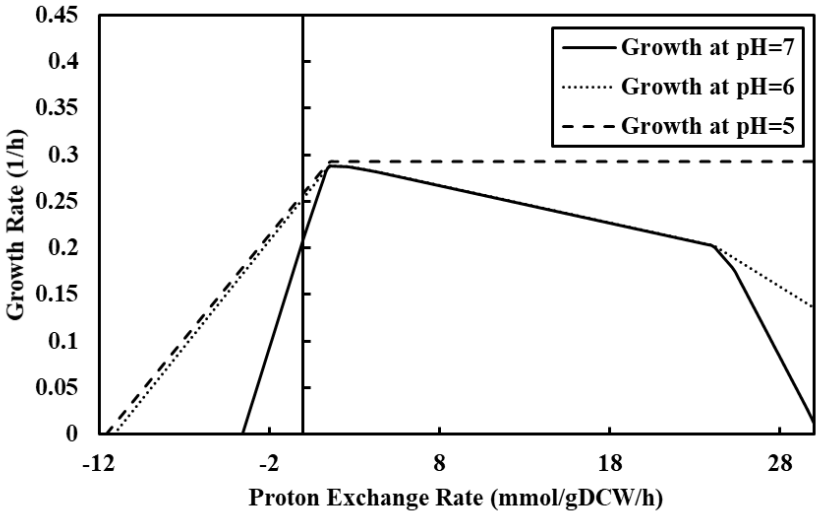


Figure 2. Flowchart of the systemic approach for finding the key reactions of ethanol overproduction and identification of the effect of pH on the metabolism.





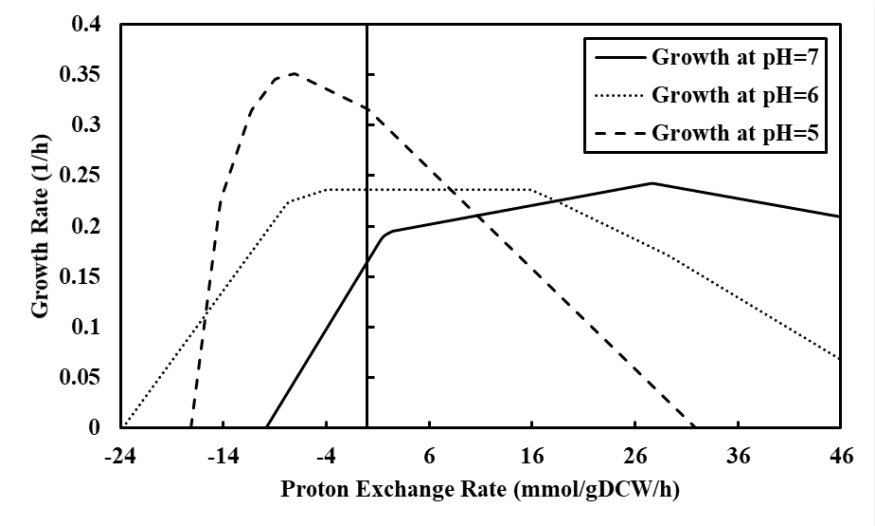
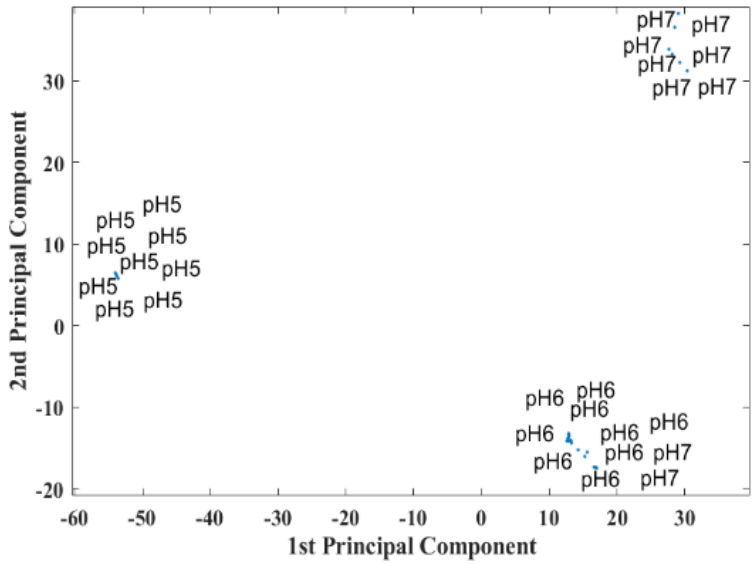


Figure 3. Robustness analysis of a) *Z. mobilis* b) *S. cerevisiae* and c) *E. coli* to the proton exchange rate for growth.



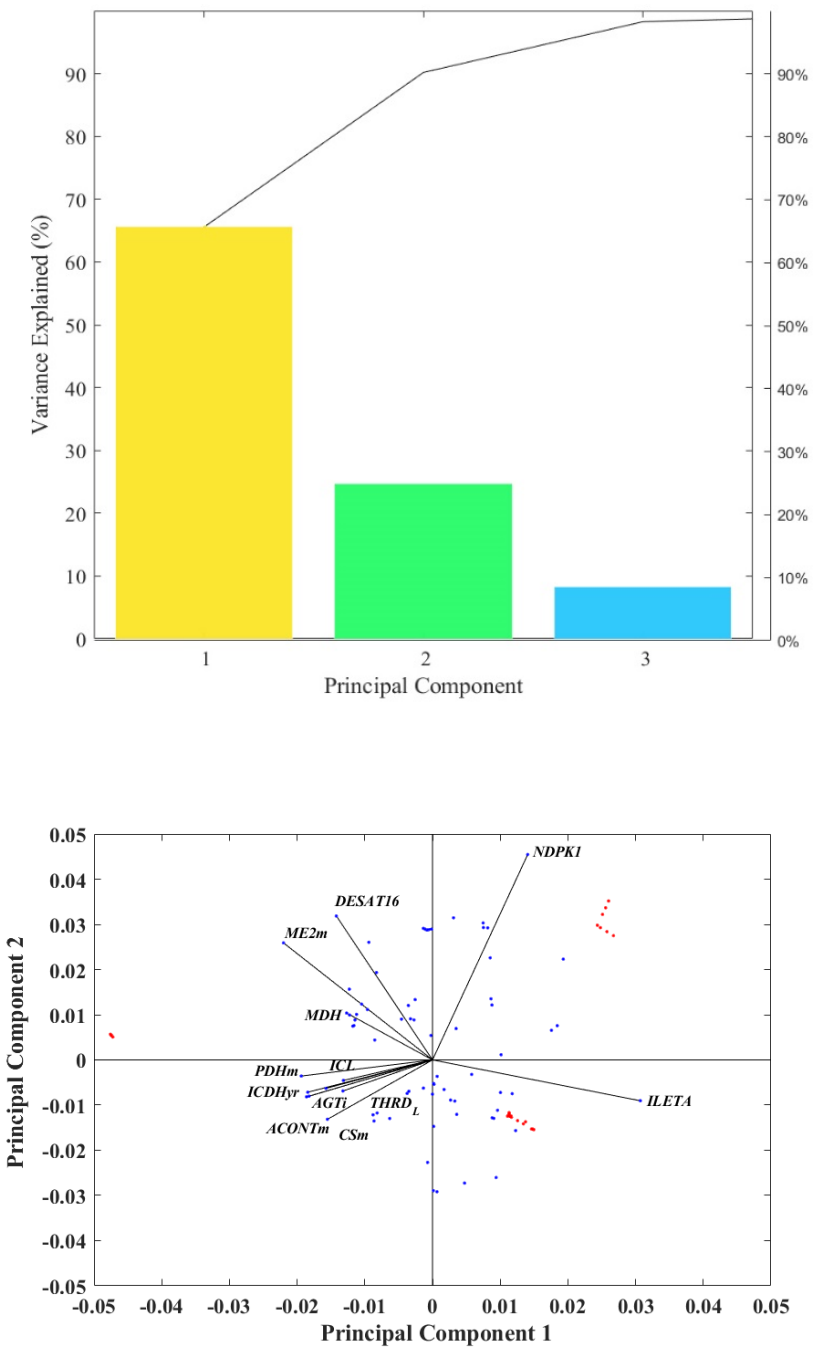


Figure 4. Principal component analysis of correlated reaction with Ethanol production. (a) The pH models plotted over the first two principal components (PCs) demonstrated significant differences between pH=5 and other pH models. (b) The first two PCs account for more than 90.18% of data variance. (c) The PCs are a combination of differentiated reactions. The score plot has identified potential reactions that are responsible for the discrimination of pH models.

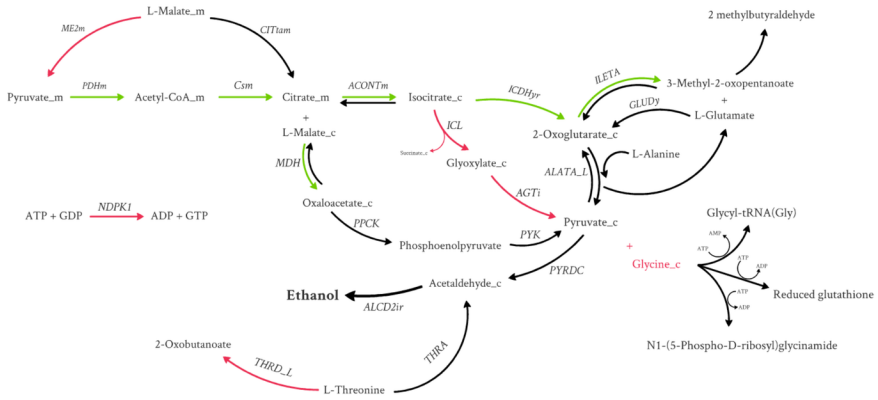


Figure 5. A comprehensive overview of the recognized reactions by the systemic approach, which are candidates for up-regulation (green arrows) and down-regulation (red arrows), in order to improve ethanol production.

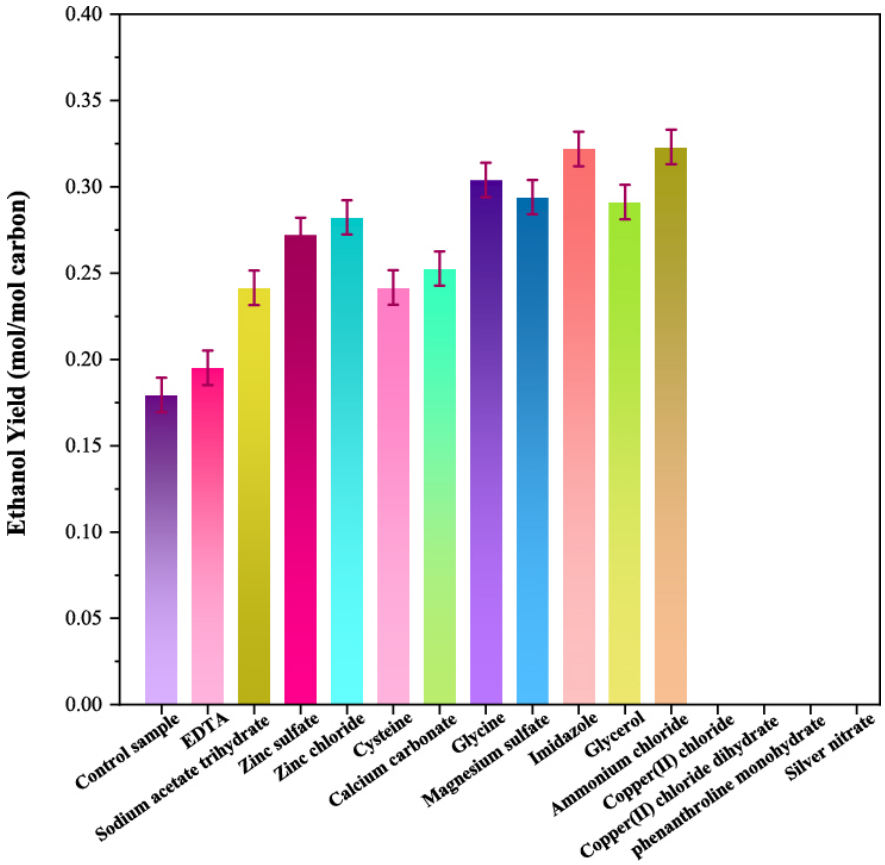


Figure 6. Effect of added regulators to the culture medium of *S. cerevisiae* compared with the control sample.

Table 1. F-value and p-value provided by the ANOVA for significant factors (A: Glycine, B: Imidazole, C: Glycerol).

Source	F-value	p-value	
Model	697.83	< 0.0014	significant
A	130.29	0.0076	
B	2642.57	0.0004	
C	346.23	0.0029	
AB	149.17	0.0066	
BC	220.9	0.0045	

Hosted file

`Graphical Abstract.pdf` available at https://authorea.com/users/364004/articles/484592-improving-ethanol-production-by-studying-the-effect-of-ph-using-a-modified-metabolic-model-and-a-systemic-approach