Paper # N-339

Application of custom-designed fermentors for extremophilic microorganisms

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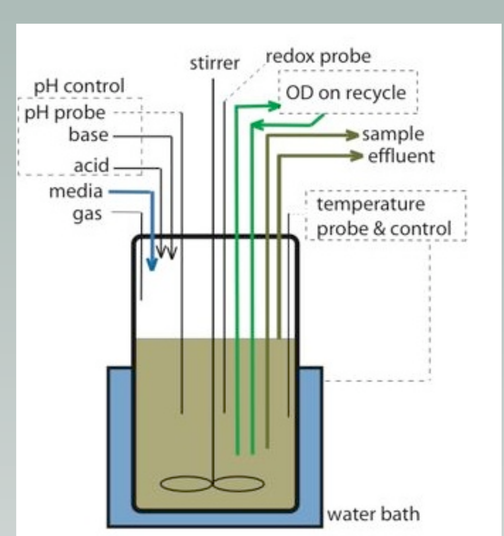
Abstract

Background: Extremophilic microorganisms may play key roles in remediation of groundwater contaminants and biofuels development. Standard fermentors are not equipped for anaerobic growth conditions, nor can stainless steel withstand the reactivity of metal-reducing organisms and their metabolic by-products. We have developed methods to grow the sulfate-reducing bacterium, *Desulfovibrio vulgaris* Hildenborough (DvH), under various, batch and continuous growth conditions for detailed physiological and molecular analyses, using custom-built fermentors.
Methods: Five-liter-volume fermentors have PEEK headplates and agitators. During growth, temperature, pH, OD, and redox potential are continuously controlled. Samples are taken for ion chromatography, phospholipid fatty acid (PLFA) analysis, direct cell counts, RT-PCR, and total cell protein. DvH is grown in batch and turbidostat modes using a defined, lactate-sulfate medium at 30ºC. Batch cultures have also been grown under various stress conditions.
Results: The maximum specific growth rate for DvH in the 5-liter fermentor is 0.11 h^{-1}. At the onset of deceleration phase, all lactate (60 mM) is depleted, and 30 mM of the initial 50 mM sulfate is metabolized. The dilution rate to maintain the culture at mid-log phase in the turbidostat is 0.15 h^{-1}. Cell densities and total proteins range from 5-10 x 10^8 cells/ml, and 80-120 µg/ml, respectively. PLFA profiles for DvH are sensitive to the growth conditions and growth phase, while preliminary results show consistent whole-cell protein patterns when visualized using SDS-PAGE. Melt curves generated by real-time PCR proved to be an excellent quality control tool to monitor culture purity.
Conclusion: Detailed characterization of DvH during growth in custom-designed pilot-scale fermentors provides insights into physiological changes during different growth phases and stress conditions, and has resulted in the development of protocols for controlled and reproducible production of high quality biomass. The experiments have also proven the utility of custom-designed fermentors for growth of extremophiles.

Description of Fermentors

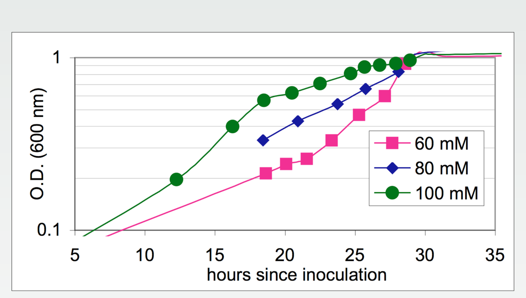


Schematic of fermentor

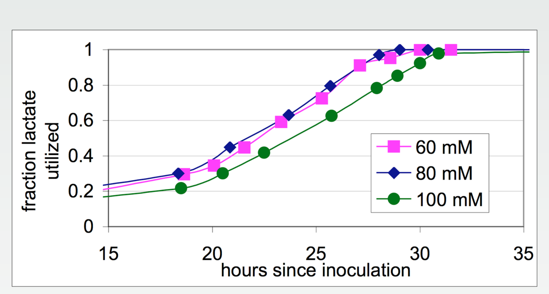
Two fermentors were run simultaneously in turbidostat mode to produce 100-liter DvH/week for the Protein Analysis Complex Project. The 10-liter medium bottle is shown between the fermentors. Effluent was continuously collected and chilled to 4°C (right image), while cells were harvested by batch centrifugation. Anaerobic headspace is maintained by flow of sterile nitrogen, or other anaerobic gas mixes.

Batch Growth - Effect of initial lactate concentration

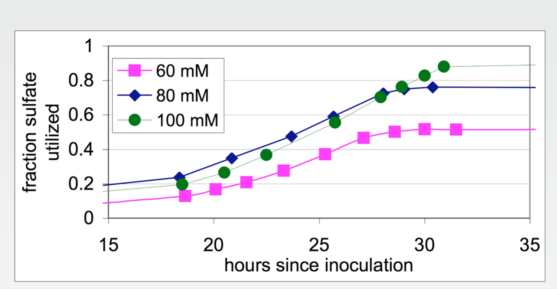
Growth of DvH in lactate/sulfate medium with 10% inoculum was compared for initial lactate concentrations of 60, 80 and 100 mM. The initial sulfate concentration was 50 mM in all cases. pH was maintained at 7.2 with 1 N HCl. The mixing rate was set to 200 RPM. Redox potential increased from -700 to -450 mV during growth phase, and furthermore to -350 mV during stationary phase.



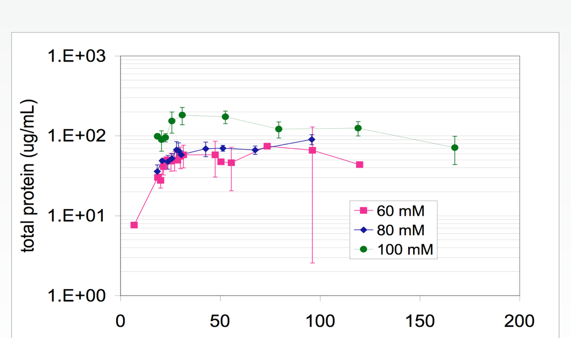
Specific growth rate increased with increasing initial lactate concentration



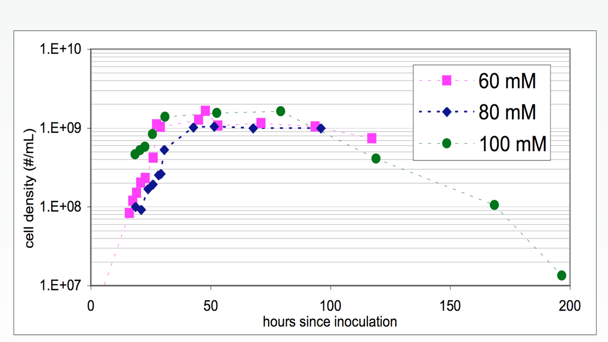
At the onset of stationary phase, all lactate was metabolized



Stoichiometric use of sulfate with initial lactate concentration was observed



Higher protein concentrations were measured for 100 mM initial lactate

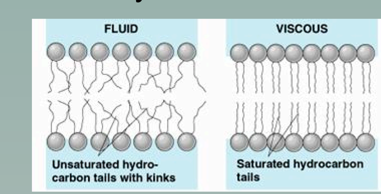


Cell densities were comparable between runs, indicating higher protein concentrations per cell with higher lactate concentrations

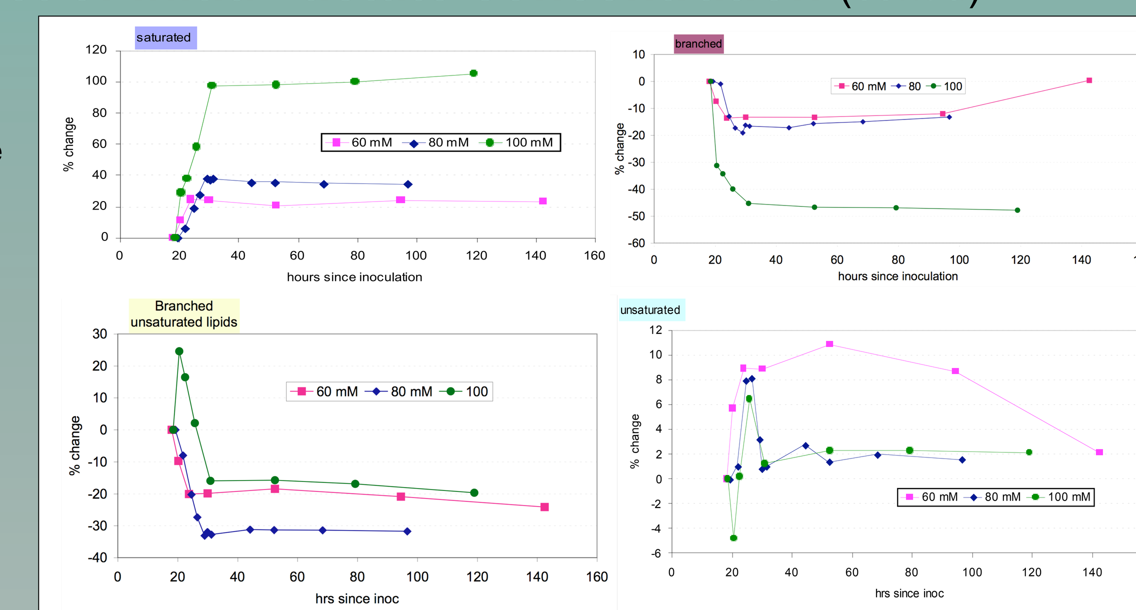
Batch Growth - Effect of initial lactate concentration (cont'd)

Phospholipid Fatty Acids:

The membrane lipid composition varies with the physiological state of the organisms. Membrane fluidity increases with a higher fraction of branched, unsaturated lipids; this increases membrane permeability.



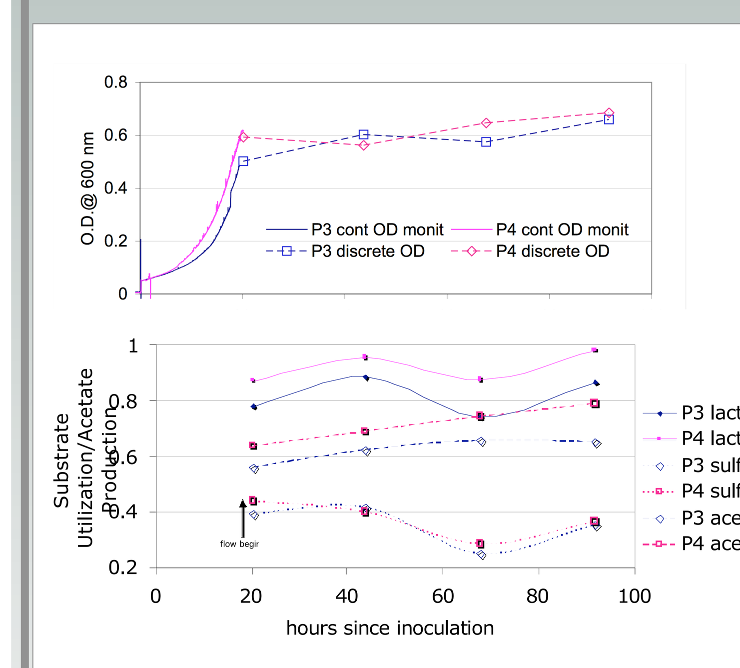
http://fajerpc.magnet.fsu.edu/Education/2010/Lectures/11_Membranes.htm

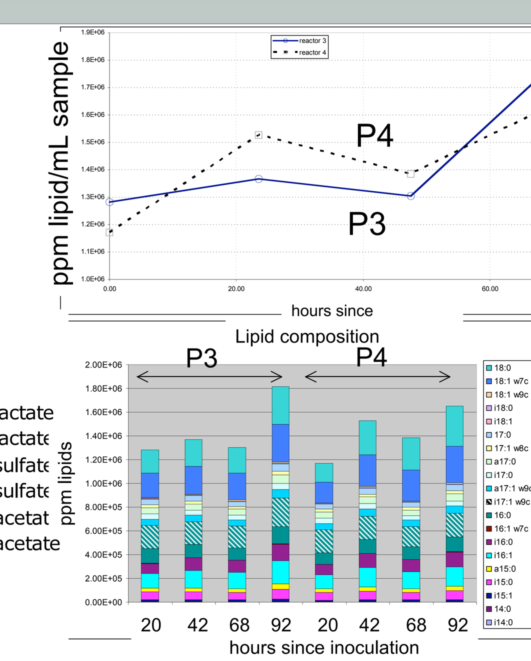


Percent change of the different types of lipids relative to the midlog phase composition: With increasing lactate concentration, production of saturated lipids during log phase is increased and branched-saturated lipids is decreased significantly. Production of branched, saturated lipids decreases during log phase and unsaturated straight chain lipids remain relatively constant but do show a moderate increase during low lactate conditions. The increase in saturated lipids during high lactate conditions will decrease the fluidity of the membrane reducing ion transport. Phospholipid decreases during stationary and death phase follow similar trends found by cell count assays – the lipids decompose upon death of the cells.

Turbidostat runs in lactate/sulfate medium

DvH was grown in two fermentors, P3 and P4, to an O.D. of 0.6 and then continuously fed LS4D medium containing 60 mM lactate and 50 mM sulfate. pH was maintained at 7.4 with the addition of 1.5 M H2SO4. At a dilution rate of 0.13 hr^{-1}, OD of ~0.6 was maintained over 70 hours or 10 reactor volumes. Redox potential was monitored and remained between -700 and -650 mV.





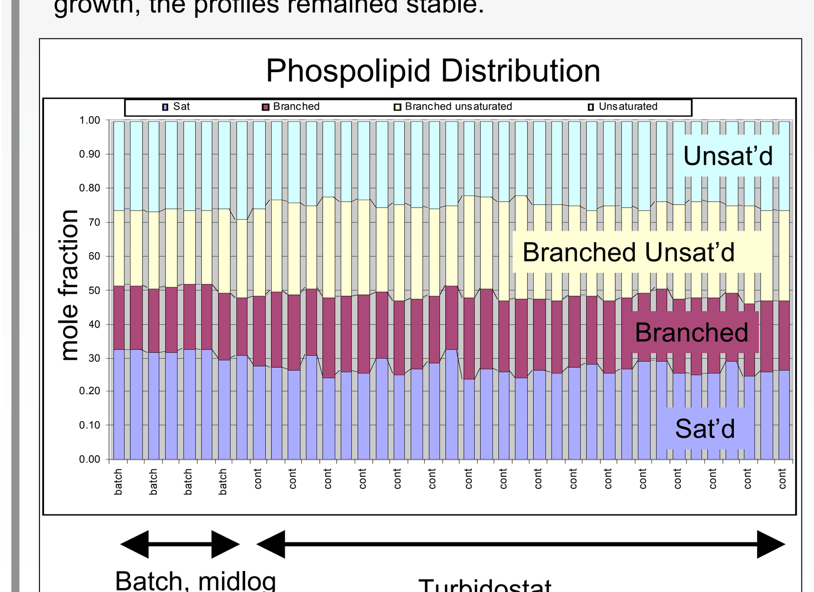
O.D., lactate and sulfate utilization, and total lipids content generally increased with time, while the lipid profile remained constant. Cell densities ranged from 2 x 10^8 to 7 x 10^8 cells/mL, and proteins ranged from 70-125 µg/mL (plots not shown)

Lipid Key:
X:YwZ, where X equals the number of carbon atoms, Y equals the number of double bonds, and Z equals the position of the first double bond counting from the methyl end.
Saturated: 14:0,16:0,17:0,18:0
Branched: i14:0,i15:0,a15:0,i16:0,i17:0,a17:0,i18:0
Branched unsat'd: i15:1,i16:1,i17:1w7c,i17:1w9c,a17:1w9c,i18:1
Unsaturated: 16:1w7c,17:1w8c,18:1w9c,18:1w7c
DvH biomarker

Culture consistency assessed by PLFA and RT-PCR

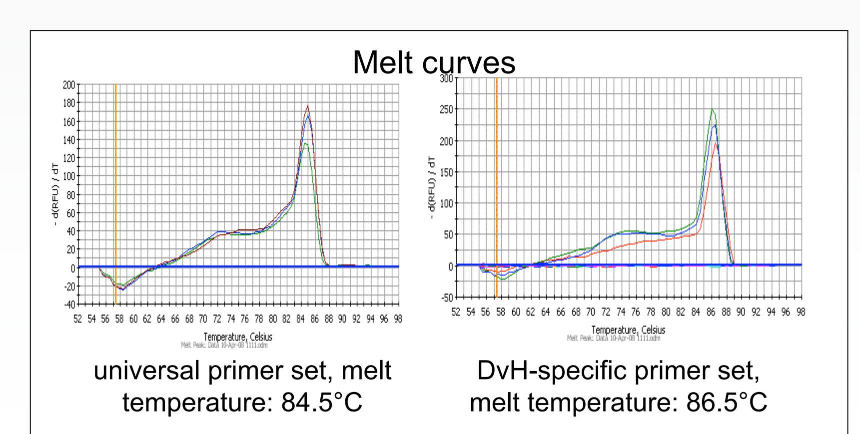
PLFA

The relative distribution of saturated, unsaturated, branched, and unbranched lipids were compared for several mid-log phase batch and turbidostat growth runs. While average lipid concentrations differed slightly between batch and turbidostat growth, the profiles remained stable.



Real-time PCR

Real time PCR provided a highly reproducible and relatively inexpensive way of high-throughput analysis of culture purity in a continuously running bioreactor. A combination of universal and DvH-specific primers targeted less than 200-bp fragments of the highly conserved 16S rRNA-genes, respectively. Using SYBR® GreenER™ (Invitrogen) for amplicon detection, genomic DNA was amplified. Melt curve analysis validated that all bioreactor samples represented only one organism, a pure culture of *Desulfovibrio vulgaris* Hildenborough.

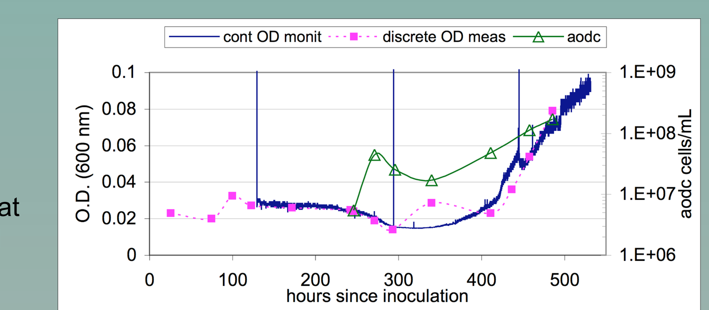


universal primer set, melt temperature: 84.5°C

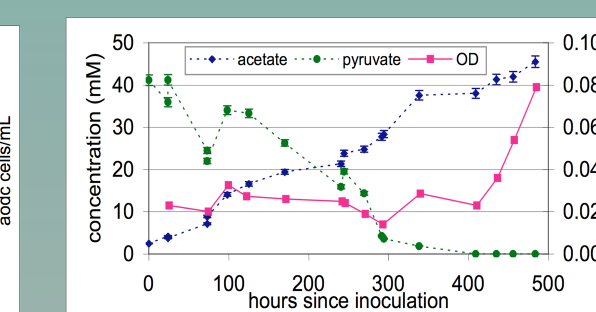
DvH-specific primer set, melt temperature: 86.5°C

Pyruvate fermentation - batch mode

DvH was grown in a defined medium with 40 mM pyruvate, and no sulfate, Tris buffer, 90% nitrogen/10% carbon dioxide headspace gas, at 150 rpm mixing rate. pH ranged from 6.7 to 7.

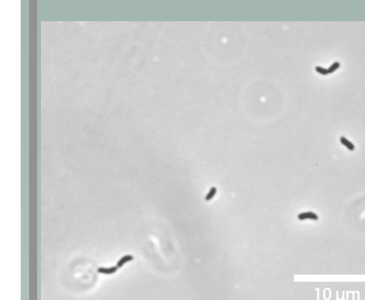


O.D. values during fermentation were 3-times lower for the same cell density compared to respiration

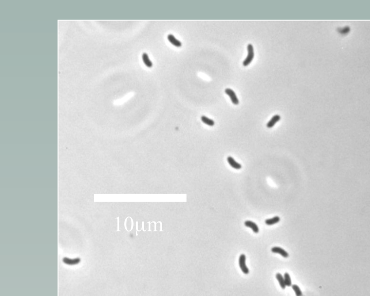


Pyruvate concentration decreased as acetate concentration increased. Cell density increased faster at lower pyruvate concentrations

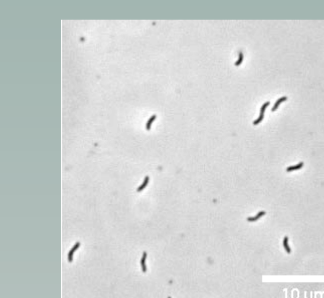
Respiration vs fermentation



DvH grown in LS4D (60 mM lactate)

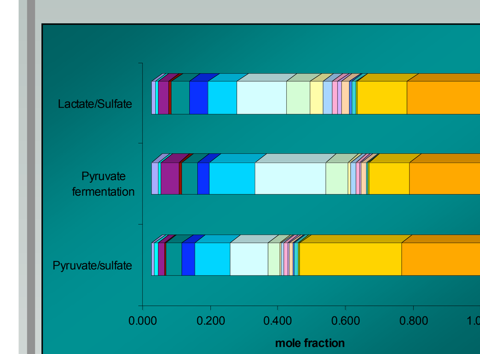


DvH grown in LS4D (100 mM lactate)

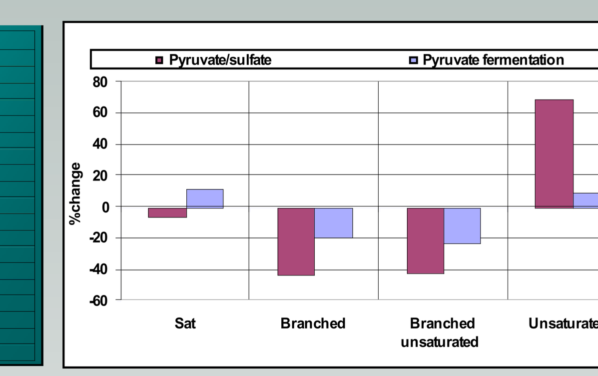


DvH grown in pyruvate medium (40 mM, no sulfate added

DvH gains energy by respiration in LS4D, while it grows by fermentation in a pyruvate-containing medium that has no electron acceptor. Fermentation produced cell morphologies that appeared thinner and longer compared to cells grown by respiration.



	Pyruvate/sulfate	Pyruvate fermentation	Lactate/Sulfate
18:0	0.262	0.235	0.244
18:1 w7c	0.300	0.122	0.149
18:1 w9c	0.006	0.003	0.002
i18:0	0.012	0.004	0.013
i18:1	0.003	0.000	0.005
17:0	0.010	0.015	0.026
17:1 w8c	0.007	0.006	0.009
a17:0	0.010	0.011	0.019
i17:0	0.007	0.014	0.027
a17:1 w9c	0.005	0.011	0.036
i17:1 w9c	0.035	0.066	0.073
16:0	0.114	0.206	0.143
16:1 w7c	0.102	0.135	0.085
i16:0	0.037	0.035	0.056
i16:1	0.049	0.048	0.053
a15:0	0.004	0.009	0.011
i15:0	0.019	0.052	0.031
i15:1	0.010	0.009	0.007
14:0	0.010	0.021	0.012



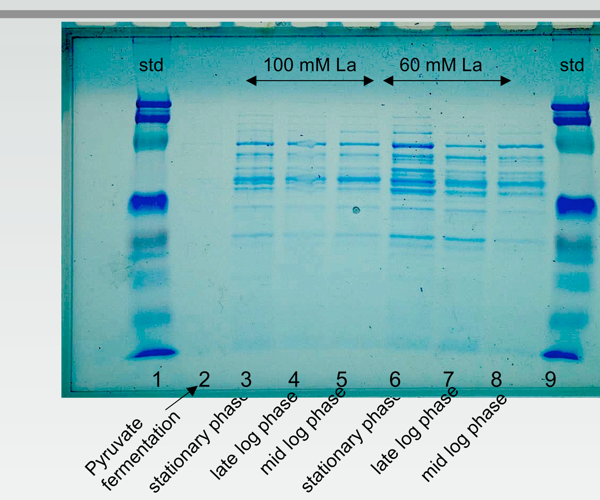
Membrane phospholipid distribution (plot on the left) changewith electron donor during respiration (compare lactae/sulfate with pyruvate/sulfate growth), as well as with respiration vs fermentation (compare pyruvate fermentation with pyruvate/sulfate growth). The change in types of lipids in percentage to lactate/sulfate growth (plot on the right) shows that the direction of change is the same for all but the saturated lipids. Larger changes, however occurred with change in electron donor compared to fermentation.

Analysis of the cytoplasmic proteins using 1D SDS-PAGE

Protein yield from pyruvate fermentation was very low.

The stationery phase samples from 100 and 60 mM lactate show most number of proteins expressed.

Similar profiles were observed if cells harvested at similar growth phases were compared, regardless of the substrate concentration.



Summary and conclusions

- Higher initial lactate concentrations increased specific growth rate in batch reactors, and produced higher protein concentrations. The fraction of saturated lipids in the cell membrane increases with lactate concentration, suggesting that the membrane becomes less fluid, and less permeable.
- Membrane lipid distributions change with growth phase, specifically production of branched, saturated lipids decreases during log phase and unsaturated straight chain lipids remain relatively constant
- From the visualization of the cytoplasmic proteins, the largest number of proteins were expressedduring stationary phase, and profiles were independent of initial lactate concentration
- In turbidostats, RT-PCR and PLFA showed culture consistency throughout 70 h of production. Small increases in cell densities, protein concentrations and total lipids were measured with time.
- Comparing pyruvate respiration vs fermentation, distinct differences were seen in DvH cell morphology and lipid profiles. Relative to growth by respiration of lactate, phospholipids distributions differed significantly for growth by pyruvate respiration, and pyruvate fermentation. Straight chain unsaturated lipids increased, and branched saturated and unsaturated lipids decreased, and the magnitude of change was much greater for growth in pyruvate respiration.

Acknowledgements

The authors are grateful to Chris Walker, Judy Wall and Bill Yen for their helpful guidance.
PCAP is part of the Virtual Institute for Microbial Stress and Survival (VIMSS) supported by the US Deparatment of Energy, Office of Science, Office of Biological and Environmental Research, Genomics:GTL Program through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the US Department of Energy.