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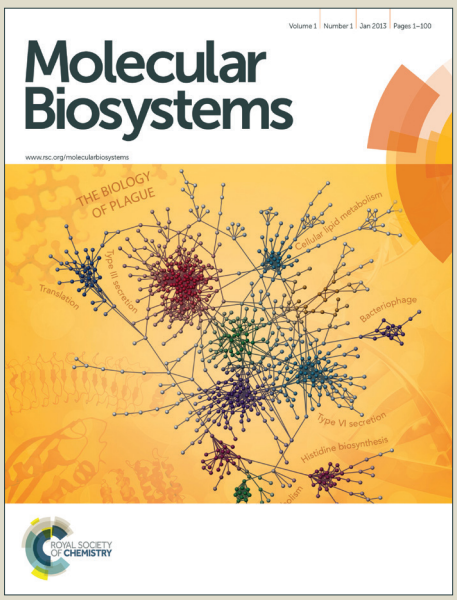

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ARTICLE

Can we predict the intracellular metabolic state of a cell based on extracellular metabolite data? †

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The analysis of extracellular metabolites presents many technical advantages over the analysis of intracellular compounds, which made this approach very popular in recent years as a high-throughput tool to assess the metabolic state of microbial cells. However, very little effort has been made to determine the actual relationship between intracellular and extracellular metabolite levels. The secretion of intracellular metabolites has been traditionally interpreted as a consequence of an intracellular metabolic overflow, which is based on the premise that for a metabolite to be secreted, it must be over-produced inside the cell. Therefore, we expect to find a secreted metabolite at increased levels inside the cells. Here we present a time-series metabolomics study of *Saccharomyces cerevisiae* growing on a glucose-limited chemostat with parallel measurements of intra- and extracellular metabolites. Although most of the extracellular metabolites were also detected in the intracellular samples and showed a typical metabolic overflow behaviour, we demonstrate that the secretion of many metabolites could not be explained by the metabolic overflow theory.

Keywords: Yeast, exometabolome, metabolic footprinting, metabolomics, metabolite profiling.

Introduction

Metabolite analysis is a very important aspect of all life sciences and of particular relevance to industrial biotechnology where cells are used as living factories to produce a wide range of chemicals.[1-3] With the development of metabolomics, metabolite analysis became the most robust, reproducible and cost-effective post-genomic tool to assess the metabolic state of living cells in a high-throughput manner.[4, 5] However, the analysis of intracellular metabolites is still a challenging task in metabolomics, mainly considering microbial, plant or animal cell cultures mostly due to a low biomass/culture medium ratio. Spent culture media are rich in extracellular metabolites and unused substrates, which pose a significant interference to the analysis of low concentrated intracellular compounds.[6, 7] Besides, the turnover rates of intracellular reactions are much higher than most reactions taking place outside the cell (e.g. uptake/secretion).[6, 7] Thus, for an accurate measurement of intracellular metabolite levels, a reliable method for quenching the cell metabolism without provoking leakage of intracellular metabolites is imperative.[6-8] Therefore, extracellular metabolites are more easily accessible for chemical analysis without the need of fast quenching procedures; and most importantly, the profile of extracellular metabolites contains a plethora of information regarding the metabolic state of the cells growing on that particular medium and environmental condition.[9]

The comprehensive profiling of extracellular metabolites, also known as metabolic footprinting or exometabolome analysis, has gained enormous popularity in last 10 years and have been widely used to assess and compare the metabolic state of different microbial systems.[10-12] However, little effort has been made to determine the relationship between intracellular and extracellular metabolite levels. Traditionally, the secretion of intracellular metabolites to the extracellular medium has been regarded as a consequence of intracellular metabolic overflow, where secretion of certain metabolic intermediates is observed due to the intracellular accumulation resulted from an overflow of specific metabolic pathways.[13-15] Thus, based on the metabolic overflow concept, a given metabolite is secreted to the extracellular medium when its intracellular levels increase, reflecting an increase in the metabolic flux of the pathway(s) associated to the production of that metabolite. But this phenomenon can only be truly observed experimentally by using dynamic metabolite level measurements of both intra and extracellular media under time series experiments, which is scarcely available. Carneiro *et al.*[16, 17] published time-series metabolomics experiments of *Escherichia coli* during the production of recombinant proteins and by assessing their data we observed that the concentrations of some extracellular metabolites

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† Electronic Supplementary Information (ESI) available: [Tables containing the list of all metabolites identified in extracellular and intracellular samples and their respective relative abundances]. See DOI: 10.1039/x0xx00000x

increased[16] despite their intracellular levels being decreased.[17] Similarly, Han *et al*.[18] also observed that the level of some extracellular metabolites increased whilst their intracellular level decreased when studying the response of *Candida albicans* to phenylethyl alcohol in a time series shake-flask experiment. Therefore, metabolic overflow must not be the sole cause of intracellular metabolite secretion during growth. To study this phenomenon, we carried out an experiment using the laboratory model yeast strain, *Saccharomyces cerevisiae* CEN.PK113-7D, which has been widely used in different omics studies.[19-22] Moreover, the change in microbial metabolism due to the environmental perturbation (specifically anaerobic to aerobic) is also widely studied and industrially relevant experimental conditions.[19, 20] Therefore, here we present a simple time-series experiment with *S. cerevisiae* growing on a glucose-limited chemostat to determine what is the relationship between the levels of intracellular and extracellular metabolites during *S. cerevisiae* response to environmental changes (anaerobic to aerobic growth).

Results and discussion

S. cerevisiae CEN.PK113-7D strain was grown on a glucose-limited chemostat (5 g/L) under anaerobic condition (**Figure 1**). Intra- and extracellular metabolomics samples (triplicates) were taken at anaerobic steady-state. Soon after oxygen was introduced to the system to reach 70% saturation, and individual samples were taken during the transition between anaerobic and aerobic steady-states (**Figure 1**). Finally, samples (triplicates) were harvested after three residential times at aerobic steady-state (**Figure 1**). Overall, we accurately identified 64 intracellular and 38 extracellular metabolites in our samples using an in house mass spectra library of metabolite standards (**Supplementary Tables 1** and **2**). Although this is still a small snapshot of the whole yeast metabolome, it is enough to establish some typical relationship between intra and extracellular metabolite levels. Not all metabolites detected in the intracellular samples were secreted to the medium, which was expected (**Figure 2** and **Supplementary Tables 1** and **2**). Despite most metabolites identified in the extracellular samples had been also detected intracellularly (**Figure 3** and **Supplementary Tables 1** and **2**), six metabolites were uniquely detected in the spent culture media (**Figure 4** and **Supplementary Tables 1** and **2**). Therefore, the intracellular levels of these six compounds were probably very low to be detected, which somehow question the concept of metabolic overflow unless their intracellular threshold is very low. Among all identified metabolites, 68 had their levels changed significantly ($p < 0.05$) between anaerobic and aerobic steady-states either in the intracellular medium, extracellular medium or in both as shown in **Figures 2 - 4**. It is clear that many metabolites such as alanine, glycine, lactate, citramalate, fumarate, and palmitate had their intracellular and extracellular levels changing in very similar pattern when comparing the time series events (anaerobic steady-state/transition/aerobic steady-state) (**Figure 3**); and many others showed similar trend when comparing the two steady-states alone such as citrate, succinate, valine, stearate, among others (**Figure 3**). However, aspartate, 2-oxoglutarate, benzoate and the fatty acid decanoate had their intracellular and extracellular levels changed in opposite directions (**Figure 3**), which does not conform to the metabolic overflow theory. The intracellular level of these four metabolites decreased significantly ($p < 0.05$) between anaerobic and aerobic growth, whilst their extracellular level significantly increased (**Figure 3**). Therefore, the yeast cells seem to have actively secreted these compounds in response to oxygen, which seems to be independent of an intracellular overflow. On the other hand, the intracellular level of glutamate, glutathione, pyruvate, GABA and others did not change significantly between anaerobic and aerobic steady-states (**Figure 4**); yet their extracellular level significantly increased (e.g. glutamate, glutathione, glutarate) or decreased (e.g. GABA, pyruvate, 2-isopropylmalate) in response to oxygen (**Figure 4**), which is also difficult to explain based on the intracellular metabolic overflow concept alone. The pattern of GABA, pyruvate, 2-isopropylmalate could be explained by their intracellular catabolism while maintaining a constant intracellular level. In contrast, the intracellular levels of hexanoate, octanoate, and the alkanes tridecane and dodecane significantly decreased in response to aerobiosis without significantly affecting their extracellular levels, showing that not always intracellular changes are reflected in the extracellular medium despite the metabolites being detected in both media.

To gain in-depth knowledge about metabolic changes of *S. cerevisiae* CEN.PK113-7D during two steady-states in response to oxygen, we performed a Pathway Activity Profiling (PAPi) analysis[23] (**Figure 5**). Our results clearly indicated a global up-regulation of at least 43 metabolic pathways that were associated with amino acid metabolism, carbohydrate metabolism, energy metabolism, lipid metabolism and other amino acid metabolism (**Figure 5**). Therefore, the metabolic activity of *S. cerevisiae* CEN.PK113-7D indeed changed in the direction of utilizing respiration pathways to produce energy in response to oxygen, which was an expected outcome. To verify this hypothesis and also to determine the key metabolites, we also performed a significance analysis using our data, which revealed the identification of five metabolites (malate, citramalate, lactate, succinate, and GABA) that belong to closely related metabolic pathways (**Table 1**). This again confirms the importance of respiratory pathways on *S. cerevisiae* growth during the aerobic steady state. Our findings were in agreement with Rintala *et al* [20] who reported a similar trend in a transcriptomics study using the same strain of *S. cerevisiae.* They compared the transcriptome with metabolic pathways and observed that the shift of the environment significantly impacted the metabolism of *S. cerevisiae* CEN.PK113-7D in the anaerobic steady-state with a down-regulation of fatty acids oxidation, peroxisome biogenesis, oxidative phosphorylation, TCA cycle and pentose phosphate pathway indicating the opposite trend of what we observed in our the aerobic steady state. Therefore, it is clear that *S. cerevisiae* CEN.PK113-7D up-regulate most of the central carbon metabolic pathways under aerobic condition.

It is noteworthy the general up-regulation of amino acid metabolism. This was an expected effect since *S. cerevisiae* under aerobic environment shift its metabolism from fermentation to respiration, increasing biomass production[24]. Moreover, the intra- and extracellular levels of most amino acids decreased, which agrees with an increased incorporation of them into structural proteins (e.g. valine, alanine and glycine; **Figure 3**). However, aspartate levels displayed an anti-metabolic overflow behaviour by showing an increased extracellular level in response to oxygen, whilst decreased levels inside the cell; which suggests a clear active secretion. Hess *et al.*[25] found that the ammonium ion is toxic for *S. cerevisiae* and *Saccharomyces bayanus* cells under potassium limitation. To detoxify, these microorganisms excrete amino acids to the extracellular environment in an attempt to remove ammonium from their cytoplasm. Therefore, Hess *et al.* study shows that an active efflux of amino acids that is not directly related to the metabolic overflow phenomenon could take place under specific environmental conditions.

In this study we found many metabolites that did not follow the metabolic overflow rule (**Figure 3**). We speculate that *S. cerevisiae* cells could use the extracellular medium as a storage space for at least four of them, from where cells could re-uptake these metabolites during the time of starvation. Pazcia *et al.*[26] also suggested a similar hypothesis where they categorised some metabolites as primary by-products that could be re-uptaken by the microbial cells at a later stage of the growth. Moreover, Giardina *et al.*[22] followed the changes in the secretome of *S. cerevisiae* under low and high glucose levels and found that some of the metabolic enzymes were present in the extracellular media depending on the growth conditions. Based on their observation, they hypothesized that extracellular media could be used as a storage place for those enzymes and, therefore, some primary metabolic reactions could take place in the extracellular medium. This would explain why some metabolites were found only in the extracellular medium and not inside the cells (**Figure 4** and **Supplementary Tables 1** and **2**). We can also assume that some metabolites could be bound to some of the enzyme that are secreted by the cells. The hypothesis of yeast cells utilising the extracellular medium as another cell compartment where metabolites could be "temporary stored' makes sense in a pure culture situation. However, it is not clear how this would work in a natural environment where there is competition for resources among microorganisms. Therefore, similar time-series metabolomics studies would be interesting to investigate when culturing *S. cerevisiae* in a mixed population of microorganisms. Nevertheless, *S. cerevisiae* is known to outcompete other microorganisms in its natural environment.[27] Giardina *et al.*[22] also suggested that some of the enzymes found extracelularly might have a different function than when inside the cell. Thereby, the extracellular metabolites could also have a different role in the extracellular medium such as cell regulation, cell-to-cell communication and quorum sensing.

Conclusions

It is not possible at this stage to speculate on which metabolites are exclusively secreted following an intracellular metabolic overflow and which ones are not. Neither can we infer any species-specific trends because the same metabolite (e.g. citramalate) showing anti-metabolic overflow behaviour in an early study with *C. albicans*[18], showed typical metabolic overflow behaviour in *S. cerevisiae* (**Figure 2**). Similarly, palmitate showed an anti-metabolic overflow behaviour in *E. coli* under fed-batch recombinant protein production[16, 17], and it behaved as typical metabolic overflow metabolite in our *S. cerevisiae* study (**Figure 2**). Moreover, we observed an active secretion of some metabolites independent of their intracellular levels. We speculate that the cell could be using the extracellular medium as a storage place to re-uptake the metabolites at a later stage. Another possibility is the use of extracellular medium as an additional cell compartment where some metabolites might have a different role than inside the cell such as cell-to-cell communication or metabolic regulation and detoxification. Nevertheless, we believe this present study clearly shows that intracellular metabolic overflow cannot explain the secretion of many intracellular metabolites to the extracellular medium, and further studies are required if we want to be able to accurately assess the metabolic state of a cell based on its extracellular metabolite profile data.

Experimental procedures

Chemicals. All chemicals used in this study were of analytical grade. Methanol, chloroform, sodium bicarbonate, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). The internal standard L-alanine-2,3,3,3-d_4, the derivatization reagent methylchloroformate (MCF), pyridine, D-glucose and, glycerol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous sodium sulfate was obtained from Fluka (Steinheim, Germany).

Yeast strain and culture conditions. *S. cerevisiae* CEN.PK113-7D was maintained on YPD agar plates, containing yeast extract (6 g/L), peptone (3 g/L), dextrose (10 g/L), and agar (15 g/L), incubated at 30°C. Pre-cultures were grown aerobically in shake flasks in a shaking incubator at 30°C and 200 rpm for ~24 h. We used a chemically defined mineral medium containing 20 g/L glucose[28], where the carbon source is the first limiting nutrient during batch growth. The continuous fermentation was carried out initially under anaerobic conditions in a 5-L Labfors 5 bioreactor system from INFORS HT (Bottmingen, Switzerland), with a working volume of 2 L. Nitrogen flow was set to 0.2 L/min. The temperature was kept constant at 30°C and pH was regulated to 5.0 using 2M NaOH. Exponentially growing pre-inoculum was used for inoculation of the bioreactor with an initial OD_{600} of 0.1. Before inoculation, the yeast cells were centrifuged (3220 *g*, 2 min) and washed once with non-inoculated medium. The fermentation started as a batch-phase culture stirring at 300 rpm (**Figure 1**). Once the glucose level was exhausted, the bioreactor was switched to continuous culture. Continuous culture was set up using the same medium described for the batch phase except with a glucose concentration of 5 g/L, KH_2PO_4 (6 g/L) and dilution rate at 0.3 h^{-1} (**Figure 1**). Continuous

culture was left for two residence times (two working volume of the bioreactor) before samples (n=3) were harvested for intra and extracellular metabolite analysis (**Figure 1**). Then, oxygen was introduced into the bioreactor by replacing nitrogen with compressed air until reaching 70% oxygen saturation (**Figure 1**). Five samples were harvested for intra- and extracellular metabolite analysis during this transition period (every 10 min). Once the working volume was recovered and aerobic steady-state was reached, aerobic continuous culture was left for three residence times before samples (n=3) were harvested for intracellular and extracellular metabolite analysis (**Figure 1**). The optical density (OD) was monitored every hour to ensure that the cells were in a "steady state".

Sampling and extraction procedure for intracellular metabolite analysis. The sampling, quenching, and intracellular metabolite extraction were based on our previously published protocol[20]. In summary, 30 mL of culture broth (n=3) were harvested and quenched from the bioreactors by rapidly mixing the samples with cold glycerol-saline solution (3:2) followed by centrifugation at -20°C. The cell pellets were resuspended in cold glycerol-saline washing solution (1:1) followed by centrifugation at -20°C. Intracellular metabolites were extracted from the cell pellets after addition of cold methanol-water solution (1:1) at -30°C and the internal standard (2,3,3,3-d_4-alanine), followed by three freeze-thaw cycles. All extraction steps were carried out at -20°C or below. Sample containing extracted intracellular metabolites were freeze-dried using a VirTis freeze-dryer from SP Scientific (Newtown Square, PA, USA). During the transition state from the anaerobic to the aerobic environment, one 30 mL sample was collected every 10 min for a total of 50 min (**Figure 1**).

Extracellular metabolite analysis. Culture broth (10 mL) was harvested from the bioreactor and was immediately filtered using a membrane filter (0.2 µm pore size). The filtrate was then separated into 3 aliquots (2 mL), and 20 µL of the internal standard (2,3,3,3-d_4-alanine) was added to each of them. The samples were frozen and subsequently freeze-dried on a VirTis freeze-dryer (SP Scientific, Newtown Square, PA, USA).

Chemical derivatization and GC-MS analysis. The freeze-dried intracellular and extracellular samples were resuspended using 200 µL of NaOH (1M) and derivatized using the methylchloroformate (MCF) method according to our standard laboratory procedure described previously.[29] The derivatized samples were analyzed by GC-MS according to the parameters established previously[29] using a GC7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a MSD5975 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Metabolite identification and data analysis. AMDIS software was used for deconvoluting GC-MS chromatograms and identifying metabolites using an in-house MCF mass spectra library. The identifications were based on both MS spectrum of the derivatized metabolite and its respective chromatographic retention time. The relative abundance of identified metabolites was determined by ChemStation (Agilent Technologies, Santa Clara, CA, USA) by using the GC base-peak value of a selected reference ion. These values were normalized by the biomass content in each sample as well as by the abundance of internal standard (2,3,3,3-d_4-alanine). The extracellular metabolites with traces amounts found also in the non-inoculated medium had their level readjusted by subtracting the respective amounts found in the non-inoculated medium samples before data normalization by biomass. A students' t-test was applied to determine whether the relative abundance of each identified metabolite was significantly different between growth conditions (anaerobic vs. aerobic steady-states). The entire data mining and data normalization were automated in R software as described previously[29, 30]. We performed a significance analysis using the web-based platform Metaboanalyst 2[31] Moreover, a Pathway Activity Profiling (PAPi) analysis[23] was undertaken to generate hypotheses about the significant metabolic changes (p<0.05) in S. *cerevisae* CEN.PK113-7D in response oxygen.

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Figure captions

Figure 1. Experiment overview. *Saccharomyces cerevisiae* was cultured in a 5-L bioreactor on glucose limited condition starting as 10 g/L of glucose (batch phase), under anaerobic conditions, 30^{o}C and pH 5. The bioreactor was switched to continuous culture once glucose level was exhausted. Continuous culture was carried out with 5 g/L of glucose and dilution rate at 0.3 h^{-1}. Samples for metabolome analysis were harvested at anaerobic steady-state (after two residence times), during the transition between anaerobic and aerobic steady states (at 10 min intervals) and at aerobic steady-state (after 3 residence times). In the figure, "intra" and "extra" refer to number of samples taken for intracellular and extracellular metabolite analysis respectively. O.D: Optical density at 600 nm.

Figure 2. Metabolites which intracellular levels (red) changed significantly ($p < 0.05$) in response to oxygen. These metabolites were not detected in the extracellular medium or their extracellular levels (green) did not change significantly. Metabolite levels shown on Y axes refer to the relative abundance of their GC-MS base peak after normalization by the internal standard (2,3,3,3-d_4-alanine) and biomass concentration. Ana: anaerobic steady-state; T1 to T5: Samples harvested during the transition between anaerobic and aerobic steady-states; Aer: aerobic steady-state. (Only representative metabolites are shown)

Figure 3. Metabolites which intracellular (red) and extracellular (green) levels changed significantly ($p <0.05$) in response to oxygen. Metabolite levels shown on Y axes refer to the relative abundance of their GC-MS base peak after normalization by the internal standard (2,3,3,3-d_4-alanine) and biomass concentration. Ana: anaerobic steady-state; T1 to T5: Samples harvested during the transition between anaerobic and aerobic steady-states; Aer: aerobic steady-state. (Only representative metabolites are shown)

Figure 4. Metabolites which extracellular levels (green) changed significantly ($p < 0.05$) in response to oxygen. These metabolites were not detected in the intracellular samples or their intracellular levels (red) did not change significantly. Metabolite levels shown on Y axes refer to the relative abundance of their GC-MS base peak

after normalization by the internal standard (2,3,3,3-d_4-alanine) and biomass concentration. Ana: anaerobic steady-state; T1 to T5: Samples harvested during the transition between anaerobic and aerobic steady-states; Aer: aerobic steady-state. (Only representative metabolites are shown)

Figure 5. Predictive metabolic pathway activities of *Saccharomyces cerevisiae* based on intracellular metabolite profiling data obtained from yeast cells growing in a glucose-limited chemostat in the presence and absence of oxygen. Metabolite pathway activity under anaerobic steady-state were set to 0 (reference blue line). Red line indicates metabolic activities in cells under aerobic steady-state. The change in relative metabolic activity was plotted using a $\log_2$ scale. Positive values indicate the metabolic pathways had their activity up-regulated in response to oxygen. Only the pathways generating statistically significant scores (p-value<0.05) are shown here.

Table 1: List of metabolites short-listed by Significance analysis using metabolomics data of *Saccharomyces cerevisiae* grown in a glucose-limited chemostat under aerobic and anaerobic conditions.

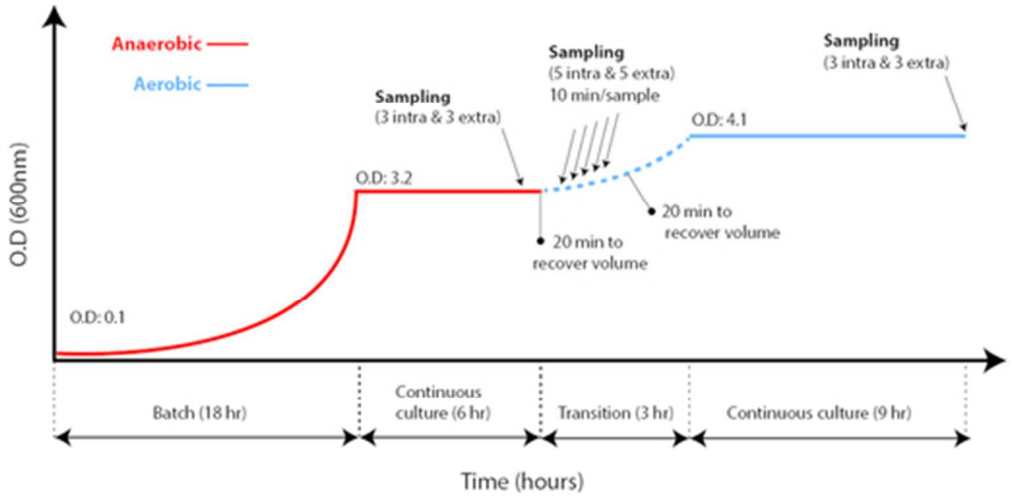

Figure 1. Experiment overview. Saccharomyces cerevisiae was cultured in a 5-L bioreactor on glucose limited condition starting as 10 g/L of glucose (batch phase), under anaerobic conditions, 30oC and pH 5. The bioreactor was switched to continuous culture once glucose level was exhausted. Continuous culture was carried out with 5 g/L of glucose and dilution rate at 0.3 h-1. Samples for metabolome analysis were harvested at anaerobic steady-state (after two residence times), during the transition between anaerobic and aerobic steady states (at 10 min intervals) and at aerobic steady-state (after 3 residence times). In the figure, "intra" and "extra" refer to number of samples taken for intracellular and extracellular metabolite analysis respectively. O.D: Optical density at 600 nm.
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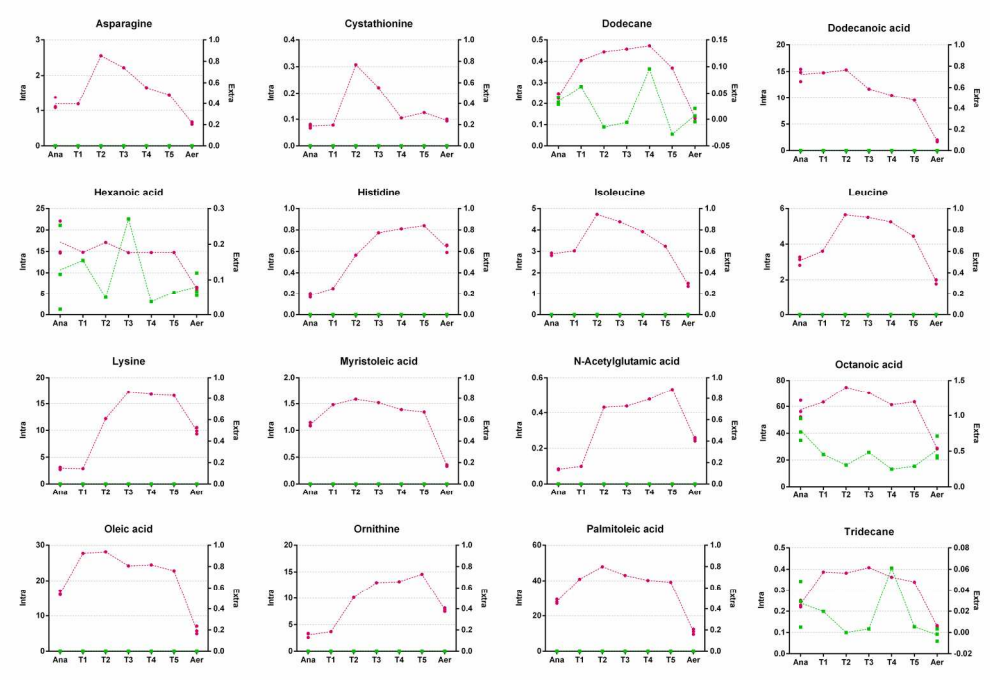

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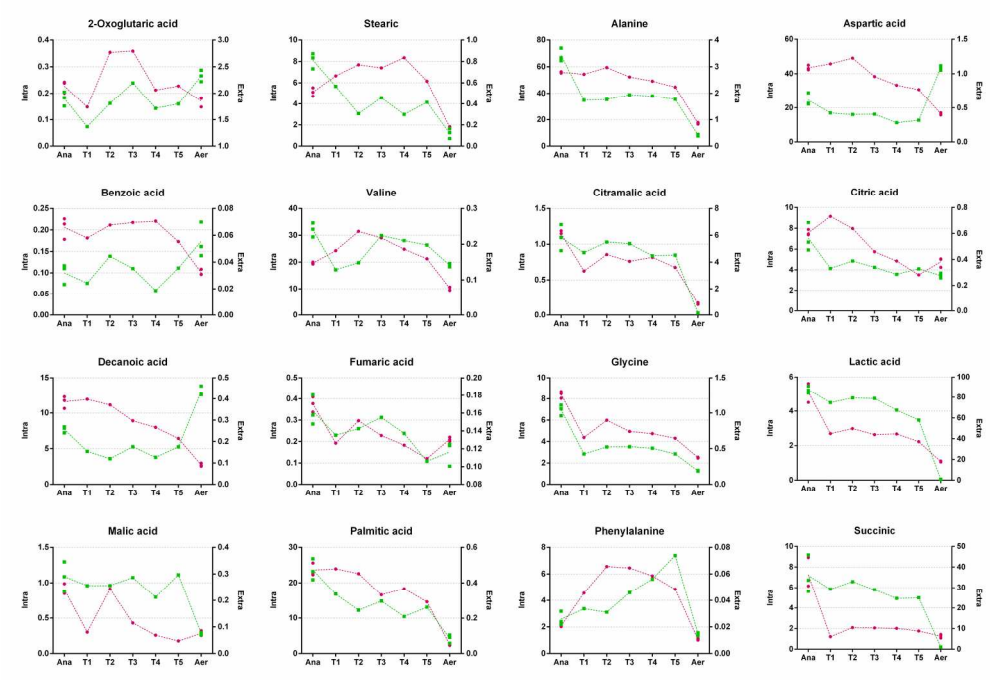

Figure 3. Metabolites which intracellular (red) and extracellular (green) levels changed significantly ($p < 0.05$) in response to oxygen. Metabolite levels shown on Y axes refer to the relative abundance of their GC-MS base peak after normalization by the internal standard (2,3,3,3-d4-alanine) and biomass concentration. Ana: anaerobic steady-state; T1 to T5: Samples harvested during the transition between anaerobic and aerobic steady-states; Aer: aerobic steady-state. (Only representative metabolites are shown)
207x142mm (300 x 300 DPI)

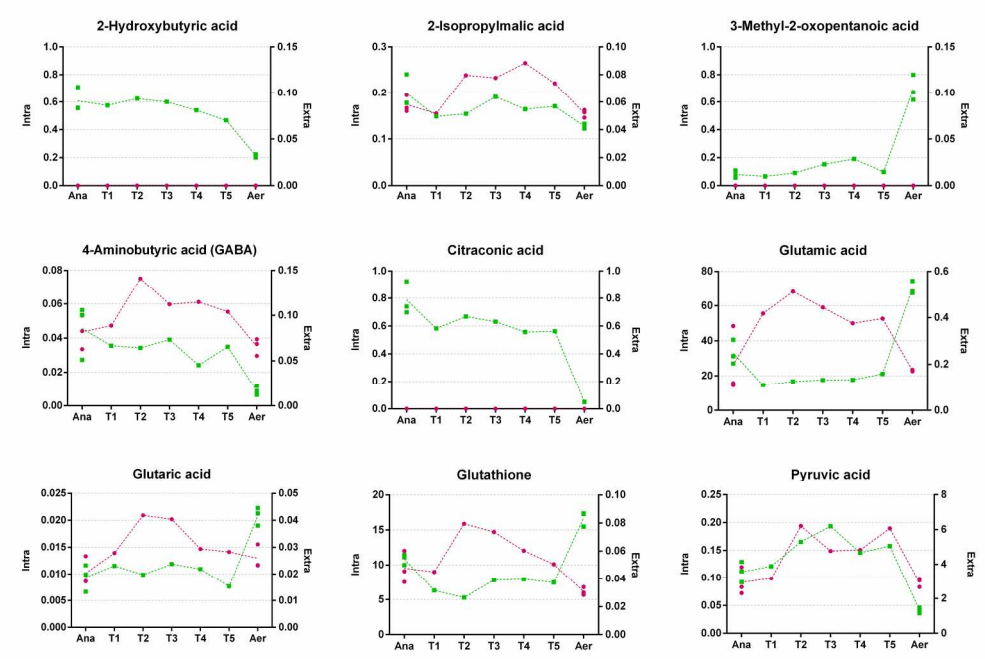

Figure 4. Metabolites which extracellular levels (green) changed significantly ($p < 0.05$) in response to oxygen. These metabolites were not detected in the intracellular samples or their intracellular levels (red) did not change significantly. Metabolite levels shown on Y axes refer to the relative abundance of their GC-MS base peak after normalization by the internal standard (2,3,3,3-d4-alanine) and biomass concentration. Ana: anaerobic steady-state; T1 to T5: Samples harvested during the transition between anaerobic and aerobic steady-states; Aer: aerobic steady-state. (Only representative metabolites are shown)
203x138mm (300 x 300 DPI)

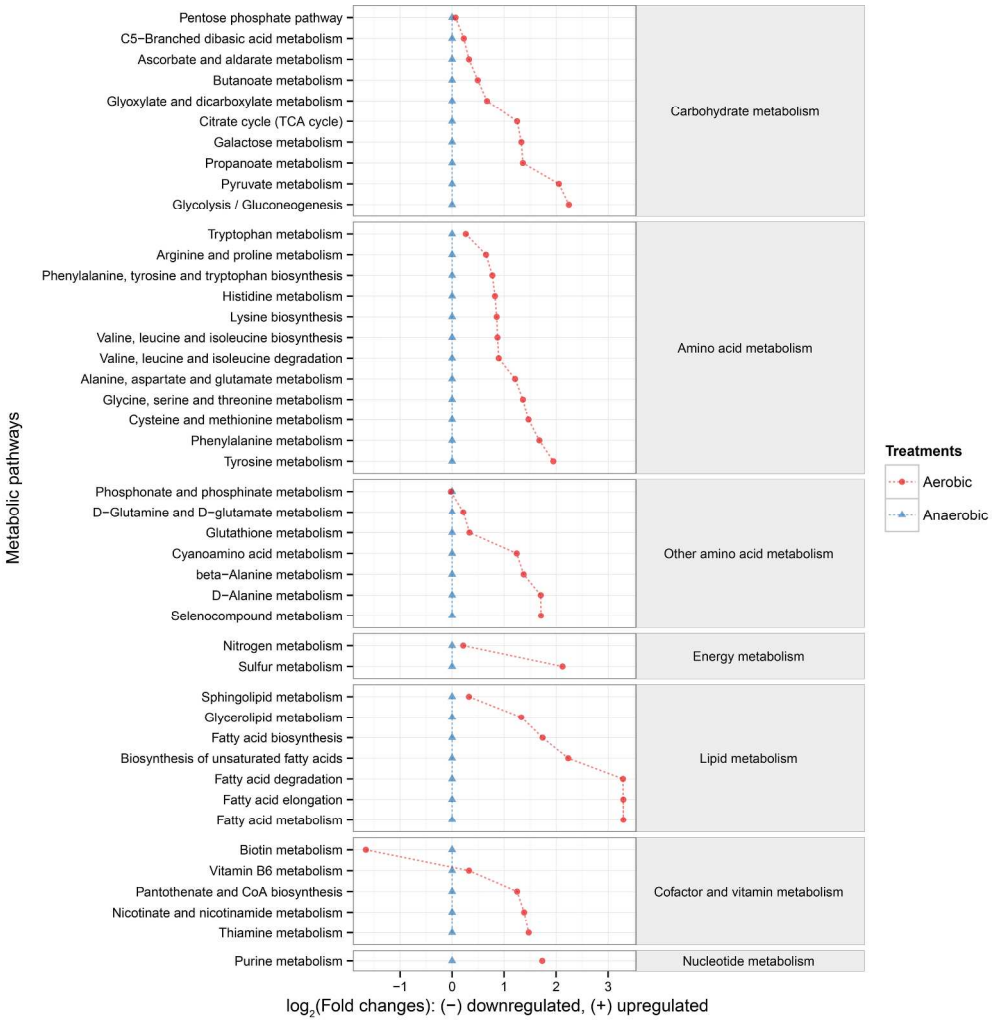

Figure 5. Predictive metabolic pathway activities of Saccharomyces cerevisiae based on intracellular metabolite profiling data obtained from yeast cells growing in a glucose-limited chemostat in the presence and absence of oxygen. Metabolite pathway activity under anaerobic steady-state were set to 0 (reference blue line). Red line indicates metabolic activities in cells under aerobic steady-state. The change in relative metabolic activity was plotted using a log2 scale. Positive values indicate the metabolic pathways had their activity up-regulated in response to oxygen. Only the pathways generating statistically significant scores (p-value<0.05) are shown here.
247x251mm (300 x 300 DPI)

Table 1: List of metabolites short-listed by Significance analysis using metabolomics data of *Saccharomyces cerevisiae* grown in a glucose-limited chemostat under aerobic and anaerobic conditions.

Metabolite	d-value	Standard deviation	Raw *p*-value	q-value	Associated pathway/s
Citramalic acid	2.1099	0.23247	0.000286	0.02	C5-Branched dibasic acid metabolism
Succinic acid	2.061	0.24447	0.000571	0.02	TCA cycle, pyruvate metabolism, Alanine, aspartate and glutamate metabolism, phenylalanine metabolism
Lactic acid	1.6944	0.33539	0.000857	0.02	Pyruvate metabolism, fructose and mannose metabolism
4-Aminobutyric acid (GABA)	1.3744	0.41311	0.003571	0.0625	Alanine, aspartate and glutamate metabolism
Malic acid	1.3025	0.42987	0.010714	0.15	TCA cycle, Glyoxylate and dicarboxylate metabolism, pyruvate metabolism