Bacteria Fermentation: Identifying Critical Components of Peptone Fermentation Media

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ABSTRACT

In any fermentation or bioproduction process it is useful to understand the compounds and processes that are involved at any point during the growth. The global IROA labeling protocol (Figure 1) provides an ideal mechanism to achieve this with unequalled sensitivity and accuracy. In this study we examine a fungal fermentation and examine the metabolic changes occurring in the cells and in the media. They both are very complex pictures as the cells move through early lag phase through log, and into senescence. Biochemically these stages are extremely well differentiated from one another. The metabolite fluxes are clearly quite different depending on the stage of fermentation. The examination of the media also demonstrates a very complex picture; not only is it straight-forward to witness the depletion of nutrient components, but to also monitor with complete certainty, the complex assortment of compounds exported by the cells not only as final products but also as adjuvants to the cells growth. In addition to completely characterizing each state, these data make it possible, in a single experiment, to pinpoint metabolic bottlenecks, nutrient depletions, and wasteful production. The IROA protocol provides an ideal way to follow any fermentation.

The primary and surprising finding of this preliminary study was the extremely dynamic interaction that was witnessed in the media, wherein a sole carbon source was fed to the yeast and the yeast transformed it so rapidly that at the first time point there were already a number of compounds derived from it. These compounds were then consumed by the yeast in later stages of its growth. Other compounds were exported into the medium at later stages, some were later consumed, and some accumulated. The IROA protocol made these changes obvious and highly quantifiable. The dynamic nature of any fermentation may easily be missed without the use of similar techniques.

BACKGROUND

Metabolic profiling is a useful analytical approach which can be applied to the rational development of industrial cell culture processes[1-8]. The most difficult aspect of most metabolic profiling experiments is the ability to separate peaks of biological origin from artifactual peaks, and once identified as biological, to accurately identify and quantitate the peaks of interest. The IROA protocol[9,10] (Figure 1), utilizes isotopically-defined media in which all nutrients are labeled with either 5%^{13}C, "C12 IROA media" (experimental), or 95%^{13}C, "C13 IROA media" (control), so that all biological compounds carry a distinct molecular signature and can be distinguished from each sample set, experimental or control, as they have differing masses. Therefore, experimental variability may be removed by prepping and analyzing control and experimental samples as a single composite sample. IROA software can readily find all biological compounds as they will have two perfectly paired peaks (see Figure 2 - each IROA envelope is half control and half experimental) and remove unlabeled natural abundance artifacts. The IROA software performs a scan-by-scan analysis of the LC-MS dataset and identifies IROA peaks based on their extended isotopic envelopes. The ratio of peak pairs quantitates the response relative to the control. The distance between these peaks readily identifies the number of carbons in the compound. IROA software also calculates molecular formula based on compound number and accurate mass. The experimental design embedded in the IROA software and the complete dataset may be submitted to the IROA portal for an automated analysis, to identify all statistically significant compounds, significant patterns of growth, and statistically significant processes involved.

OBJECTIVES

Apply the Basic IROA protocol, software and portal to grow and characterize two cultures of Saccharomyces cerevisiae in order to test the ability of IROA to discriminate between fermentation phases by examination of the biochemical responses that are present in each phase.

METHODS

Sample Generation: Following the Basic IROA protocol, two yeast cultures (Saccharomyces cerevisiae S288C)[11] were maintained in log phase growth by daily transfer (50 ul into 50 ml fresh media) for a two day period prior to the experiment in media containing the sole carbon source. Prior to the experiment the glucose was unlabeled, upon starting the experiment the carbon source in the fresh media contained either 5% U-^{13}C glucose* ("experimental") or 95% U-^{13}C glucose* ("control"). After one day of labeled media growth, the experiment was initiated by transfer into fresh 5% U-^{13}C glucose media, and aliquots of six experimental time points during the fermentation were collected from the 5% U-^{13}C glucose fermentation "experimental"), immediately centrifuged, decanted, and both the supernatant and pellet put on ice. The six time points, and the aliquot volumes taken were: T=+2hr (early lag – 3.3 ml), T=+4hr (late lag – 3 ml), T=+8hr (early log – 2 ml), T=+24 (late log – 1ml), +48 (early stationary – 1 ml), and +72hr (late stationary – 1 ml). Twenty-four hours after their second transfer into 95% U-^{13}C glucose media, an equal number of 1 ml aliquots were taken from the 95% U-^{13}C glucose ("control") mother flask. All aliquots were specifically taken from each 50 ml mother flask during shaking to assure settling did not cause an uneven distribution within the samples. The media samples were created by mixing equal (500 ul) volumes of experimental and control supernatants. The pelleted samples were washed by suspension in buffered saline, and repelleted. The washed control samples were resuspended and added to the experimental pellets, the pooled sample was vortexed, and the mixed sample repelleted. These pooled IROA pellet and media samples frozen at -80° C until analysis. *Produced especially for IROA Technologies by Cambridge Isotope Laboratories (CIL).

METHODS

Sample Preparation: Pooled cell pellet samples were homogenized in a mini-ball mill. Into each sample 400 ul of 40% aqueous methanol, ~270 mg of 0.7 mm zirconia beads (Biospec.com), and two 3mm borosilicate beads (Kimble Chase) was added. Samples were then homogenized for 10 minutes (at the maximum rate) and centrifuged for 10 min at 4750 RPM. 300 uL of supernatant was withdrawn to a filter-plate, and filtered. The resulting filtrate was dried under a gentle 30° nitrogen stream. The dried sample was brought up in 50 ul of distilled water for analysis. Pooled media samples were filtered and treated as above.

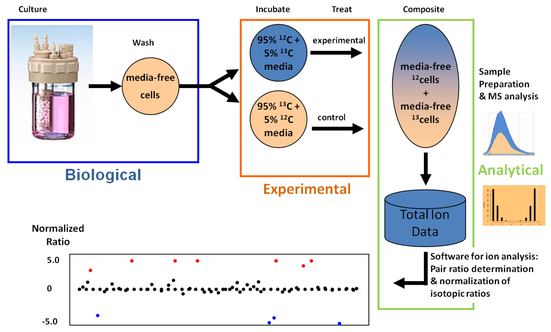

Figure 1: The "Basic" IROA method grows isotopically labeled cells which are then treated experimentally. When pooled cells are processed the signals for the compounds from both the control and experimental cells may be distinguished and differences between the ratio of their areas are directly indicative of the ratio of the respective sizes of their metabolic pools. Pooling the cells reduces sample-to-sample variance, and increases data quality. Outliers to the normalized ratios are metabolic pools that are impacted by the experimental treatment.

Mass Spec Analysis: Samples were stored at 4° C during mass spec analysis. Mass Spec analysis was performed on an Agilent 1200 LC/6530 QTOF LC-MS system. The LC system consisted of an Agilent 1200 Binary Pump, an h-ALS-SL cooled sample compartment, and a TCC-SL column heating compartment. An injection of 7 µL for positive mode 12µL for negative mode of sample was injected and chromatographed as follows: A Waters (Millford, MA, USA) Acquity UPLC HSS-T3 column (1.8 µm, 2.1 x 50 mm) with the Accquity VanGuard HSS T-3 pre-column (1.8 µm) was used. The column was heated to 40 C for separation of metabolites. For positive mode, the mobile phase was A: house distilled water with 0.1% Formic acid, and B: Methanol (JT Baker, LC-MS grade) with 0.1% Formic acid. For negative mode the mobile phase was: A: distilled water with 0.1% Ammonium Bicarbonate (Sigma) and B: 95% Methanol, 5% distilled water, 0.1% Ammonium Bicarbonate. The LC gradient was: 1% B (isocratic, 0.5 min), linear gradient from 1-99%B, (1.5 min), 99%B (isocratic, 4 min), 1%B (ramp and hold 3 minutes to return to starting conditions.) The flow rate for both positive and negative mode was: 0.35 ml/min.

Data Processing: The Agilent raw datafiles ".D" file we exported as mzdata files which were imported directly into the IROA Software Program and IROA portal.

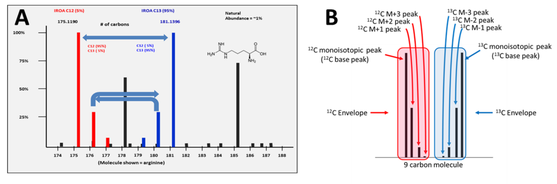

Figure 2: With the IROA protocol where the percentage of ^{13}C is increased to 5%, the M+1 for a six-carbon molecule is significantly larger, namely 32%. Likewise, when the percentage of ^{13}C is increased to 95%, then the M-1 for a six-carbon molecule is also 32% the height of the monoisotopic peak. The number of carbons in a biological molecule can be also determined by the distance between the two monoisotopic peaks, ^{12}C and ^{13}C. Since the relative height of the M+1, the relative height of M-1, and the distance between the monoisotopic peaks all provide confirmation of this fact, this results in a triply redundant quality control check point. Also, the shape of the entire isotopic envelope is different for every number of carbons. Moreover, knowledge about the number of carbons present in an unknown molecule the ability to calculate the remaining elements is significantly enhanced; indeed for molecules with masses below 450 the formula is generally unique; artifacts and noise will contain no labeling signatures, no false peaks, and removing sample-to-sample variance including ion suppression.

RESULTS

All time-points (2, 4, 8, 24, 48 and 72 hours) were measured relative to the 24 hour, or mid-log-phase state, and were biochemically differentiated from one another. In this study 455 IROA peaks were identified between positive mode (289 compounds) and negative mode (166 compounds) analyses of the yeast pellet extracts. The media extracts yielded fewer compounds, as expected, 120 total IROA peaks (62 from positive mode, and 58 from negative mode). A variety of bioinformatics techniques including: Nonnegative Matrix Factorization (NMF), Principal Component Analysis (PCA), and Random Forest (RF) all yielded clean separations of each time-point. Linear regressions on the compounds showed many significant compounds (p <.01); 83 of 120 and 338 of 445 were significant; the top 40 were arbitrarily selected with p values <2.1E-6 (media) or <7.3E-7 (pellets). Elevated glutamine levels were consistent with T48/T72 elevated levels of adenosine monophosphate and conversion of kynurenine to NAD (de novo synthesis of AMP and NAD via kynurenine and de novo NAD+ biosynthesis pathways)[12-13] as well as increased levels of n-acetylglutamate and ornithine (key intermediates in the energetically more economical "acetyl recycling" arginine biosynthetic pathway)[14].

Random Forest (RF), a supervised ensemble learning method for classification, when applied to this dataset had less than a 5% OOB error in its identification over six groups (Figure 3A & B), and defined a number of important compounds in each group. Principal Components Analysis (PCA – Figure 3 C & D – unsupervised), cleanly separated the samples representing the media and the pellets.

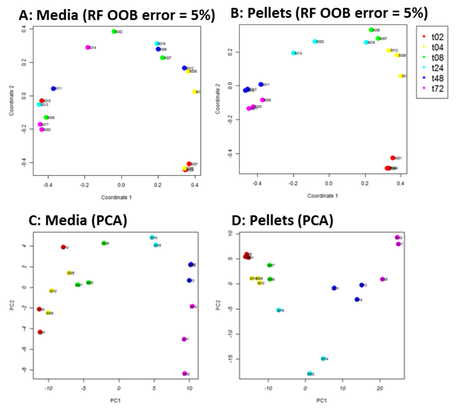

Figure 3 A & B: Random Forest and C&D: Principal Components Analysis

The 40 most significant compounds all had p values <2.1E-6 (media) or <7.3E-7, and this subset was an arbitrarily large enough group to be representative of the major trends but small enough that it could be analyzed as a single body (see Figure 3). The correlation matrices (Figure 3 A & B) of these compounds over all phases, organized hierarchically (Figure 3 C & D), suggests the 40 most significant compounds cluster into two groups and that of the compounds in both are either highly correlated with one another or anti-correlated with one another.

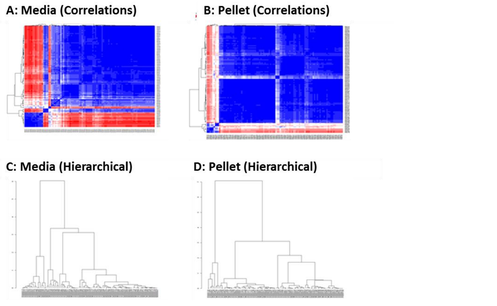

Figure 4: Relationships within the 40 most statistically significant metabolite responses.

In Figure 5, compounds A, B, and C demonstrate the most common pattern in which the production of the compound increases throughout the fermentation. Compounds D, E, and F demonstrate a common consumption pattern, reflecting early glucose activity. G, H, & I represent other patterns found. The strong red and blue patterns seen in the correlation matrices (Figure 3) are primarily the result of the first two patterns. The white areas are representative of these other temporal types.

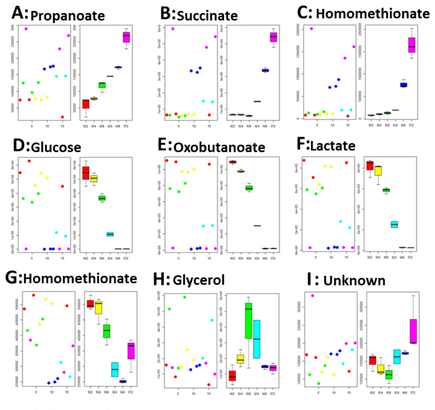

Figure 5: Temporal Patterns of metabolites found in media

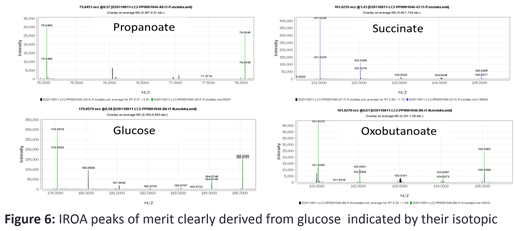

Figure 6: IROA peaks of merit clearly derived from glucose indicated by their isotopic patterns.

Random forest identified top significant time course metabolites measured relative to 24-hour, mid-log phase as seen in Figure 8: A-media glucose (key carbon nutrient), glucosamine, citrate[d] (TCA cycle intermediate), hydroxy glutamate, propanoate, fructose 6-phosphate (Pentose phosphate pathway)[c], oxobutanoate; B-cell pellets) hypoxanthine[a], leucine (amino acid)[a], phenylalanine (amino acid)[a], oxidized glutathione[a], adenosine monophosphate[b] (de novo NAD biosynthesis), ornithine (biosynthetic intermediate)[b], glutamine[c] and n-acetyl-glutamate (precursors in the synthesis of glutamate and AMP), succinate (TCA pathway)[d], cytidine[e], guanosine[e], uracil, kynurenine. (a) levels tend to rise in all forms of starvation; (b) levels tend to rise in carbon starvation but fall in nitrogen starvation; forms of starvation; (d) tend to rise in yeast starvation, and (e) levels are known to rise in nitrogen starvation but to fall in carbon starvation. Results correlated with studies reported by Brauer *et al*.[15]

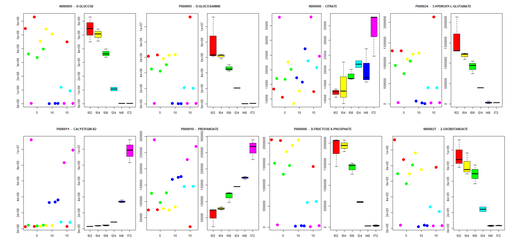

Figure 8A : Media

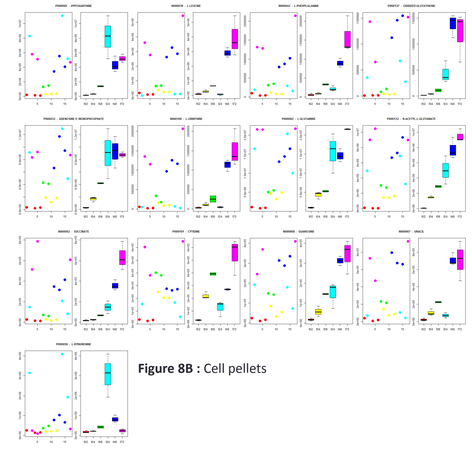

Figure 8B : Cell pellets

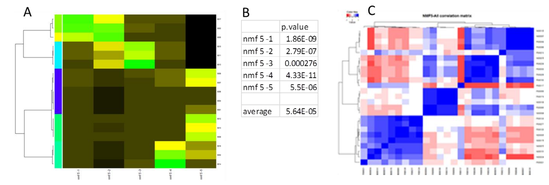

Figure 8: NMF (k=5) of the compounds found in the cells interact as five significant subsystems.

CONCLUSIONS

This study was preliminary in nature and a test of the ability to discriminate between fermentation phases by examination of the biochemical responses that are present in each phase. The biochemistry of each stage of the fermentation was easily discovered using the IROA protocol. In a fully non-targeted analysis 455 compounds were discovered and their temporal patterns were easily discovered. The analysis of the media demonstrated that there is a complex interplay of compounds continuously produced, and continuously consumed by the fermenting organism. Contrary to initial assumptions the media appears not to be initially consumed and then a dumping ground for cellular waste but rather a medium in which a cell is able to adapt and use its environment as a strategy for growth.

REFERENCES

1. Creek DJ, Nijagal B, Kim DH, Rojas F, Matthews KR, Barrett MP. 2013. Metabolomics guides rational development of a simplified cell culture medium for drug screening against *Trypanosoma brucei*. Antimicrob Agents Chemother. 57(6): 2768-79.
2. Hasunuma T, Sanda T, Yamada R, Yoshimura K, Ishii J, Kondo A. 2011 Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae.* Microbial Cell Factories 10(2):1-13.
3. Schilling BM, Abu-Absi S, Thompson P. 2012 BioProcess Intl. 10(1): 42-49.
4. Goudar CT, Biener R, Oiret JM, Konstantinov KB. 2007. Metabolic Flux estimation in mammalian cell cultures. Meth in Biotech 24: 301-317.
5. Fazenda ML, Dias JML, Harvey LM, et al. 2013. Toward better understanding of an industrial cell factory: investigation the feasibility of real-time metabolic flux analysis in *Pichia pastori* Microbial Cell Factories 12(51):1-14.
6. Selvarasu S, Ho YS, Chong WP, et al. 2012. Combined in silico modeling and metabolomics analysis to characterize fed-batch CHO cell culture. Biotechnol Bioeng.109: 1415–1429.
7. Chong WP, Goh LT, Reddy SG, et al. 2009. Metabolomics profiling of extracellular metabolites in recombinant Chinese Hamster Ovary fed-batch culture. Rapid Commun. Mass Spectrom. 23: 3763–3771.
8. Dietmair S, Hodson MP, Quek LE, et al. 2012. Metabolite profiling of CHO cells with different growth characteristics. Biotechnol Bioeng 109:1404–1414.
9. de Jong FA, Beecher C. 2012. Addressing the current bottlenecks of metabolomics: Isotopic Ratio Outlier Analysis (IROA®), an isotopic-labeling technique for accurate biochemical profiling, Bioanalysis. 4(18):2303-14.
10. Stupp GS, Clendinen CS, Ajredini R, Szewc M, Garrett T, Menger RF, Yost RA, Beecher C, Edison A. 2013. Isotopic Ratio Outlier Analysis Global Metabolomics Of *Caenorhabditis elegans*. Anal Chem. 85(24): 11858-11865.
11. Mortimer RK and Johnston JR. 1986.Genealogy of principal strains of the Yeast Genetic Stock Center. Genetics 113: 55-43.
12. Wilson WA, Hawley SA, Hardie DG. 1966.Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. Curr Biol. 6(11):1426-34.
13. Panozzo C, Nawara M, Suski C, Kucharczyka R, Skoneczny M, Bécam AM, Rytka J, Herbert CJ. 2002 Aerobic and anerobic NAD+ metabolism in Saccharomyces cerevisiae. FEBS Lett. 517(1-3): 97-102.
14. Cunin, R, Glansdorff N, Pierard A, Stalon V. 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol Rev 50:314-352.
15. Brauer MJ, Yuan J, Bennett BD, Lu W, Kimball E, Botstein D, Rabinowitz JD. 2006. Conservation of the metabolic response to starvation across two divergent microbes. PNAS. 51: 19302-19307.

[1] http://iroatech.com/portal/