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Fermentation Monitoring of a Co-Culture Process with *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* Using Shotgun Proteomics

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Abstract

Co-culture processes present the opportunity to produce value-added products from economical raw materials, but there lacks a high-throughput fermentation monitoring technique to study the temporal physiology of fermentation organisms in co-culture processes. In this study, we applied shotgun proteomics to investigate a co-culture process of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis*, and we monitored the fermentation until glucose depletion. Three time points were taken for proteomics analysis at 11.5 hour, 18.5 hour and 32 hour, representing transition into diauxic shift. Using label-free quantitation, we observed cellular dynamic within 20-hour time frame. We distinguished the proteome between two yeasts, and the most abundant proteins of *S. stipitis* and *S. cerevisiae* contained expected processes of glycolytic enzymes, histones, heat shock proteins, ribosomal proteins and F_1F_0-ATPase. After glucose depletion, we identified up-regulations of *S. stipitis* malate synthase and isocitrate lyase as key enzymes in glyoxylate cycle and gluconeogenesis. Increased expression of *S. stipitis* histone 2B was observed in diauxic shift, and histone acetylation was suggested by up-regulation of acetyl-CoA synthetase. Without the presence of xylose, we observed induction of NAD(P)H-dependent D-xylose reductase (Xyl1p) as early as 11.5-hour before glucose depletion. We also observed the expression of D-xylulose reductase after glucose depletion without xylose induction. Further study is needed to investigate the cause of derepression signals for xylose oxo-reductive pathway. Without cellulose induction, the up-regulation of *S. stipitis* endo-1,4-beta-glucanase suggested *S. stipites*' strategy in diversifying carbon choices after glucose repression. This research demonstrated the application of shotgun proteomics in high-throughput monitoring of complex co-culture system and able to elucidate the temporal physiology of *S. stipitis*.

Keywords: *Saccharomyces cerevisiae; Scheffersomyces stipitis; Pichia stipitis*; Shotgun proteomics; Co-Culture; Mudpit; Fermentation monitoring

Introduction

Co-culture processes present the opportunity to establish stable and profitable biotechnological bioprocesses and produce value-added products from economical raw materials such as agricultural residues [1]. Mixed culture systems have demonstrated promise in hydrogen, methane, ethanol and polyhydroxyalkanoates productions from renewable resource, increasing potential revenue and reducing environmental impacts [2-4]. The use of controlled mixed fermentation using *Saccharomyces* and non-*Saccharomyces* yeasts has been implemented in winemaking to alter both chemical and the aromatic composition of wines [5]. The synergistic interaction between different yeasts provides a tool for the implementation of new fermentation technologies [5]. In biofuel research, the co-culture process of *Saccharomyce cerevisiae* and *Scheffersomyces stipitis (Pichia stipitis)* has been extensively studied with attempts to achieve a simple, one-batch process to ferment glucose and xylose using lignocellulosic feedstock [6,7]. The co-culture process poses technical obstacles such as low ethanol yields associated with different optimal oxygen transfer rate required by each organism. Novel fermentation apparatus using two fermentors and two microfiltration modules to achieve optimal fermentation condition for each yeast [7]. However, before optimizing a co-culture process such as ones described by Taniguchi et al. [7], there lacks a robust, high-throughput fermentation monitoring technique to monitor the temporal physiology of each organism in more complex process such as co-culture fermentation. In recent years, fermentation technology has turned '-omics' for solutions in process development [8]. Proteomics quantification of yeast high gravity ethanol fermentations was investigated to determine the dynamic of metabolic pathways (e.g. the deactivation of secondary metabolites pathway) [9]. Shotgun proteomics can provide the physiological backgrounds needed for non-model organisms such as *S. stipitis* and allow observation of the global profile of the organism's proteome. The analysis of the proteome allows the evaluation of cellular processes at greater depth with a much simpler technique than 2D-PAGE-MS [10]. In this study we demonstrated the application of shotgun proteomics in monitoring a co-culture process using *S. cerevisiae* S288C and *S. stipitis* CBS 6054. The yeast proteome of three time points were analyzed based on metabolic and regulatory pathways. Without the presence of xylose, we observed the induction of xylose reductase (Xyl1p, *S. stipitis*) as early as 11.5-hour before glucose depletion, and D-xylulose reductase (Xyl2p, *S. stipitis*) as early as 18.5-hour after glucose depletion. . We distinguished the proteome between two yeasts, identified the most abundant proteins of *S. stipitis* and *S. cerevisiae*, and determine the dynamic of *S. stipitis* proteome during the co-culture process.

Materials and Methods

Microorganism and culture condition

Saccharomyces cerevisiae S288C and *Scheffersomyces stipitis (syn. Pichia stipitis* CBS 6054) were obtained from American Type Culture

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Collection (ATCC, Manassas, VA, USA) and maintained on YPD or YPX agar slants accordingly. YPX substituted xylose with glucose in YPD. Pre-inoculums of *S. cerevisiae* and *S. stipitis* were grown separately at 26°C, 100 rpm and harvested at mid-exponential phase. Two biological replicates of the co-culture process were carried out in a 250mL Erlenmeyer flask with 100mL working volume at 26°C and 100rpm. The medium composition for the co-culture process was 20g/L glucose, 20g/L peptone and 10g/L yeast extract.

Fermentation parameters analysis

Glucose, ethanol, acetic acid and glycerol were measured using enzymatic kits (Megazyme, Wicklow, Ireland). OD610 was monitored throughout growth with a UV-VIS spectrophotometer. Colony-forming unit (CFU) was used to obtain viable yeast counts of *S. stipitis* and *S. cerevisiae*. *S. stipitis* and *S. cerevisiae* were enumerated by adapting methods as described in Laplace and colleagues [11]. In brief, *S. stipitis* and *S. cerevisiae* were distinguished from each other by plating on medium containing erythromycin (1g/L), 1% yeast extract, 2% peptone, 2% glycerol or 2% glucose. *S. stipitis* was able to grow on media containing erythromycin (1 g/L) with 2% glycerol, whereas *S. cerevisiae* was inhibited by the mitochondrial inhibitor.

Cells lysis and protein extraction

Three protein samples from each biological replicate were taken at 11.5 hour, 18.5 hour and 32 hour. ~10 mg of wet-mass cell pellet for each sample was obtained and processed through single tube cell lysis and protein digestion [12,13]. The protein concentrations after the extraction were ~6 mg/mL for each sample after extraction. Tris/10mM $CaCl_2$ at pH 7.6 and 6M Guanidine/10mM Dithiothreitol (DTT) (Sigma-Aldrich Canada, Oakville, Ontario) were used to lyse cells and extrude proteins. In brief, the mixture was placed on the rocker for the first hour and vortexed every 10 minutes, and then incubated for 12 hours at 37°C. The mixture was spun down at 10,000 g. The supernatant was discarded and the pellet was diluted with 6-fold 50mM Tris/10mM $CaCl_2$. ~5 µg of sequencing grade trypsin (Promega, Madison, WI, USA) was added to each sample and digested for 12 hours at 37°C by gentle rocking. The same amount of trypsin was added a second time and incubated at 37°C for another 12 hours. 1M DTT was added to a final concentration of 20mM and incubated for another hour with gentle rocking at 37°C followed by centrifugation at 10,000 g for 10 minutes. The supernatant was kept and samples were cleaned and de-salted via solid phase extraction with Sep-Pak Plus cartridges (Waters Limited, Mississauga, Ontario), concentrated, solvent exchanged, filtered, aliquoted and frozen at -80°C [14].

2D-LC MS/MS

Each extracted and digested sample was analyzed twice for technical replicate in 2D-LC MS/MS. Samples were loaded onto the back column of split phase 2D column [15] (~3-5cm Luna SCX 5 µm and ~3- 5cm C_{18} Aqua 5µm 100A) (Phenomenex, Torrance, CA) using a pressure cell and then connected to a front column with integrated nanospray tip (~15 cm of Aqua C_{18} Aqua 5µm 100A, Phenomenex, Torrance, CA; 150 µm with 15 µm tip, New Objective, Woburn, MA). Samples were analyzed via 2-D LC-nanoESI-MS/MS on a LTQ XL (ThermoFisher Scientific, San Jose, CA) as previously described [13]. Samples were analyzed via 13-hr runs (6 steps with 1 wash step) [14,16].

Proteome informatics

Proteome Discoverer 1.1 (ThermoFisher Scientific, San Jose, CA) and SEQUEST algorithm [17] was used to correlate peptides spectra with *S. stipitis* protein sequence (filtered models containing 5839 entries downloaded from the Joint Genome Institute; accessed on 05/15/2011) and *S. cerevisiae* protein sequence (5884 entries downloaded from the *Saccharomyces* Genome Database; accessed on 05/15/2011) [6,16]. Proteome Discoverer 1.1 was used to filter SEQUEST results and sort peptides into protein identification based on modified method of Verberkmoes et al. [17] (minimum Xcor of at least 1.5 [+1], 2.3 [+2], 2.8 [+3]; minimum probability score of at least 2 [+1], 7 [+2] and 7.5 [+3]; minimum two fully tryptic peptides) [13]. We enabled protein grouping, counted only rank 1 peptides and only in the top scored proteins. Each protein identified had at least one unique peptide. Reverse protein sequences were included in the search database to estimate the overall false-positive rates of protein identification (false-positive rate = $2[n_{rev}/(n_{rev} + n_{real})]*100$; n_{rev} = the number of peptides identified from the reverse database; n_{real} = the number of peptides identified from the real database) [18].

Data analysis

KEGG and GO annotations of *S. cerevisiae* and *S. stipitis* (also KOGs) were downloaded from *Saccharomyces* Genome Database and Joint Genome Institute [19,20]. The identified proteomes were examined manually with each biological function. In-house Perl scripts were written to perform the functional counts of GOs and KOGs. The metabolic pathways were visualized using KEGG Mapper [21]. For comparison of protein abundance levels, normalized spectral abundance factors (as label free estimation of relative protein abundance) (NSAF) were determined as described in Florens et al. [22]. This method estimates protein abundance by first dividing the spectral count for each protein by the protein length and then dividing this number by the sum of all length-normalized spectral counts for each organism and multiplying by 100 [22,17]. Differentially expressed proteins between 11.5-hour and 32-hour were determined using spectral count and the software QSpec with log. fold change of 3 and up-regulated FDR = 0.01 [23].

Results and Discussions

Overview of proteome

This study represented the first attempt to monitor the temporal physiological profile of a co-culture process using *S. cerevisiae* and *S. stipitis* with shotgun proteomics. Key metabolites, including ethanol, glucose, acetic acid and glycerol were measured and monitored throughout the co-culture fermentation (Figure 1 and 2). The fermentation lasted for 36 hour in both biological replicates. The highest ethanol yielded ~9 g/L. We observed low acetic acid (< 0.5 g/L) throughout the process, but glycerol was observed at moderate level (<1.15 g/L). Glucose was consumed to near depletion (< 1 g/L) at ~16 hour, indicating diauxic shift for both organisms. We inoculated batch co-culture fermentation with the pre-inoculums and obtained initial cell density of $8.07x10^5$ CFU for *S. cerevisiae* and $7.23x10^5$ CFU for *S. stipitis* in biological replicate 1 at time zero. In biological replicate 2 we obtained initial cell density of $7.87 x10^5$ CFU for *S. cerevisiae* and $1.10 x10^6$ CFU for *S. stipitis*. Before 11.5 hour, both *S. cerevisiae* and *S. stipitis* multiplied at near equal rate. In biological replicate 1, *S. stipitis* outgrew *S. cerevisiae*, reaching $1.06x10^8$ CFU for *S. stipitis* and $6.20x10^7$ CFU for *S. cerevisiae* at the end of the fermentation. However, in biological replicate 2, the population of *S. stipitis* and *S. cerevisiae* fluctuated back and forth, failing to determine the dominant specie. This study further demonstrated the challenge in producing consistent batches in co-culture process. Based on the growth curve of each organism, the maximal growth rates of both *S. cerevisiae* and *S. stipitis* occurred at 11.5 hour and the maximal glucose consumption rate was also observed

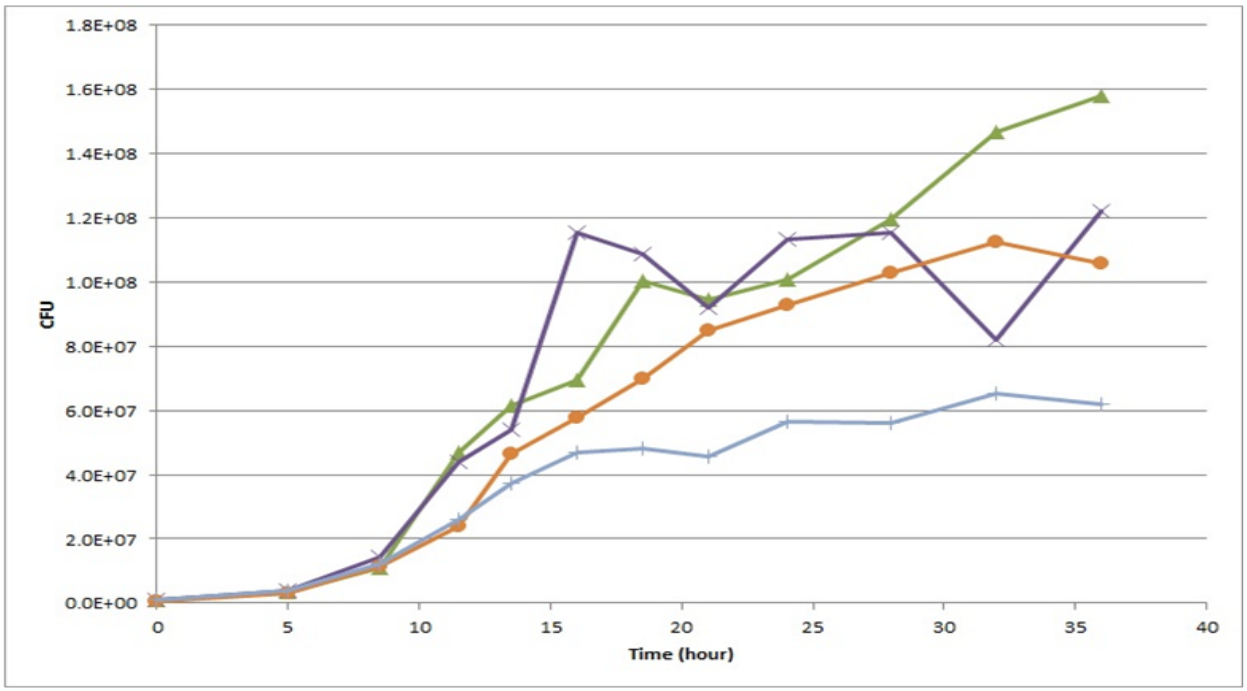

Figure 1: Viable cell counts of the co-culture process of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* in both biological replicate 1 and 2. Proteomics samples were taken at 11.5 hour, 18.5 hour, 32 hour, representing transition into diauxic shift. Fermentation was terminated at 36-hour (● *S. stipitis* CFUs Biological 1; ▲ *S. stipitis* CFUs Biological 2; + *S. cerevisiae* CFUs Biological 1; X *S. cerevisiae* CFUs Biological replicate 2).

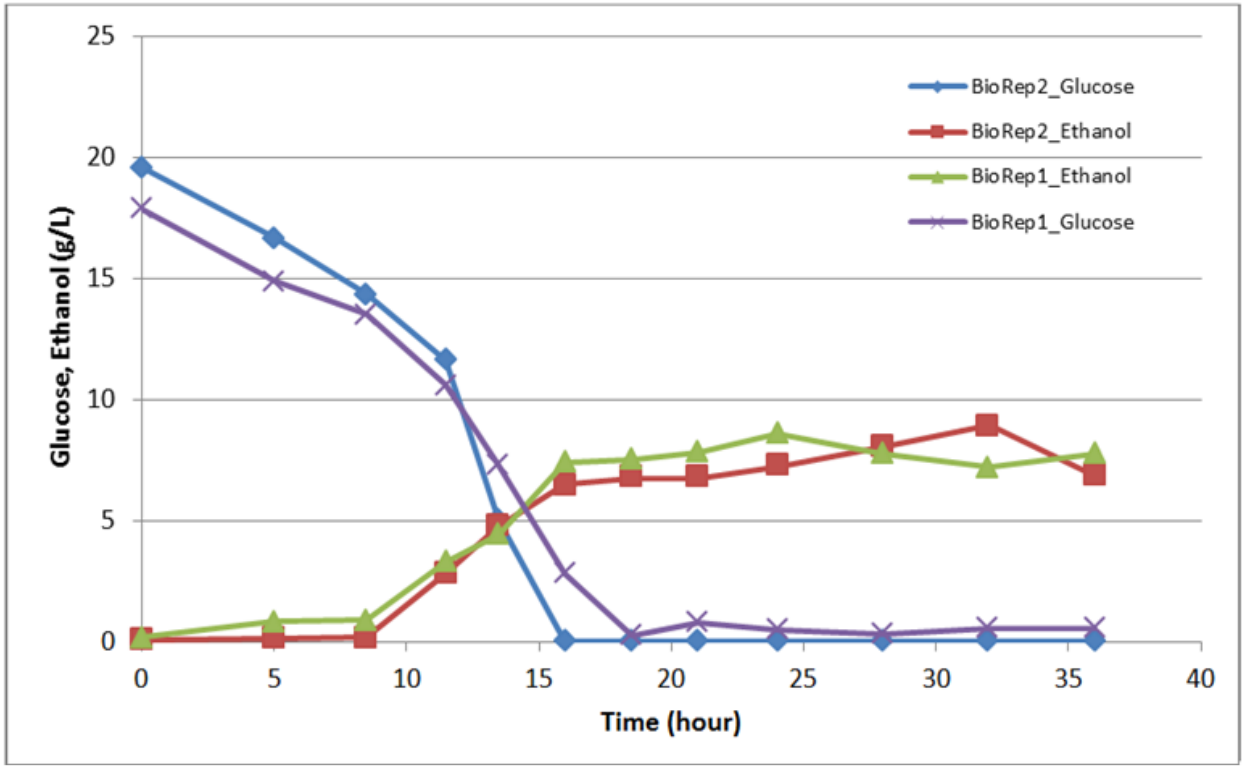

Figure 2: Monitoring glucose and ethanol concentrations during the co-culture process of *Saccharomyces cerevisiae* and *Scheffersomyces stipitis*. Data shown included both biological replicate 1 and 2.

at 11.5 hour, corresponding to the exponential growth phase of both yeasts. We collected proteomics samples from two flask fermentations at three time points (11.5 hour, 18.5 hour and 32 hour) to monitor the co-culture process. The shotgun approach was used to identified and matched peptide mass data to the sequenced *S. stipitis* and *S. cerevisiae* sequence databases. In biological replicate 1, combining all time points and technical replicates for each time point, we identified a total number of 1390 proteins throughout the co-culture process (650 proteins from *S. stipitis* and 740 proteins from *S. cerevisiae* (Table 1). In biological replicate 2, combining all time points and technical replicates for each time point, we identified a total number of 1499 proteins throughout the co-culture process (666 proteins from *S. stipitis* and 833 proteins from *S. cerevisiae*; Table 1). The false discovery rates of 2D-LC-MS/MS runs were calculated between 0.16% to 0.57%. Table 1 summarized spectral counts and the CFU for each organism in each time point in both biological replicates. We observed that although both *S. cerevisiae* and *S. stipitis* CFU were at approximately 1:1 ratio (~$2.5x10^7$ CFU each) at 11.5 hour, the total peptides counts were not equal between two yeasts. The difference between total peptides counts demonstrated the difference in cell size and mass between *S. stipitis* and *S. cerevisiae*, where *S. stipitis* is smaller than *S. cerevisiae* in cell mass per cell. *S. stipitis* exhibited higher CFU than *S. cerevisiae* during the fermentation, but higher spectral counts in *S. cerevisiae* were observed. Looking at the spectral counts, CFU and protein identified, we found an increase in *S. cerevisiae* CFU did not necessary result in identifications of higher proteins and spectral counts, but an increase in *S. stipitis* CFU resulted in higher spectral counts and minor increase in numbers of protein identification. This implied other variables, which could be time dependent, interacted with spectrum identification capacity of the mass spectrometry. One possible explanation could be the competition for electro-spray ionization between *S. cerevisiae* and *S. stipitis* peptides. Another explanation was the difference in extraction and digestion efficiency between two organisms. We calculated normalized spectral abundance factors (NSAFs) to estimate the relative protein abundance. By comparing the technical replicates of each dataset, we obtained highly reproducible results in each biological replicate, with the linear regression line fell between R^2 = 0.81 and 0.89 for *S. cerevisiae* in biological replicate 1(as illustrated in Figure 3 for 18.5 hour time point) and between R^2 = 0.85 and 0.90 for *S. stipitis* (data not shown).

Most abundant proteins in *s. cerevisiae* and *s. stipitis*

Proteomics data was further analyzed below by using both biological replicates if not stated. Similar to the most abundant proteins found using GFP fusion library and high-throughput flow cytometry, *S. cerevisiae* glycolytic enzymes were among the most abundant proteins in the cell from exponential to diauxic shift [24]. These included pyruvate kinase (Cdc19p), 3-phosphoglycerate kinase (Pgk1p), glyceraldehyde-3-phosphate dehydrogenase (Tdh1p, Tdh2p, Tdh3p), enolase II (Eno2p) and fructose 1,6-bisphosphate aldolase (Fba1p). Alcohol dehydrogenase (Adh1p) and F1 sector of mitochondrial F1F0 ATP synthase (Atp2p) were also highly abundant in *S. cerevisiae* proteome, indicating the importance of oxidative phosphorylation. The fact that the other enzymes of the tricarboxylic acid cycle were not switched off during fermentative growth can be explained by their involvement in amino acid biosynthesis, gluconeogenesis, heme biosynthesis or *de novo* fatty acid synthesis.

The most abundant proteins identified in *S. stipitis* also came from glycolysis and gluconeogenesis. Similar to *S. cerevisiae*, the glycolytic enzymes observed in most abundance included glyceraldehyde-3-phosphate dehydrogenase (Tdh2p and Tdh3p), enolase I (Eno1p), phosphoglycerate mutase (Gpm1.1p), fructose-bisphosphate aldolase (Fba1p), phosphoglycerate kinase (Pgk13p) and pyruvate kinase (Pyk1p). *S. stipitis* cells also produced high amount of alcohol dehydrogenase (Adh1p), which reduces acetaldehyde to ethanol. We observed high abundance of F_1F_0-ATPase complex alpha and beta subunits (Atp1p, Atp2p) located in mitochondrial cellular components. We also identified high abundance of mitochondrial malate dehydrogenase (Mdh1p) involved in tricarboxylic acid (TCA) cycle, indicating respiro-fermentative. Proteins involved in chromatin assembly, protein translation and folding were also high in abundance. These included heat shock protein (HSP70 family Ssa2p), histone H4 and 2B (Hhf1p and Htb2.1p) and ribosomal proteins (L14B, 40S S14-B, S7-A and 60S L4360S). The resemblance in both *S. cerevisiae* and *S. stipitis* proteomes showed that highly abundant proteins were housekeeping proteins and essential for viability. In yeast cells, glycolytic enzymes constitute a major fraction (30 to 60%) of the soluble proteins [25], and in rapidly growing cells, 60% of total transcription is devoted to ribosomal RNA [26].

Functional categorization and analysis of s. *cerevisiae* and *s. stipitis* proteome

A comparison between *S. stipitis* and other more related yeast such as C. albicans would almost certainly show a higher degree of conservation, but because regulation has been best studied in *S. cerevisiae*, that is a standard frame of reference. Therefore in this study regulation of *S. stipitis* was inferred and compared with *S. cerevisiae* if not otherwise stated. The proteome of *S. cerevisiae* and *S. stipitis* observed in the co-cultural process contained housekeeping processes such as oxidative phosphorylation, glycolysis, non-oxidative branch of the pentose phosphate pathway, gluconeogenesis, biosynthesis of amino acids and aminoacyl-tRNA, protein synthesis and proteolysis, fatty acid metabolism and cell division. An attempt was made to categorize *S. stipitis* proteome based on GO terms for direct comparison between *S. stipitis* and *S. cerevisiae*, but the incomplete nature of the GO entries for *S. stipitis* proved impractical (data not shown). The incomplete

	Number of Proteins Identified Biological Replicate 1			**Total proteins measured from each organism**
	11.5 hour	18.5 hour	32 hour	
Scheffersomyces stipitis	322	456	574	650
Saccharomyces cerevisiae	535	594	539	740
Total proteins in each time-point	857	1050	1113	1390
	Number of Proteins Identified Biological Replicate 2			**Total proteins measured from each organism**
	11.5 hour	18.5 hour	32 hour	
Scheffersomyces stipitis	444	412	577	666
Saccharomyces cerevisiae	626	637	660	833
Total proteins in each time-point	857	1050	1113	1499

Table 1: Numbers of *Scheffersomyces stipitis* and *Saccharomyces cerevisiae* proteins identified from shotgun proteomics of the co-culture process in both biological replicates.

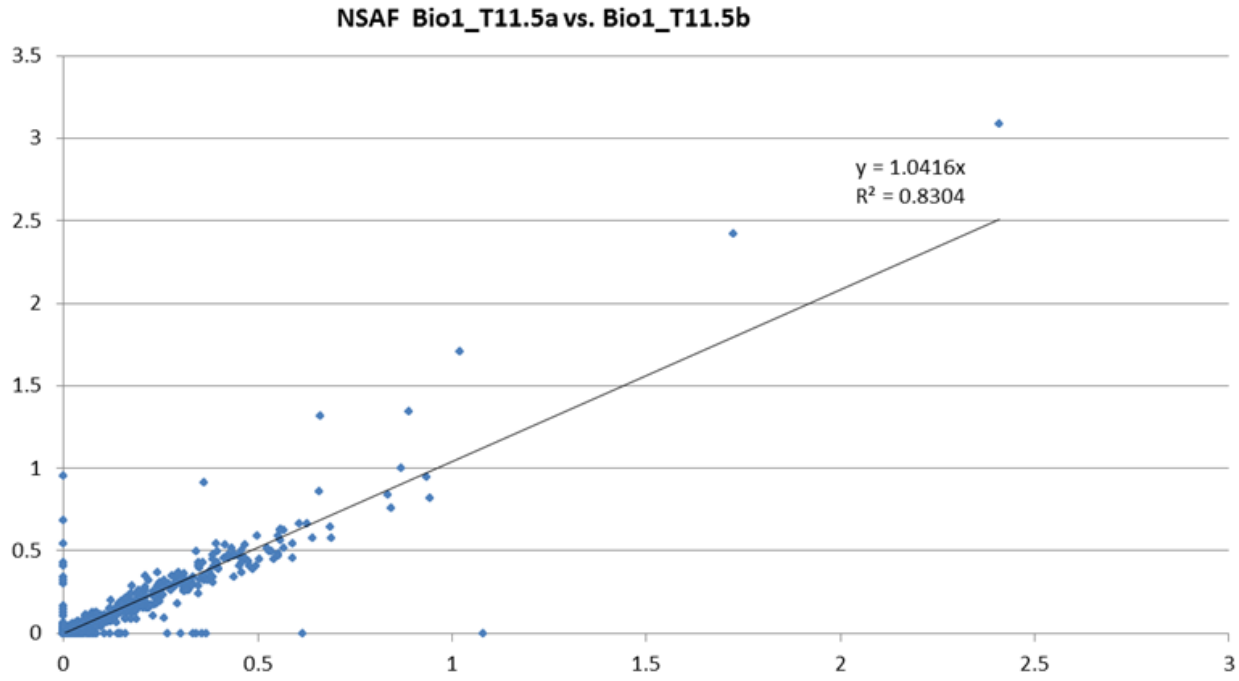

Figure 3: Representative linear regression based on NSAFs of *S. cerevisiae* using technical replicate *a* and technical replicate *b* at 18.5 hour in biological replicate 1.

nature of the GO annotations for *S. stipitis* directed us to categorize *S. stipitis* proteome based on KOGs. In analyzing the *S. stipitis* proteome, the majority of KOGs functions fell into the categories of carbohydrate metabolism, protein synthesis and modification. Technical replicates of each time point showed reproducible counts of each KOGs term. Even with the higher counts observed in later time points, each KOGs category still constituted the same relative ratio to the whole data set (data now shown). The majority of GO terms also fell into protein synthesis and carbohydrate metabolism in *S. cerevisiae*. The ratio of each category relative to the whole data set was consistent between time points. Technical replicate of each time point also showed reproducibility. All time points shared similar distribution of biological function in terms of KOGs for *S. stipitis* and GO Biological terms for *S. cerevisiae*.

Distinctive physiology in temporal proteome

In analyzing the proteome of *S. stipitis*, certain stress proteins were observed before diauxic shift, and these included thioredoxin peroxidase (acts as antioxidant) and glycogen synthase (Gsy1p). The expression of Gsy1p was observed as early as 11.5 hour in both *S. cerevisiae* and *S. stipitis*. It was possible that heat shock, nitrogen starvation or the 2% glucose medium (insufficient glucose) caused the de-repression of Gsy1p [27,28]. Earlier studies suggested *S. cerevisiae* and *S. stipitis* shared similar amino acid biosynthesis pathway. In *S. stipitis* proteome, we observed proteins involved in a list of amino acid biosynthesis pathways that are under general amino acid controls before diauxic shift. These included acetolactate synthase (Ilv2p), dihydroxyacid dehydratase (Ilv3p, 32 hour only), mitochondrial ketol-acid reductoisomerase (Ilv5p) and 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase isoenzyme (Aro4p). Hans and colleagues observed the most drastic alternations of intracellular amino acid pools occurred during the diauxic shift and at the entry into the stationary phase [29]. We also observed principal enzyme of glyoxylate cycle before diauxic shift, including malate synthase (Mls1.1p) and isocitrate lyase (Icl1p), a key enzyme for growth on ethanol and acetate in yeast. Earlier studies in *S. cerevisiae* showed that *ICL1* expression is tightly regulated and growth on ethanol requires the glyoxylate pathway for replenishing C4 compounds to the tricarboxylic acid cycle. *ICL1* was also induced by growth on ethanol and repressed by growth on glucose [30]. The most peculiar finding in our co-culture process was the observation of the enzymes involved in oxo-reductive pathway for xylose utilization. We observed NAD(P)H-dependent D-xylose reductase (Xyl1p) as early as 11.5- hour before glucose depletion. We also observed D-xylulose reductase as early as 18.5-hour after glucose depletion. Xylose was not presented in the media (confirmed by enzymatic assay). We observed aldehyde dehydrogenase (Ald5p) at 32 hour, but *S. stipitis* was not known to produce acetate. The function of Ald5p remained to be examined. Our metabolic data supported the expression of DL-glycerol-3-phosphatase (Gpp1p) and suggested anaerobic and osmotic stress for *S. stipitis*. Other proteins which suggested stress included Gph1p, glycogen (starch) synthase (Gsy1p), thiol-specific antioxidant protein (Tsa1p), NADPH-dependent methylglyoxal reductase (Gre2p) and thioredoxin reductase (Prx1p) [31,32].

Dynamic of *S. stipitis* during fermentation

Using the spectral counts and the software QSpec (log. fold change cutoff = 3; FDR up-regulated = 0.01) [23], we identified up-regulations of six *S. stipitis* proteins and one *S. cerevisiae* protein from 11.5 hour to 32 hour (Table 2). During the diauxic shift at 32-hour, we observed up-regulation of *S. stipitis* malate synthase and isocitrate lyase, the key enzymes involved in glyoxylate cycle. The depletion of glucose and the transition to growth on ethanol and acetate also triggered the up-regulation of *S. stipitis* phosphoenolpyruvate carboxykinase in gluconeogenesis. Interestingly, *S. stipitis* histone 2B did not exhibit

Organism Accession	Bio1 11.5hr	Bio2 11.5hr	Bio1 32hr	Bio2 32hr	Fold Change (log)	Z statistics	FDR	FDR Up-regulated	Description
S. stipitis 89873	0	0	41	26	3.185	4.94	0.021	0.006	Acetyl-coenzyme A synthetase
66312	0	0	10	40	3.228	5.28	0.01	0.002	Endo-1,4-beta-glucanase
73488	0	0	49	32	3.465	5	0.018	0.005	Histone 2B
53620	0	0	45	64	3.78	6.27	0.001	0	Malate synthase, glyoxysomal
62080	0	0	96	88	4.411	6.47	0	0	Isocitrate lyase
71471	0	0	58	123	4.941	5.32	0.009	0.002	Phosphoenolpyruvate carboxykinase
S. cerevisiae YBL075c	0	0	0	122	3.989	5.95	0.002	0	Stress-Seventy subfamily A

Table 2: Up-regulated proteins of *S. stipitis* and *S. cerevisiae* from 11.5 hour to 32 hour. Differentially expressed proteins were determined using spectral counts and the software QSpec with the cutoff of 3 log fold change and FDR Up-regulated of 0.01.

down-regulation and increased histone acetylation was suggested by up-regulation of acetyl-coA synthetase. With glucose repression no longer existed at 32 hour, we also observed up-regulation of *S. stipitis* endo-1,4-beta-glucanase, a family 5 glycoside hydrolase, without cellulose in the growth media. Another unexpected finding in our proteomics data was the observation of the expressed enzymes involved in oxo-reductive pathway of xylose utilization in *S. stipitis*. NAD(P)H-dependent D-xylose reductase (Xyl1p) and D-xylose reductase (Xyl2p) are repressed under glucose culture [33]. However, we observed *S. stipitis* Xyl1p expression as early as 11.5- hour before glucose depletion. We also observed *S. stipitis* Xyl2p expression as early as 18.5 hour after glucose depletion. Xylose was not present in the media (confirmed by enzymatic assay) and the mechanism for signal derepression of these two enzymes is unknown. Earlier studies have suggested that reducing ATP production can also bring about a partial derepression of xylose assimilation [34]. Further study is needed to investigate and verify the derepression signal for Xyl1p and Xyl2p. We hypothesize that the derepression signal came from the effect of co-culture, which could be a competitive advantage for *S. stipitis* [35] as a hedging bet to utilize other potential carbon sources. Along with the up-regulation of *S. stipitis* endo-1,4-beta-glucanase without cellulose induction, our data demonstrated S. stipites strategy in diversifying potential carbon sources once glucose no longer existed.

Conclusions

In this study, we demonstrated a shotgun proteomics to monitor a co-culture processing with *S. cerevisiae* and *S. stipitis*. Shotgun proteomics successfully distinguished, identified distinct physiology of both yeast during the co-culture process, and able to elucidate the temporal profile of *S. stipitis* proteome. The most abundant proteins of *S. stipitis* and *S. cerevisiae* came from the expected process such as glycolytic enzymes, histones, heat shock proteins, ribosomal proteins and F_1F_0-ATPase. Without the presence of xylose, the induction of NAD(P)H-dependent D-xylose reductase and D-xylulose reductase were unexpected. Up-regulation of *S. stipitis* endo-1,4-beta-glucanase, histone 2B and acetyl-coA synthetase provided insight into the dynamics of non-model yeast *S. stipitis* during the co-culture process.

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