Experimental Approach For Large-Scale Proteome Measurements From Small Amounts (low mg) Of Microbial Cultures

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OVERVIEW

- The evolution in development in mass spectrometry and liquid chromatographic separation provides a deeper molecular view of microbes.
- To date, most approacches of proteome characterization require large quantities of biomass (~500mg-2 g) for sample preparation and protein identification, however our objective is to apply a single tube lysis to 500 mg of biomass (wet cell pellet).
- Single tube lysis of small biomass for bacterial isolates, microbial communities like the acid mine drainage biofilm (Figures 1 and 6), and the coupling cell sorting to proteomics are all drivers in the development of the methodology.

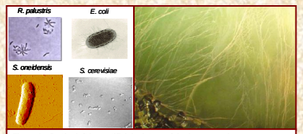

Figure 1: On the left is a artificial mixture of 4 microorganisms (*Rhodopseudomonas palustris*, *Escherichia coli*, and *Sacchromyces cerevisiae*) and on the right is a photograph of a natural Biofilm community located in California[1] that are used to test the ability of guanidine as a chemical lysis agent.

INTRODUCTION

- The determination of the identity and metabolic state of microbes is critical for a number of applications, ranging from environmental to biomedical. Some examples include:
  - An acid mine drainage biofilm community
  - The microbial community of human digestive tract
- Most methods of proteome analysis require large quantities of biomass (~500mg-2 g) for sample preparation and protein identification.
- Our experimental approach differs from traditional lysis techniques by providing the ability to identify the proteomic state of microorganisms from just a few milligrams of starting cellular material and is advantageous because:
  - Minimizing sample loss with sample remaining in a single tube
  - Reduction in the amount of sequencing grade modified trypsin
  - Smaller sample cultures to better mimic natural growth conditions
  - Permits multiplex growth assays
  - Potentially enables cell sorting coupled to "shotgun" proteomics

EXPERIMENTS

- Samples of microbial isolates, mixed microbial cultures, and natural microbial mixtures were as follows:
  - *Shewanella oneidensis* MR-1 wild-type (under aerobic conditions)
  - *Rhodopseudomonas palustris* CGA0100 (photoheterotrophic conditions)
  - 4 component artificial mixture (*S. oneidensis*, *R. palustris*, *Escherichia coli*, and *Sacchromyces cerevisiae*) were combined prior to lysis.
  - A natural acid mine drainage biofilm microbial community was collected from Richmond Mine (Ram et al, Science, 2005).
- Lysis and proteome preparation of microbial samples
  - *S. oneidensis* cells lysed originally by traditional method, sonication: the Control Sample (Figure 2).
    - 4 grams of cells are lysed using a sonic probe (5 pulses for 30 sec with a 30 sec cooling period on ice)
    - Cellular debris removed by low speed centrifugation (5000 x g for 10 min twice)
    - Cellular lysate quantitated with the BCA assay, aliquoted and frozen at -80 °C until digestion
    - Control sample digested with standard trypsin protocol and desalted (Sep-Pak C_{18} cartridges)
  - *S. oneidensis*, *R. palustris*, 4 micro-organism mixture, and Biofilm community using Guanidine lysis.
    - Cells lysed using buffered solution of 6 M Guanidine-HCl/10 mM DTT in 50 mM Tris-HCl, 10 mM $CaCl_2$
    - Lysis samples left at 37 °C overnight, following morning guanidine was diluted 6-fold in the Tris/$CaCl_2$ buffer
    - Trypsin added at an optimized ratio to reduce the identification of autotryptic peaks
    - Samples of the higher mass range ≥ 1 mg wet cell pellet desalted with Sep-Pak C_{18} cartridges
    - Samples of lower mass range < 1mg were de-salted on column
    - All lysed samples were analyzed by mass spectrometry (Figure 2)
    - Biomass ranges were from ~0.5 mg all the way to ~500 mg
- 24 hour Multidimensional nanoHPLC-MS/MS analysis of samples
  - Separation was accomplished by 2-D nano chromatography using strong cation exchange as the first dimension and C_{18} reverse phase as the second dimension of separation.
    - a LCQ Deca XP Plus 3-D ion trap (Thermo Finnigan) and Ultimate HPLC pump (LC Packings) were operated in the data dependent mode for all the samples
    - a LTQ linear trapping quadrupole (Thermo Finnigan) and Ultimate HPLC pump were operated in the data dependent mode for the *S. oneidensis*, *R. palustris*, 4-microbe mixture, and Biofilm community
    - a LTQ-Orbitrap hybrid (Thermo Electron) and Ultimate HPLC pump were operated in the data dependent mode for the Biofilm samples. Full scans (60K resolution) and MS/MS scans (30K) were acquired in the Orbitrap.
- Proteome Bioinformatics
  - Peptide identification was using the SEQUEST algorithm with a two unique peptide cut-off for protein identification and X-corr values 1.8 (+1), 2.5 (+2) and 3.5 (+3) and only fully tryptic peptides.
  - For some of the analyses, the algorithm DBDigger was used for peptide identification with a two unique peptide cut-off for protein identification and Xcorr values of 25 (+1), 30 (+2), and 45 (+3). Only fully tryptic peptides were considered.

MICROORGANISM SAMPLES

Table 1: Small sample lysis with 6M Guanidine technique.

Organism		Biomass size	Cleanup	Instrument
S. oneidensis	Gram-negative	50 mg	Sep-Pak	LCQ
S. oneidensis	Gram-negative	25 mg	Sep-Pak	LCQ
S. oneidensis	Gram-negative	10 mg	online-desalt, Sep-Pak	LCQ
S. oneidensis	Gram-negative	5 mg	online-desalt, Sep-Pak	LCQ, LTQ
S. oneidensis	Gram-negative	1 mg	online-desalt, Sep-Pak	LCQ, LTQ
S. oneidensis	Gram-negative	0.5 mg	online-desalt	LTQ
R. palustris	Gram-negative	25 mg	online-desalt	LCQ
R. palustris	Gram-negative	5 mg	online-desalt, Sep-Pak	LCQ
R. palustris	Gram-negative	1 mg	online-desalt, Sep-Pak	LCQ, LTQ
4-microbe mixture	Gram-negative and eukaryote	25 mg	online-desalt	LCQ
4-microbe mixture	Gram-negative and eukaryote	5 mg	online-desalt	LCQ
4-microbe mixture	Gram-negative and eukaryote	1 mg	online-desalt	LCQ
AMD Biofilm	bacteria and archea	300 mg	Sep-Pak	LCQ
AMD Biofilm	bacteria and archea	50 µL	Sep-Pak	LTQ-Orbitrap

LYSIS AND MASS SPECTROMETRY RESULTS

Flow chart of traditional and single-tube lysis methods

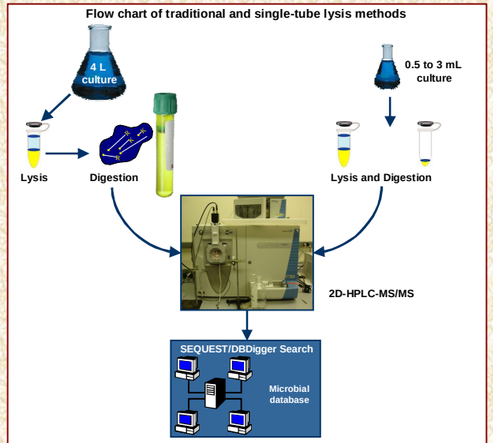

Figure 2: Flow Chart of the two different lysis methods used in this study. The first (on the left) is our traditional method of lysis using a microprobe and sonicating the cells in order to break them apart. This method requires at least 500 mg of wet cell pellet. The second method is adding 6 M Guanidine to the cell pellet and incubating overnight followed by trypsin digestion all performed in a single tube.

Table 2: Total proteins identified for each sample of different organisms.

Organism	Biomass size	Instrument	Proteins Identified	
			Sequest	DBDigger
S. oneidensis	4 g	LCQ	754	782
S. oneidensis	50 mg	LCQ	700	730
S. oneidensis	25 mg	LCQ	782	832
S. oneidensis	10 mg	LCQ	698	743
S. oneidensis	10 mg	LCQ	562*	585*
S. oneidensis	5 mg	LCQ	791	851
S. oneidensis	5 mg	LCQ	697	590*
S. oneidensis	5 mg	LTQ	1472	1673*
S. oneidensis	1 mg	LCQ	829	869
S. oneidensis	1 mg	LCQ	915*	585*
S. oneidensis	1 mg	LTQ	1308	1546
S. oneidensis	0.5 mg	LTQ		918*
R. palustris	25 mg	LCQ		793*
R. palustris	5 mg	LCQ		1394^
R. palustris	1 mg	LCQ		1571^
R. palustris	1 mg	LTQ		2146*
4-microbe mixture	25 mg	LCQ		888*
4-microbe mixture	5 mg	LCQ		1020*
4-microbe mixture	1 mg	LCQ		849*
AMD Biofilm	300 mg	LCQ		455^

* online-desalting of sample
* total protein number count of replicate runs

Table 3: *R. palustris* Protein Quantification

Wet Cell Pellet	Measured Conc.	Protein Yield
0.5 mg	305.27 µg/mL	61.0%
1.0 mg	393.90 µg/mL	39.4%
5.0 mg	1197.53 µg/mL	24.0%
25 mg	6650.65 µg/mL	26.6%

Table 3 Results:

- Wet cell pellet is the amount used to evaluate protein extraction.
- Measured Concentration is the average protein concentration extracted and measured with a BCA analysis.
- Protein Yield is the Measured Concentration divided by the Wet Cell Pellet weight.
- Preliminary results suggest smaller amounts (500 mg and 1 mg) lyse more efficiently.

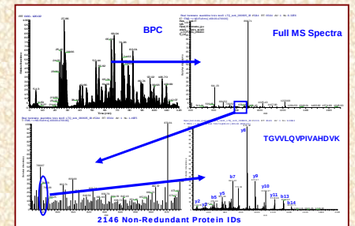

Figure 3: This very low abundant peptide was triggered for MS/MS following the full MS (top right spectra) that eluted at 65 min. The peptide corresponds to a doubly charged ion, which was identified as belonging to LysS (RPA4685, lysyl tRNA synthetase) protein from *R. palustris*.

- Multidimensional HPLC-tandem mass spectrometry is sensitive and can identify low-abundance peptides that would be overlooked using other methods as shown in Figure 3 with the peptide from LysS, which was identified along with 2,145 other proteins in *R. palustris* using the LTQ.
- This method provides a less biased lysis procedure in comparison to sonication (See Figure 4).
- Therefore, this method of lysis is useful for identifying members from mixtures of microorganisms that other methods of lysis discriminate against.

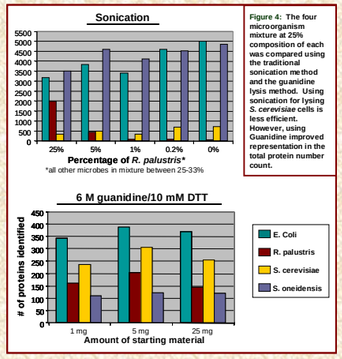

Figure 4: The four microorganism mixture at 25% composition of each was compared using the traditional sonication method and the guanidine lysis method. Using sonication for lysing *S. cerevisiae* cells is less efficient. However, using Guanidine improved representation in the total protein number count.

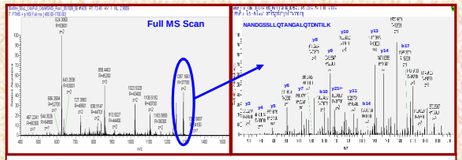

Figure 5: A 50 mL sample of AMD Biofilm cells was analyzed by a LTQ-Orbitrap hybrid mass spectrometer using two dimensional HPLC nano-ESI. This peptide was identified with Sequest as being from a flagellin from the Leptospirtium group III microorganisms within the community. In the full MS scan there was a mass accuracy of 0.5 ppm. The mass accuracy of the in the MS/MS spectrum were between 0.37 to 6.8 ppm. The high mass accuracy of the LTQ-Orbitrap increases the confidence in detection and identification of peptides from complex mixtures like the AMD Biofilm.

TRYPSIN OPTIMIZATION

Table 4: Digestion comparison with immobilized trypsin beads and traditional lyophilized trypsin

Trypsin Type	*R. palustris* Peptide Count	*R. palustris* Protein Count	Trypsin Spectral Count
Promega Modified Sequencing Grade	6079	819	8
Trypsin Beads	5194	677	0

Table 5: Identification of autotryptic cleavage peptides using lyophilized trypsin

Organism	Biomass size	Instrument	Trypsin (mg)	Sequence Coverage	Peptide Count	Spectra Count
S. oneidensis	4 g	LCQ	120	17.8%	4	32
S. oneidensis	50 mg	LCQ	40	17.3%	4	49
S. oneidensis	25 mg	LCQ	40	17.3%	4	21
S. oneidensis	10 mg	LCQ	40	17.3%	4	37
S. oneidensis	5 mg	LCQ	20	17.3%	4	53
S. oneidensis	1 mg	LCQ	20	48.1%	10	124
S. oneidensis	1 mg	LTQ	10	50.2%	10	308
S. oneidensis	0.5 mg	LTQ	10	50.6%	13	482
R. palustris	25 mg	LCQ	40	18.2%	7	27
R. palustris	5 mg	LCQ	40	24.2%	6	17
R. palustris	1 mg	LCQ	40	51.5%	18	116
AMD Biofilm	300 mg	LCQ	40	NA*	NA*	NA*

* The protein database contains a trypsin-like serine protease protein that precludes the ability to identify auto-tryptic peaks.

- We tested two different methods for improvement or elimination of auto-tryptic peptide identifications with the small biomass samples.
  - The first method tested was a comparison between using covalently bound trypsin beads and sequencing grade modified trypsin (See Table 4).
    - The same samples were analyzed, but the number of *R. palustris* proteins identified dropped from ~820 with modified lyophilized trypsin to ~677 proteins with the beads.
  - The second method tested whether decreasing the amount of trypsin used would decrease the autotryptic peptides but not sacrifice peptide cleavage of the proteome (Table 5).
    - There is not much of a decrease in the number of autotryptic peptide identifications using a LCQ mass spectrometer between adding 40 or 20 µg of trypsin to 1 mg of cells. 10 µg of trypsin added to 1 mg of *S. oneidensis* with the LTQ drops the autotryptic spectral count by 20.
- Use of trypsin beads decreases the number of proteins identified, but decreasing the amount of trypsin added to the sample leads to a decrease in the identification of autotryptic peptides.

CONCLUSION

- Preliminary results of using Guanidine for chemical lysis of microorganisms.
  - We have the ability to identify the same number and constitution of proteins starting with either 4 g of cells or 1 mg of cells.
    - There is an overlap of ~68% of the proteins identified between cell pellets of 1-5 mg and 4 g.
  - Newer instrumentation like the LTQ-orbitrap allows a deeper characterization of a microbial or community proteome (Figure 3).
  - Single tube lysis provides less bias in microorganism lysis of our 4 microorganism artificial mixture (Figure 4).
  - Use of less trypsin (in lyophilized form) works more effectively than trypsin beads in our studies.
    - Tables 4 and 5 depict the loss in protein identifications using the trypsin beads.
  - Comparison of using Guanidine versus a recently published method using TFE[2].
    - Trifluoroethanol (TFE) is an organic solvent that will lyse cells, extract, and denature proteins in a similar manner to Guanidine used in this study. We are currently comparing these techniques.
  - Demonstration of single tube lysis provides the ability to investigate the metabolic dynamics of a naturally occurring biofilm in a spatial manner

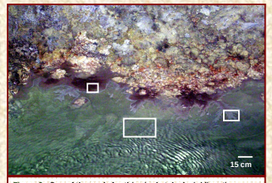

Figure 6: One of the goals for this single tube lysis/digestion method is to develop the ability to study the spatial community dynamics of this biofilm and other natural microbial communities. (AMD biofilm image obtained from: Dr. Jill Banfield)

REFERENCES

1. Ram *et al.* 2005. Science 308: 1915-1920.
2. Wang *et al.* 2005. Journal of Proteome Research 4: 2397-2403.

ACKNOWLEDGMENTS

- M. Thompson and B. Erickson acknowledge support from the ORNL-UTK Genome Science and Technology Graduate School.
- Research support was provided by the U.S. Department of Energy Office of Science, Environmental Remediation Sciences Program.
- This research was supported by grants from the DOE Microbial Genome Program (JFB), NSF Biocomplexity Program (JFB), DOE Energy Biosciences Program (RCB), and DOE Genomes to Life Program (RLH).
- We thank Dr. David Tabb (ORNL) and the Yates Proteomics Laboratory at Scripps Research Institute for DTASelect/Contrast software, the Institute for Systems Biology for proteome bioinformatics tools used in analysis of the MS data, and Dr. Frank Larimer at ORNL for providing computer resources.
- Oak Ridge National Laboratory is managed and operated by the University of Tennessee-Battelle, L.L.C., under contract DE-AC05-00OR22725 with the U.S. Department of Energy.