

## Research Article

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**Extracellular acidity and oxygen availability conjointly control eukaryotic cell growth via modulation of cytoplasmic translation****Ökaryotik hücre büyümesinin sitoplazmik çevirimin düzenlenmesi yoluyla denetiminde asidite ve oksijen sinerjisinin rolü**<https://doi.org/10.1515/tjb-2019-0130>

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**Abstract**

**Background:** Oxygen availability and extracellular acidity both have a strong impact on growth and cultivation characteristics of eukaryotes, however they are often considered in isolation, whereby a single parameter is varied at a time to identify its impact, rendering the investigation of synergistic effects created by two or more factors non-achievable. This study identified the synergistic effect between environmental pH and oxygen levels on the physiological and cellular characteristics of the simplest eukaryote, *Saccharomyces cerevisiae*.

**Materials and methods:** The physiological, transcriptomic, and metabolic responses of yeast were investigated during batch growth in a 2×2 factorial design setting; environmental pH and oxygen availability were either controlled at their optimal settings, or allowed to follow their own course during cultivation.

**Results:** Synergistic effects had a significant impact on yeast physiology, which was provoked further by both the modulation of gene expression by transcription, and the modification of metabolite pools. Genes involved in cytoplasmic translation, the extracellular and intracellular amino acid and their precursor metabolite pools were significantly responsive to concurrent variations in these two factors.

**Conclusion:** The synergistic effect of extracellular acidity and oxygenation on eukaryotic landscape of growth-associated events was significantly more pronounced than their individual effects.

**Keywords:** *Saccharomyces cerevisiae*; Environmental acidity; Growth control; Oxygenation; Transcriptomics;

Metabolic analysis; Amino acid pool; Cytoplasmic translation.

**Öz**

**Amaç:** Büyüme ortamındaki oksijen müsaitliği ve asidite ökaryotik hücrelerin büyüme ve kultivasyon özellikleri üzerinde önemli etkiye sahiptir ancak bu özelliklerin etkileri çoğunlukla diğer parametrelerden ayrı olarak tek başlarına incelenir ve değerlendirilir, ve bu da çoklu etkileşimlerin (sinerjinin) etkilerinin belirlenmesini olanaksız kılar. Bu çalışma, ortam pH ve oksijen seviyeleri arasındaki sinerjiin en basit ökaryotik hücre olan *Saccharomyces cerevisiae*'nin fizyolojik ve hücresel özellikleri üzerindeki etkisini belirlemeyi amaçlamaktadır.

**Gereç ve Yöntem:** Maya hücrelerinin kesikli üretimde fizyolojik, transkriptomik ve metabolik yanıtları 2×2 faktöryel tasarım ile incelenmiştir; ortam pH değeri ve oksijen müsaitliği ya optimal değerlerinde kontrol edilmiş, ya da kultivasyon süresince kendi başlarına değişimlerine olanak sağlanmıştır.

**Bulgular:** Sinerjik etkileşimlerin maya fizyolojisi üzerinde istatistiksel açıdan anlamlı bir değişime yol açtığı gözlemlenmiştir ve bu değişim hem hücresel gen anlatımının yazılım seviyesinde düzenlenmesi hem de amino asit havuzlarının ayarlanması ile gerçekleşmektedir. Sitoplazmik çevirimde rol alan genler ile hücre içi ve hücre dışı amino asit ve amino asit öncül metabolit havuzları, bu iki çevresel faktörün etkileşimine istatistiksel açıdan anlamlı cevaplar vermiştir.

**Sonuç:** Hücre dışı asidite ve oksijenlenme arasındaki sinerjinin büyüme ile bağıntılı ökaryotik etkinlikler üzerinde, bu parametrelerin tek başlarına yarattıkları etkiden daha belirgin ve istatistiki olarak anlamlı bir şekilde daha yoğun sonuçlar doğurduğu gözlemlenmektedir.

**Anahtar Kelimeler:** *Saccharomyces cerevisiae*; çevresel asidite; büyümenin kontrolü; oksijenlenme; transkriptom

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analizi; metabolik analiz; amino asit havuzu; sitoplazmik çevirim.

## Introduction

The budding yeast *Saccharomyces cerevisiae* has long been a loyal workhorse of industrial biotechnology, with evidence for its use dating back several millennia. The emergence of the study of the yeast cellular metabolism in the 19<sup>th</sup> century accompanied targeted bioprocess development, and this marks the first modern applications of biotechnology [1]. Yeast has substantial commercial relevance as a cell factory in numerous applications ranging from the production of bioethanol [2] to vanillin [3] to resveratrol [4] to chemotherapeutic agents [5]. Yeast has enjoyed this rightful reputation due to the availability of extensive tools and information [6–9], which ensued from being the first eukaryotic system, whose genome has been completely sequenced [10].

Understanding the control of growth [11] and the optimal growth conditions of Baker's yeast [12, 13] has been considered in depth as a result of the commercial interest in fermentation technologies associated with its utilisation [14]. The effect of parameters such as pH [15, 16] and oxygen availability [17–19] on growth and fermentation characteristics of yeast were extensively studied, and variations in either parameter has been employed as a control or modification mechanism for fermentation processes [20, 21]. However, the synergetic effects between these parameters on growth and fermentation characteristics, nor on the cellular landscape, were not investigated. Combined effect of pH and oxygen availability was only considered in evaluating the productivity of genetically engineered yeast strains [22, 23] but not from a bioprocess perspective.

Extracellular pH of yeast cells was shown to be connected to some extent, and at times be correlated with the intracellular pH [24], which is one of the major controllers of cellular growth in *S. cerevisiae* [25] with a tight control mechanism to maintain intracellular acidity within a tolerable range [26]. This phenomenon was observed in other eukaryotes [27, 28], too, indicative of at least a certain degree of conservation across eukaryotic systems.

Recent studies on biotechnologically relevant systems focus on understanding of the tight interconnection between intracellular pH and oxygen availability with the aim of exploiting these systems more efficiently as cell factories [29]. This would then allow the tailoring of optimised cultivation conditions that take the cellular requirements of the system of interest into consideration, and

thus facilitate the design of bioprocesses with improved yield and productivity. Current bioprocess design practice relies dominantly on the investigation of factors contributing to the cultivation performance by varying only one parameter at a time [30], although approaches, which take synergistic effects between design parameters into consideration, also exist [31].

In a recent study, we showed that the synergy between extracellular pH and oxygen availability had an impact on the amino acid metabolism in the Baker's yeast during balanced growth in response to sustained low-dose exposure to the macrolide, rapamycin [32]. On the other hand, the interactive impact of these parameters has not ever been investigated on non-treated cultures growing under non-stressing conditions. Cell culturing or fermentation processes ranging from culturing in shake flasks to bioreactors with advanced process control implementation all inherently incorporate the synergy between these two factors into experimental design. In one extreme, simple processes where neither parameter is controlled lie; on the other extreme, bioreactors that allow tight control over both parameters reside. However, the cellular response to these two most commonly employed modes of operation, which are exploited to bring populations to pseudo steady state phase of growth, have not been investigated at the transcriptional, metabolic, or at the phenotypical level even for the simplest eukaryote, *S. cerevisiae*.

This work explores the significance and prevalence of synergetic relationships between two major growth parameters; extracellular pH and oxygen availability, on the cultivation performance and cellular response of *S. cerevisiae*, the simple eukaryotic cell. The environmental pH and dissolved oxygen ( $\text{dO}_2$ ) level of the cultures were either controlled at their optimal settings ( $\text{pH}=5.5$  and  $\text{dO}_2>90\%$ ), or left to follow their own course during fermentation, allowing the exploration of synergistic as well as single-parameter effects on cellular physiology, gene expression by transcription, and metabolite levels.

## Materials and methods

### Data compilation and the experimental background for the datasets

The data was collected from duplicate batch cultivations of homozygous *hoΔ/hoΔ* strain of *S. cerevisiae* BY4743 (*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/met15Δ0/+ ura3Δ0/ura3Δ0*) grown in synthetic medium (Table 1) [33]. The dissolved oxygen ( $\text{dO}_2$ ) saturation was

**Table 1:** Synthetic medium composition.

Compound	Concentration (g/L)
Glucose	20
$\text{NH}_4\text{SO}_4$	3.13
$\text{KH}_2\text{PO}_4$	2
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.55
NaCl	0.1
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.09
Uracil	0.02
Histidine	0.02
Leucine	0.1
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.00007
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.00001
$\text{H}_3\text{BO}_3$	0.00001
KI	0.00001
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.00005
Inositol	0.062
Thiamine/HCl	0.014
Pyridoxine	0.004
Ca-pantothenate	0.004
Biotin	0.0003

maintained at above 90% in air+ cultures by sparging the cultures with air at a rate of 0.1 vvm, and was monitored in air– cultures. pH was controlled at 5.5 in pH+ cultures, and was only monitored in pH– cultures. The samples for transcriptome and metabolite analyses were collected at  $\text{OD}_{600}$  range of 0.6–0.8. Biomass density was determined gravimetrically. Microarray analysis was carried out by as described in the GeneChip® Expression Analysis Technical Manual (relevant kits, chips, and instrumentation: Affymetrix Inc., USA). The tricarboxylic acid (TCA) cycle intermediate and amino acid profiles were acquired from the Agilent 6430 Triple Quadrupole LC-MS system (USA). The glucose, glycerol, ethanol and ammonium content of the supernatant was determined enzymatically. The unprocessed raw data files for transcriptome analysis were accessed from the European Bioinformatics Institute ArrayExpress Data Repository [34] under the Accession number E-MTAB-6628. The submitted data was in compliance with the MIAME [35] standards, with fully accessible experimental details. The growth characteristics, endometabolic and exometabolic amino acid and TCA cycle intermediate profiles were accessed from [32].

## Microarray data preprocessing

The raw image files (.cel extension) were processed and normalised with their quality assessed by the dChip software [36]. The baseline array for normalization was

selected as the array having the best call rate, which was 59.7% for this dataset. The expression values were obtained using the model-based expression modelling method and conducting mismatch probe background subtraction calculated from the difference between the perfect match and mismatch values. The smoothing method was running median. All single, array and probe outliers were checked and datasets were ensured to have <1% array outliers and <0.5% single outliers. The expression values were  $\log_2$  transformed prior to data analysis.

## Data analysis

The significance of physiological characteristics and exometabolic metabolite measurements was evaluated employing 2-way ANalysis Of VAriance (ANOVA) for data with replicates followed by Tukey's Honest Significant Difference (HSD) test for multiple testing correction. For the transcriptome analysis and endometabolic measurements Student's t-test was employed for statistical analysis whenever sample size  $\geq 3$ , followed by the Benjamini-Hochberg test for multiple testing correction of the false discovery rate (FDR). Two-way ANOVA for data with replicates was employed as required for datasets where samples size <3, followed by Tukey HSD test as post-hoc analysis for multiple comparisons. Significance was evaluated at the confidence level of  $\alpha = 0.05$ . Hierarchical clustering of samples was carried out using Hierarchical Clustering Explorer (HCE) 3.5 [36], with Pearson correlation employed as the distance metric and average linkage employed as the linkage metric. Princeton GO tools Generic GO Term Finder was employed for Gene Ontology Enrichment Analysis (<http://go.princeton.edu/cgi-bin/GOTermFinder>) accessed on (02/2018) [37]. Bonferroni correction was applied for p-values and FDR was included in the evaluation. Gene ontology associations inferred from electronic annotation (IEA) were excluded from analysis.

## Results

### Extracellular pH and oxygen availability jointly control cellular growth

The physiological characteristics of the batch cultures where (i) the cells were grown in a pH– controlled and aerated environment (air+ pH+), (ii) in environments where neither of these parameters were controlled (air– pH–), or (iii) and (iv) only one parameter was controlled (air+ pH–,

air- pH+), were investigated. Growth rate, mid-exponential phase and steady state biomass accumulation, uptake of main carbon (glucose) and nitrogen (ammonium) sources, and the secretion of main by-products (glycerol and ethanol), amino acids, and TCA cycle intermediates were analysed to shed light on how cellular physiology was affected by synergistic effects between oxygen availability and extracellular pH.

The pH of the controlled cultures was maintained in the range of 5.44–5.51 at all times, whereas the extracellular pH was observed to drop down to the range of 3.56–3.84 during the exponential phase of growth in pH- cultures. The pH decreased further down to the range of 2.81–2.96 as those cultures reached stationary phase. The dissolved oxygen content of the air+ cultures remained always above 90%. Conversely, the dissolved oxygen content was in the range of 45–63% during the mid-exponential phase of the cultures that were fully saturated with oxygen at the time of inoculation, and were later operated as micro-aerated systems. The dissolved oxygen content of these cultures were observed to drop down to below 5% as they reached stationary phase. The overhead oxygen content of all cultures was measured as  $20.34 \pm 1.03\%$  for the duration of

the cultivation indicating that the positive pressure in the vessels did not yield to oxygen escape from dissolved to gas state; the overhead oxygen content did not vary significantly with air supplementation regime, except for marginally low overhead oxygen measured during the stationary phase of air- cultures (Table 2).

The specific growth rate ( $\mu_s$ ) at exponential phase of cultivation varied by 26% under different conditions, and it was the lowest ( $\mu_s = 0.31 \text{ h}^{-1}$ ) in air- pH- cultures. The time for the cultures to reach mid-exponential phase significantly increased with high oxygen availability (p-value = 0.032). The optical density (OD) of the cultures at mid-exponential phase was significantly affected by the interactive effect of extracellular pH and oxygen availability (p-value = 0.043), whereas the OD measured at stationary phase (prior to the diauxic shift) was significantly affected by the environmental pH only (p-value = 0.002). In both instances, the OD measurements were lower in pH- cultures. The biomass densities of the cultures were significantly affected by the interactive effect of culture pH and oxygenation both at the mid-exponential phase and at stationary phase (p values =  $6.43 \times 10^{-12}$  and  $7.96 \times 10^{-5}$ , respectively) (Table 3).

**Table 2:** Fermentation parameters of the batch yeast cultures.<sup>a</sup>

Parameter <sup>b</sup>	Air+ pH+	Air+ pH-	Air- pH+	Air- pH-
Culture pH at M	5.44 ± 0.00	3.75 ± 0.12	5.45 ± 0.01	3.57 ± 0.01
Culture pH at S	5.48 ± 0.04	2.82 ± 0.01	5.45 ± 0.00	2.93 ± 0.04
dO <sub>2</sub> sat. at M (%)	92.75 ± 8.27	97.35 ± 0.07	53.95 ± 12.37	56.55 ± 2.62
dO <sub>2</sub> sat. at S (%)	93.30 ± 5.52	100.05 ± 1.48	35.10 ± 9.63	3.00 ± 1.56
Overhead O <sub>2</sub> at M (%)	20.60 ± 0.10	21.30 ± 5.52	18.30 ± 2.94	21.50 ± 2.07
Overhead O <sub>2</sub> at S (%)	20.53 ± 0.06	20.13 ± 0.85	19.91 ± 1.41	19.80 ± 0.19

<sup>a</sup>Values for duplicate cultures provided as average ± standard deviation. <sup>b</sup>M, mid-exponential phase; S, stationary phase; sat., saturation.

**Table 3:** Growth characteristics, macro-nutrient uptake and by-product secretion of the batch yeast cultures.<sup>a</sup>

Parameter <sup>b</sup>	Air+ pH+	Air+ pH-	Air- pH+	Air- pH-
$\mu_s$ at M ( $\text{h}^{-1}$ )	0.36 ± 0.01	0.39 ± 0.04	0.35 ± 0.03	0.31 ± 0.00
Time to reach M (h)	8.84 ± 0.01	7.68 ± 0.25	10.03 ± 0.59	9.23 ± 1.02
Time to reach S (h)	29.11 ± 0.01	29.14 ± 5.98	24.23 ± 2.76	28.17 ± 4.91
OD <sub>600</sub> at M	0.87 ± 0.04	0.63 ± 0.01	0.69 ± 0.01	0.67 ± 0.10
OD <sub>600</sub> at S	1.60 ± 0.01	1.44 ± 0.05	1.59 ± 0.06	1.34 ± 0.00
Bio at M (g/L) <sup>c</sup>	0.41 ± 0.15	0.74 ± 0.12	0.71 ± 0.18	0.17 ± 0.08
Bio at S (g/L) <sup>c</sup>	1.43 ± 0.17	1.30 ± 0.45	1.52 ± 0.27	0.60 ± 0.11
Glu by M (g/L)	6.22 ± 0.28	7.41 ± 0.48	7.82 ± 0.42	6.32 ± 0.31
NH <sub>4</sub> <sup>+</sup> by M (g/L)	0.75 ± 0.00	0.75 ± 0.00	0.78 ± 0.01	0.74 ± 0.01
Eth by M (g/L)	1.58 ± 0.03	1.43 ± 0.06	2.00 ± 0.04	1.15 ± 0.03
Gly by M (g/L)	0.20 ± 0.00	0.12 ± 0.01	0.39 ± 0.00	0.27 ± 0.00

<sup>a</sup>Values for duplicate cultures provided as average ± standard deviation. <sup>b</sup>M, mid-exponential phase; S, stationary phase; Bio, biomass density; Glu, glucose consumed; NH<sub>4</sub><sup>+</sup>, ammonium consumed; Eth, ethanol secreted; Gly, glycerol secreted. <sup>c</sup>Gravimetric measurements of biomass was conducted in five replicates for each culture; results represent 10 measurements for each condition.

The uptake of the macronutrients acting as the primary carbon and nitrogen sources was significantly affected by an interaction between the two environmental factors under investigation ( $p$ -values = 0.008 and 0.047 for glucose and ammonium, respectively). The amount of glucose consumed by the yeast populations varied by 25%, whereas the amount of ammonium consumed by the cultures varied only by 0.5%, although the differences between the cultures under different conditions were apparent, and significant in both cases. The ethanol and glycerol secretion of yeast cultures were significantly different ( $p$ -values =  $2.60 \times 10^{-4}$  and  $1.60 \times 10^{-2}$  for ethanol and glycerol, respectively), and extracellular pH and oxygen availability at mid-exponential phase exhibited an interaction effect on the amount of by-product secreted. Both by-products were detected at lower concentration in the pH- cultures than in pH+ cultures (Table 3).

The uptake of histidine or leucine, to which the strain employed in the study was auxotrophic, was not significantly different between cultures. Leucine and histidine supplementation in the medium was in excess of the cellular requirements by design, thus their uptake only created a marginal and insignificant difference. On the other hand, the extracellular concentration of seven amino acids (alanine, arginine, asparagine, aspartate, cysteine, methionine, and threonine) as well as five TCA cycle intermediates (citrate, isocitrate, malate, cis-acotinate, and pyruvate) were significantly different between cultivation conditions. Since the cultures were not supplemented with either of these small molecules, all were considered as metabolites; the cellular by-products

metabolically produced and secreted in differing ratios in response to different cultivation conditions here explored. The differences in arginine, cysteine, methionine, threonine, citrate, and malate concentrations were significantly affected by the interactive effect between the two environmental factors under investigation. Extracellular alanine and isocitrate concentrations were significantly responsive to the  $dO_2$  level, whereas asparagine, aspartate, cis-acotinate, and pyruvate concentrations were significantly responsive to extracellular pH only (Table 4). The differences in the extracellular amino acid and TCA cycle intermediate metabolite concentrations revealed the directionality of material flow through metabolic fluxes of these populations grown under different environmental conditions, and highlighted the preferential production and secretion of different families of amino acids, some of which employ TCA cycle intermediates as precursors for biosynthesis (Figure 1). Aspartate synthesized from the TCA cycle intermediate oxaloacetate acted as the major precursor amino acid for the amino acids, whose exometabolic profiles displayed significant differences in response to different environmental conditions investigated in this study.

### Differences in growth characteristics were traceable in endometabolic and transcriptomic profiles of yeast populations

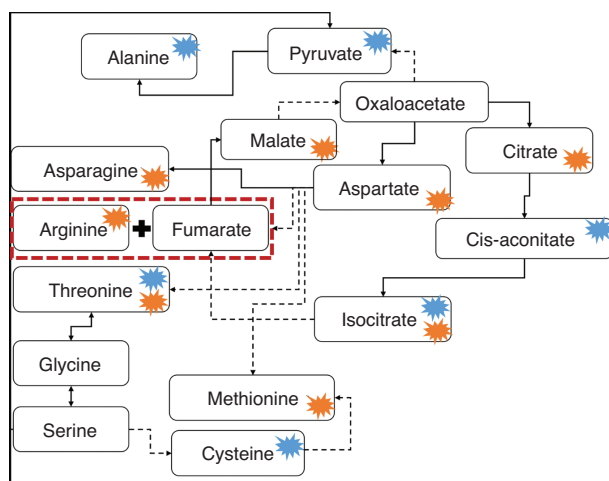
The differences observed in the growth characteristics and the physiology of the yeast populations in response

**Table 4:** Significant differences in extracellular amino acid and TCA cycle intermediate concentrations for the batch yeast cultures.<sup>a</sup>

Metabolite	Effect <sup>b</sup>	Response observed	p-Value
Alanine	Air	Higher in air+ cultures	$8.19 \times 10^{-3}$
Arginine	Air/pH	Lower in pH- cultures for air+ cases Higher in pH- cultures for air- cases	$4.97 \times 10^{-4}$
Asparagine	pH	Lower in pH- cultures	$4.99 \times 10^{-3}$
Aspartate	pH	Lower in pH- cultures	$5.42 \times 10^{-5}$
Cysteine	Air/pH	Higher in pH- cultures for air+ cases	$3.70 \times 10^{-2}$
Methionine	Air/pH	Lower in pH- cultures for air- cases	$1.73 \times 10^{-3}$
Threonine	Air/pH	Lower in pH- cultures for air+ cases Higher in pH- cultures for air- cases	$2.33 \times 10^{-7}$
Citrate	Air/pH	Lower in pH- cultures for air- cases	$1.07 \times 10^{-4}$
Isocitrate	Air	Higher in air+ cultures	$7.93 \times 10^{-4}$
	pH	Lower in pH- cultures	$3.89 \times 10^{-3}$
Malate	Air/pH	Lower in pH- cultures for air+ cases	$3.22 \times 10^{-3}$
Cis-acotinate	pH	Lower in pH+ cultures	$8.10 \times 10^{-3}$
Pyruvate	pH	Lower in pH+ cultures	$1.39 \times 10^{-3}$

<sup>a</sup>p-Values were determined by 2-way ANOVA (for samples with replicates), followed by the Tukey HSD test as an ad-hoc analysis. Pairwise comparisons are reported for the air/pH interaction effect. <sup>b</sup>Air, air only; pH, pH only; Air/pH, air – pH interaction effect.





**Figure 1:** Extracellular distribution of amino acids from TCA cycle precursors.

The amino acids and their TCA cycle intermediate precursors are depicted in rounded rectangles connected either by solid lines indicating a single metabolic step or by dashed lines indicating multiple metabolic steps. The reversibility of the steps is indicated by the direction of the arrows. The red dashed box groups two metabolites produced from aspartate in the same metabolic process. The blue banners denote metabolite concentrations that are significantly high at  $dO_2 > 90\%$  due to aeration effect, at low acidity due to pH effect, or at low acidity due to an interaction effect between culture pH and aeration levels. The orange banners denote metabolite concentrations that are significantly low at low acidity due to pH effect, or due to an interaction effect between culture pH and aeration levels.

to extracellular pH and dissolved oxygen availability led to an in-depth investigation of the accompanying intracellular changes of these cell populations in order to gain insight on the underlying molecular mechanisms, which are responsible for inducing these differences in the culture characteristics.

For this purpose, we first investigated how the intracellular TCA cycle intermediate and amino acid concentrations varied in these cultures. Significant differences were observed around the amino acid metabolism with the intracellular concentration of 14 of the 20 amino acids measured (alanine, arginine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, and valine) as well as of four TCA cycle intermediates ( $\alpha$ -ketoglutarate, malate, cis-aconitate, and pyruvate) being altered between cultivation conditions. The significant differences in alanine, arginine, glutamate, histidine, leucine, methionine, proline, serine, and valine concentrations, as well as in  $\alpha$ -ketoglutarate, malate, and pyruvate concentrations were caused by an interactive effect of both culture parameters investigated as factors; extracellular acidity and

oxygen availability. Furthermore, the differences in cis-aconitate concentration was only responsive to differences in oxygen availability and the differences in glycine, lysine and threonine concentrations were only responsive to differences in extracellular acidity. Interestingly, the differences in glutamine and isoleucine concentrations were responsive to differences in either extracellular acidity or oxygen availability alone, but these two factors did not have an interactive effect on the concentration of these intracellular metabolites (Table 5). Glutamate, which employed  $\alpha$ -ketoglutarate as its TCA cycle precursor was identified as the key amino acid in repurposing the intracellular amino acid pools available within yeast (Figure 2).

The transcriptional landscape of the yeast populations growing in different pH and  $dO_2$  controlling and monitoring regimes were investigated in order to identify the changes in gene expression events accompanying or underlying the differences observed in the exometabolic and the endometabolic profiles of these cultures. The effect of controlling dissolved oxygen or the pH of the culture was investigated by pooling the cultures such that the effect of either factor was investigated irrespective of the other, or by conducting an analysis of variance to identify the unique effect of each factor on the transcriptional profiles as well as the interaction (i.e. the synergy) between the factors.

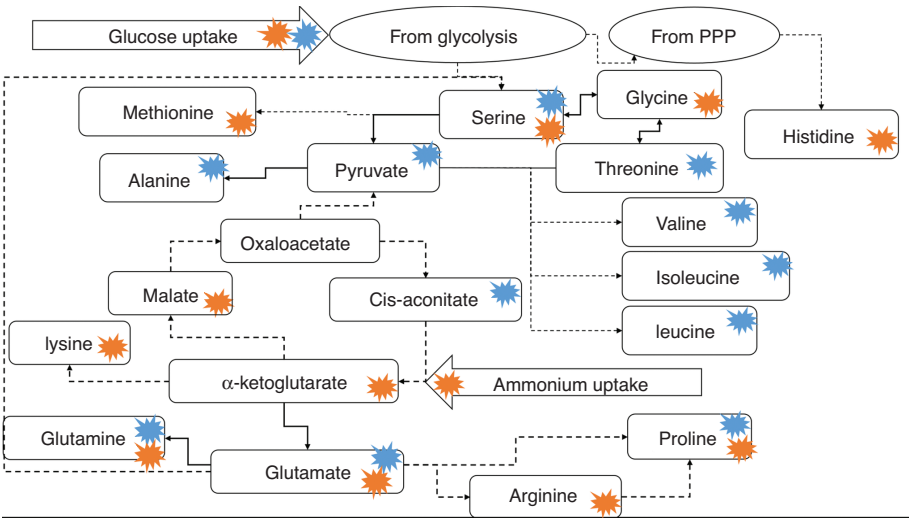
Pooling experiments by a single factor, irrespective of the other factor proved to be a very stringent criterion to identify differential expression at a threshold of p-value  $< 0.05$  post FDR correction (see Materials and methods for details) between air+ and air-, or between pH+ and pH- cultures. In fact, this was the primary indicator of the fact that the synergistic effects between the two factors had an important contribution to explaining the transcriptional landscape underlying these observations at the phenotypic and metabolic levels. The expression of only three genes were significantly affected as a response to dissolved oxygen availability, and these genes were significantly associated with establishing protein localization to membrane (p-value = 0.0118). The expression levels of 433 genes were responsive to controlling extracellular pH, although these genes were not significantly associated with a specific molecular function or biological process. However, the set was enriched for genes that are located in membrane-bound organelles (p-value =  $4.48 \times 10^{-6}$ ), and a subset of those genes were significantly associated with membrane rafts (p-value = 0.0168).

Analysis of variance showed that the expression level of 156 genes was significantly affected only by the level of aeration of the culture, and of 286 genes only by the

**Table 5:** Significant differences in intracellular amino acid and TCA cycle intermediate concentrations for the batch yeast cultures.<sup>a</sup>

Metabolite	Effect <sup>b</sup>	Response observed	p-Value
Alanine	Air/pH	Higher in pH- cultures for air- cases	$1.99 \times 10^{-2}$
Arginine	Air/pH	Higher in pH+ cultures for air+ cases	$1.10 \times 10^{-2}$
Glutamate	Air/pH	Higher in pH- cultures for air- cases Higher in pH+ cultures for air+ cases	$1.27 \times 10^{-3}$
Glutamine	Air	Higher in air+ cultures	$4.94 \times 10^{-3}$
	pH	Higher in pH+ cultures	$1.42 \times 10^{-3}$
Glycine	pH	Higher in pH+ cultures	$8.44 \times 10^{-3}$
Histidine	Air/pH	Higher in pH+ cultures for air+ cases	$8.24 \times 10^{-3}$
Isoleucine	Air	Higher in air+ cultures	$2.63 \times 10^{-3}$
	pH	Higher in pH- cultures	$6.40 \times 10^{-4}$
Leucine	Air/pH	Higher in pH- cultures for air- cases	$2.14 \times 10^{-3}$
Lysine	pH	Higher in pH+ cultures	$1.42 \times 10^{-2}$
Methionine	Air/pH	Higher in pH+ cultures for air- cases Higher in pH+ cultures for air+ cases	$1.49 \times 10^{-2}$
Proline	Air/pH	Higher in pH+ cultures for air+ cases	$3.16 \times 10^{-2}$
Serine	Air/pH	Higher in pH- cultures for air- cases Higher in pH+ cultures for air+ cases	$3.65 \times 10^{-4}$
Threonine	pH	Higher in pH- cultures	$9.24 \times 10^{-3}$
Valine	Air/pH	Higher in pH- cultures for air- cases	$6.75 \times 10^{-3}$
Malate	Air/pH	Higher in pH+ cultures for air- cases Higher in pH+ cultures for air+ cases	$1.14 \times 10^{-2}$
$\alpha$ -ketoglutarate	Air/pH	Higher in pH+ cultures for air- cases	$3.31 \times 10^{-3}$
Cis-acotinate	Air	Higher in air- cultures	$6.72 \times 10^{-3}$
Pyruvate	Air/pH	Higher in pH- cultures for air+ cases	$4.94 \times 10^{-3}$

<sup>a</sup>p-Values were determined by 2-way ANOVA (for samples with replicates), followed by the Tukey HSD test as an ad-hoc analysis. Pairwise comparisons are reported for the interaction effect. <sup>b</sup>Air, air only; pH, pH only; Air/pH, air – pH interaction effect.



**Figure 2:** Intracellular distribution of amino acids from TCA cycle precursors. The amino acids and their TCA cycle intermediate precursors are depicted in rounded rectangles connected either by solid lines indicating a single metabolic step or by dashed lines indicating multiple metabolic steps. The reversibility of the steps is indicated by the direction of the arrows. The blue banners denote metabolite concentrations that are significantly high at  $dO_2 > 90\%$  due to aeration effect, at low acidity due to pH effect, or at low acidity due to an interaction effect between culture pH and aeration levels. The orange banners denote metabolite concentrations that are significantly low at low acidity due to pH effect, or due to an interaction effect between culture pH and aeration levels. Metabolic pathways are represented in ovals. The block arrows represent the uptake of glucose and ammonium as the main carbon and nitrogen source macronutrients, respectively. The banners indicate, using the same colour codes, the significant differences in the amount of metabolite consumed in response to differences in the control of aeration and extracellular acidity.

extracellular pH of the cultivation environment. The transcription of 50 more genes were differentially affected by the interaction between the extracellular pH and dissolved oxygen availability. The 156 genes whose expression levels were affected only by the level of dissolved oxygen of the culture were not significantly associated with any biological process or molecular function, nor with any specific subcellular compartment of the cell, as classified by the Gene Ontology. On the other hand, a subset of the 286 genes, whose transcription levels were affected by extracellular pH, was significantly associated with actomyosin contractile ring organization ( $p\text{-value}=5.48\times 10^{-3}$ ), a cytokinesis-associated phenomenon. The subset of genes whose transcription was significantly altered in response to an interaction effect created by both oxygen availability and extracellular acidity were involved in growth-associated cellular mechanisms. These genes were significantly associated with cytoplasmic translation ( $p\text{-value}=0.0155$ ); more specifically, they were genes encoding proteins that constitute the cytoplasmic ribosome structure ( $p\text{-value}=2.20\times 10^{-4}$ ), and unsurprisingly, the gene products were significantly associated with the ribosome ( $p\text{-value}=4.00\times 10^{-4}$ ).

## Discussion

This paper investigated the synergetic effect of extracellular pH and oxygen availability on batch cultures of the Baker's yeast, and explored the underlying transcriptional and metabolic changes, which instigated the measured phenotypic responses of these cultures. The most striking phenotypic response was observed in the growth-associated parameters. Maintaining high oxygen levels and mildly acidic extracellular environment throughout the duration of the cultivation was observed to assist growth. This was apparent both by the high growth rates achieved, hence indicating faster growth, and in the high cell populations and biomass densities reached.

These differences that were observed in growth characteristics were complemented by differences in the ability of these cultures to consume (or uptake) macronutrients such as glucose and ammonium, and produce (and subsequently secrete) metabolic by-products such as ethanol and glycerol. Furthermore, the physiological response could also be captured by differences in the production and secretion of metabolic intermediates, particularly those of TCA cycle intermediates and amino acids. The extracellular concentrations of pyruvate, citrate, cis-acotinate, and isocitrate were responsive to differences in pH

and oxygen availability of the environment, as were those of alanine, methionine, cysteine, threonine, aspartate, arginine, and asparagine. These amino acids were able to employ only pyruvate and oxaloacetate as their biosynthetic precursors, along with ammonium. This could potentially imply that the upper TCA cycle was active and was coupled with dynamic secretion activity, which could be responsible for the exometabolic profiles observed for these cultures.

Broadly speaking, the concentration of TCA cycle intermediates and amino acids in the extracellular environment varied antagonistically in pH+ and pH- cultures. The amino acids that displayed significantly different exometabolic profiles across different conditions were of neutral character, except for arginine, which is strongly basic. On the other hand, the TCA cycle intermediates mentioned above, along with pyruvate, are able to support and maintain a low pH environment, since they all are acidic molecules. Whether the naturally occurring secretion of acidic compounds into a potentially compromised environment, where the pH was not controlled, escalated the severity of the adversely acidic conditions, or whether it is the susceptibility of the environment that induced over-secretion of acidic metabolites yet remains an open question.

A similar investigation of the endometabolic profiles highlighted the differences in the biosynthesis of compounds contributing to the intracellular amino acid pools, which would be essential for translation. These amino acids would be likened to currency amino acids required in the cell for growth and proliferation in contrast to those that were secreted out, which in a way, could serve as potential savings that could later be taken up from the extracellular environment. The concentration levels of 14 different amino acids were observed to vary in response to different levels of extracellular pH and oxygen availability, possibly indicating high adaptability and a global organisation of the amino acid biosynthetic routes to meet the demands of the cell exposed to different environmental conditions. Glutamate possibly served as the biosynthetic precursor of all of these amino acids, and it was likely to be produced from the TCA cycle intermediate  $\alpha$ -ketoglutarate, given the metabolic findings under the investigated conditions.

The differences observed around the TCA cycle and the amino acid metabolism at the metabolic level were not accompanied by differences at the transcriptional level of the genes associated with these metabolic pathways. This indicated that the transcriptional profiles of the cell populations, at the time of steady growth, were robust, and that the cell populations sustained relatively



constant transcription levels of central metabolic pathway enzymes despite substantial differences in environmental conditions. On the other hand, transcriptional differences were observed in the expression levels of genes involved in cytoplasmic translation, pointing towards the relevant cellular adjustments related to translation processes handled at the transcriptional level.

The synergistic effects of pH and oxygen availability would have been very difficult to determine during stationary phase of growth as yeast population growth is impeded primarily by the limitation in nutrient resources, in carbon in the case of the particular medium employed in this study, rather than low environmental pH or a limitation in extracellular oxygen levels. The stress imposed on the system would then instigate the manifestation of a three-factorial design problem, at the minimum; one including oxygen availability, pH and limiting nutrient availability investigated at different levels.

In conclusion, the synergy between environmental parameters emerged as the key effector of optimal growth and proliferation for eukaryotic cells, even at the level of the simplest eukaryote. This study explored the synergy between environmental pH and oxygen availability affecting yeast growth. It showed that this synergy, rather than the stand-alone effect of individual factors was responsible for most of the physiological changes, which were accompanied by an extensive rewiring of the cellular events manifesting both at the transcriptional level and at the metabolic level through the modulation of cytoplasmic translation coupled with the reorganisation in amino acid and their precursor pools. Design of experiments is often a crucial step in optimisation of bioprocesses to improve production systems in biotechnological, and particularly, in metabolic engineering applications. Yeast, a popular biotechnological workhorse, and an efficient cell factory, was thus shown to be impacted significantly and substantially by the synergy between cultivation parameters. This highlights the importance of selecting experimental design tools, which consider interaction effects in their evaluation, in order to exploit these biotechnologically relevant systems more efficiently to achieve improved bioprocess design leading to superior production performance.

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