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Metabolome Studies of Stress Responses in *Saccharomyces Cerevisiae*

- implementation of sampling and analytical protocols

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Submission date: August 2011
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ACKNOWLEDGEMENTS

This master thesis was carried out at the Department of Biotechnology, Faculty of Natural Science and Technology at the Norwegian University of Science and Technology (NTNU), leading to the degree of Master of Science in Biotechnology.

My profound thanks to my supervisor, Associate Professor Per Bruheim, for introducing me to the field of metabolomics, as well as valuable guidance, feedback and support whenever needed. My gratitude is also given to Kåre Kristiansen for support regarding GC-MS and fermentation and to PhD Candidate Stina Katrine Lien for great support under the process for building the DRS library. Appreciation is also given to laboratory technician Siri Stavrum and the Orakeltjenesten at NTNU Gløshaugen.

Immense appreciation is given to my friends and family for inestimable support and encouragement.

Trondheim, August 2011

Anne Marte Haug

ABSTRACT

As a part of cancer research and therapy, it is important to study metabolic responses caused by DNA damaging agents. Whereas earlier studies have focused on changes at protein levels caused by DNA damaging agents, this project focuses on establishment of sampling and cultivation protocols for metabolome analysis, using *Saccharomyces cerevisiae* as a model organism growing in exponential phase. Sampling and cultivations protocols were optimized before cultures were stressed by DNA damaging agent. At the time of sampling, cells and extracellular media were separated by filtration prior to quenching and extraction in 75% boiling ethanol. Solvent evaporation under reduced pressure and ambient temperature were used for metabolite concentration. Yeast cultures were exposed to osmotic stress and two concentrations of the DNA damaging agent 5-fluorouracil. Samples were taken prior to stress exposure, at the time of exposure and in intervals after stress exposure. Metabolite extracts were analyzed by GC-MS single quadrupole in respect of amino acids, citric acids cycle intermediates and fatty acids. Generally, citric acid cycle intermediates were most influenced by stress agents, followed by amino acids and fatty acids. GC-MS single quad was the only analytical method applied. For a more complete metabolic profiling, including other groups of metabolites, LC-MS/MS should be applied. This analytical method will cover groups, such glycolytic intermediates, which can not be analyzed by GC-MS, due to their volatility.

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1 INTRODUCTION

1.1 *Saccharomyces cerevisiae* as a Model Organism

Microbes are less complex systems to be studied and show advantages over higher organisms. Gene regulation, metabolic network and physiology are therefore much more studied in microbial cells compared to higher eukaryotic systems (1). One of the main criteria for model organism is the compliance to experimental manipulation. *Saccharomyces cerevisiae (S.cerevisiae)*, also referred to as yeast, fulfills the criteria as well as it grows on defined media, which enables control of chemical and physical environment. Use of *S.cerevisiae* as a model organism, to understand different biological aspects of the eukaryotic cell, has increases over the last several decades due to its compliant properties (2).

In 1996, *S.cerevisae* was the first eukaryotic specie to have its complete genome sequenced (3). By complete sequencing of the yeast genome, the more complex human genome can be understood. Similarities in human and yeast amino acid sequence are seen in nearly half of the proteins known to be defective in human heritable diseases. Suggestions are purposed that the majority of the yeast has human homologs. However, the human genome specifies proteins which are not seen in yeast (3).

Forster et al (2003) show that <30% of metabolites are involved in only two enzymatic reactions, while 12% is involved in >10 reactions and 4% is involved in >20 reactions. This reveals that metabolic network is closely linked and analysis of single metabolites is needed for an overall understanding of interaction of the metabolic activity in a cell.

1.2 Metabolite Analysis

Metabolomics are defined as a systematic analysis of large number of low molecular weight compounds from a biological system (4). As a term, it includes quantification of extracellular and intracellular metabolite concentrations (5). Metabolite levels of an organism will reflect responses to genetic or environmental changes in a biological system (6). For metabolite analysis, a large number of metabolites must be extracted, the extract must be representative for the whole culture, the metabolites must be separated from other cell components and the metabolic activity must be stopped immediately after sampling (7). Because all criteria rarely are fulfilled simultaneously, inactivation and extraction methods are often optimized to fulfill the criteria in the best manner.

1.2.1 Inactivation, Extraction and Concentration of Intracellular Metabolites

Rapid inactivation of metabolism is needed to keep the *in vivo* metabolite concentration at a constant level from time of sampling to time of analysis (6). For analysis of intracellular metabolites, cells and media are separated. Intracellular metabolites are thereby concentrated and disturbance from natural extracellular metabolites are avoided (7). Filtration and centrifugation are two commonly used methods for separation of cells and media. After separation, intracellular metabolites are made available for analysis by extraction process. High temperatures, extreme pH, organic solvents, mechanical stress or a combination can be used as extraction methods (8). Extraction solutions re-dilute the metabolites, and freeze-drying, also called lyophilization, and solvent evaporation are two available methods for metabolite concentration. Sample preparation from sampling until the last concentration is referred to as quenching and extraction methods or protocols.

In 1976, Saez and Lagunas presented a protocol which combined cell separation and quenching by filtration and washing the cells with 60% methanol. De Koning and Van Dam (1992) revealed that filtration could not be applied for metabolites with short turn over times, and presented an improved protocol which proposed direct quenching in 60% cold methanol followed by centrifugation. Direct quenching followed by centrifugation is still the most widespread method for rapid sampling for microbial cultures (4).

Common criteria for an ideal metabolite extraction are complete extraction so the complete intracellular pool is accessible, effective inactivation of enzymes to prevent metabolite changes under sample preparation and absence of extensive degradation of metabolites by the procedure itself (8-10). Examples of different extraction methods studied are freeze-thaw in liquid nitrogen (11), chloroform (9), direct quenching and extraction by 75% boiling ethanol (10), chloroform; methanol buffer, boiling ethanol, perchloric acids, potassium hydroxide, methanol; water and pure methanol (6) and hot water, boiling ethanol, chloroform; methanol, freeze-thaw in methanol and acidic-acetonitril methanol (8). Different extraction methods favor some classes of compounds and even discriminations within classes do appear (6). A combination of extraction must be applied to cover the diversity of chemical and physical properties of metabolites. The choice of sample preparation methods must therefore be coherent with the aim of study and metabolites of interest (5).

When sampling preparations includes several steps, addition of intern standards are useful for determination of metabolite recoveries and to register which stages causes the largest losses of metabolites (8).

1.2.2 Analytical Methods

1.2.2.1 Enzymatic Reactions

Phosphorylated hydrocarbon is relatively similar in regard of their polarity and structure, which challenge the analysis. Enzyme assays can easily separate and quantify glycolytic intermediates, as well as being a quick and simple method. Concentrations are proportional to production or use of NADH or NADPH, measured by increase or decrease of absorbance at 340nm respectively. Metabolite concentration in extracts can be estimated by comparing change in absorbance with change in absorbance from assays with standards. Enzyme assays for analysis of glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), dihydroxyacetone phosphate (DHAP), fructose-1,6-biphosphate (F-1,6-BP) and pyruvate were used in this project. All the above metabolites are part of the glycolysis, and quantifications will reflect the central metabolism. Reaction mechanism for each metabolite is explained below.

Glucose-6-phosphate

Glucose-6-phosphate dehydrogenase catalyzes the oxidation of glucose-6-phosphate to 6-phosphoglucono-δ-lactone while NAD^+ is reduced to NADH, resulting in increased absorbance. Reaction mechanism is shown in Figure 1.

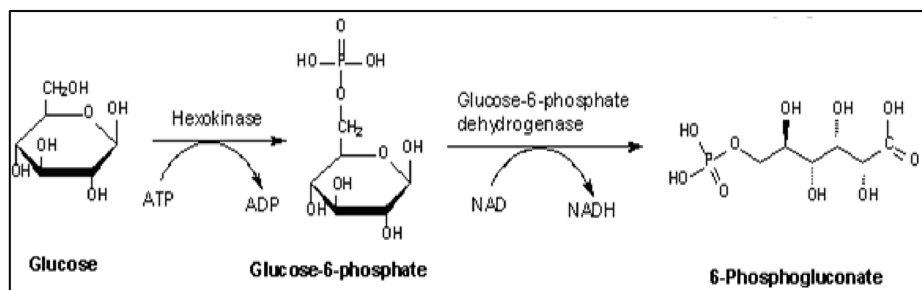

Figure 1-Reaction mechanism of production of 6-phosphogluconate

Fructose-6-phosphate

Glucose-6-phosphate isomerase catalyzes the isomerization of fructose-6-phosphate to glucose-6-phosphate. For quantitative measurements the reaction is coupled with the reaction in Figure 1, because no production or use of NADH is observed in the isomerization reaction. The reaction mechanism of fructose-6-phosphate to glucose-6-pjosphate is shown in Figure 2.

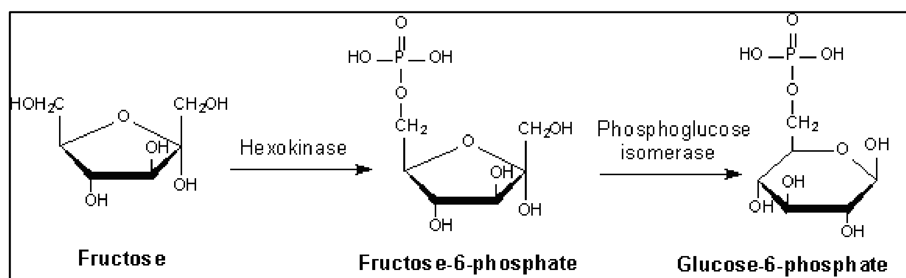

Figure 2-Reaction mechanism of production of glucose-6-phosphate from fructose-6-phosphate

Dihydroxyacetone phosphate

Glycerol-3-phosphate dehydrogenase catalyzes production of glycerol-3-phosphate from DHAP. NADH is used and absorbance decreases as the reaction proceed. The enzyme is delivered in combination with triose-phosphate isomerase, which catalyzes the formation of glyceraldehyde-3-phospahte from DHAP. This enzyme is also referred to as triose phosphate dehydrogenase. No change in absorbance is seen in the latter reaction mechanism. Both reaction mechanisms are shown in Figure 3.

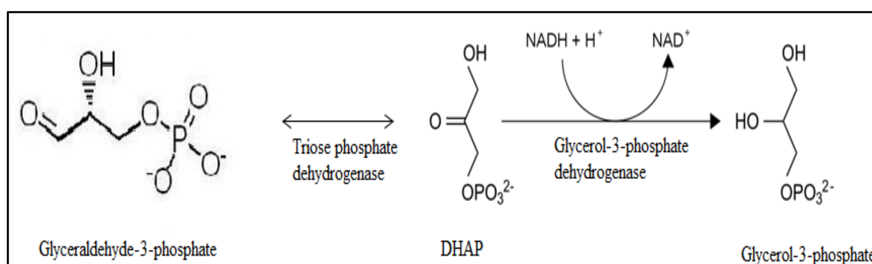

Figure 3-Reaction mechanism of production of glyceraldehyde-3-phosphate and glycerol-3-phospahte from DHAP.

Fructose-1,6-biphosphate

Fructose-1,6-biphosphate aldolase converts fructose-1,6-biphosphate to glyceraldehyde-3-phosphate and DHAP. No production or use of NADH is involved in the reaction, and it is therefore coupled to the reaction in Figure 3 for quantitative measurements. The reaction mechanism of production of glyceraldehyde-3-phosphate and DHAP from fructose-1,6-biphosphate is seen in Figure 4.

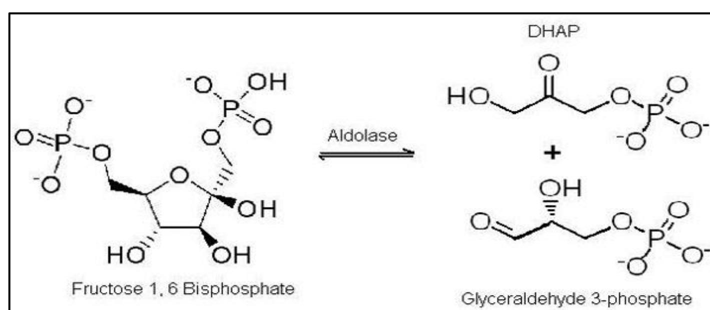

Figure 4- Reaction mechanism of the production of glyceraldehyde-3-phosphate from fructose-1,6-biphosphate

Pyruvate

Lactate dehydrogenase (LDH) catalyzes the production of lactate from pyruvate, as seen in Figure 5. Pyruvate concentration is estimated by the decreased absorbance of NADH.

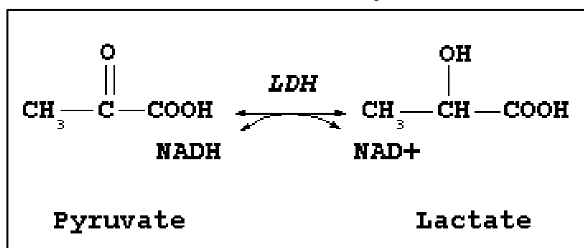

Figure 5-Reaction mechanism of production of lactate from pyruvate. Lactate dehydrogenase is abbreviated LDH

1.2.2.2 Gas Chromatography

The principle of chromatography is the migration of analytes through a column. Analytes are carried on mobile phase and interacts with stationary phase in the column. Interactions between mobile phase and stationary phase determine the retention time of analyte. Retention time is the time taken for an anlayte to pass through a column. In gas chromatography (GC) mobile phase is gas, typically nitrogen or helium and stationary phase is a solid. Volatility of each analyte determines the rate through the column and so does its solubility in stationary phase (12). For analyzes the sample is introduced to the injector, where evaporation takes place, before entering the column by the carrier gas. By changing the temperature in the column, analytes are separated by their volatility. At the end of the column, the analytes enter the detector, for example mass spectrometer. Key elements of a gas chromatograph are shown in Figure 6.

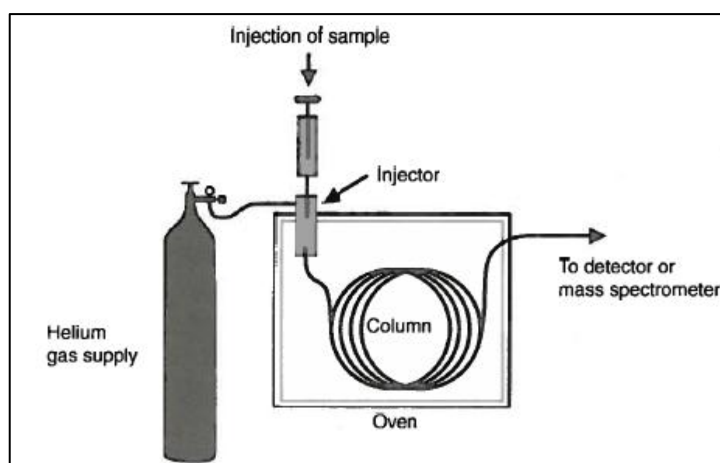

Figure 6- The key element of a gas chromatograph (13)

For GC analyses analytes must be volatile. In metabolomics nonvolatile metabolites are of interest such as as amino acids, sugars, small organic acids, polar metabolites and apolar metabolites. Metabolites can be made volatile by derivatization, covering carboxylic, hydroxylic and apolar side groups are covered with either methyl group or silylan (13).

1.2.2.3 Mass Spectrometry

Mass spectrometry (MS) as a detector provides high sensitivity, can give both structural and chemical information and can analyze complex samples, which makes it a versatile detector in the field of chromatography. Any compound carrying charge, or which can be charged and evaporated can be analyzed for mass to charge ratio (m/z), which is the core principle in MS. Over the years, there has been extensive development of MS, improving the sensitivity, expanded the range of molecules that can be determined, and reducing the prize as well as they are more easily to operate nowadays (13). Three units compose a MS; ionization, separation and detection. (13). The ion source transfers the samples into gas phase, if not already evaporated as in GC, ionizes the samples and

transfers it to vacuum. Electron ionization is the most common ionization technique. The metabolites are exposed to high-energy electrons, leaving positive ions, M^+. The ions carry much more energy than the parent molecule, and are frequently fragmented. Fragmentation is reproducible and characteristic for single metabolites, and can be compared to reference spectra for identification (12). Under ionization molecule-molecule collision is avoided by applying high vacuum. The newly made ions are lead to the analyzer by an acceleration plate with higher potential. A repeller plate inside the ionization source controls the electric field. The positions of acceleration and repeller plate are shown in Figure 7. High temperature prevents condensation and high vacuum removes non-ionized compounds as well as carrier gas. (13).

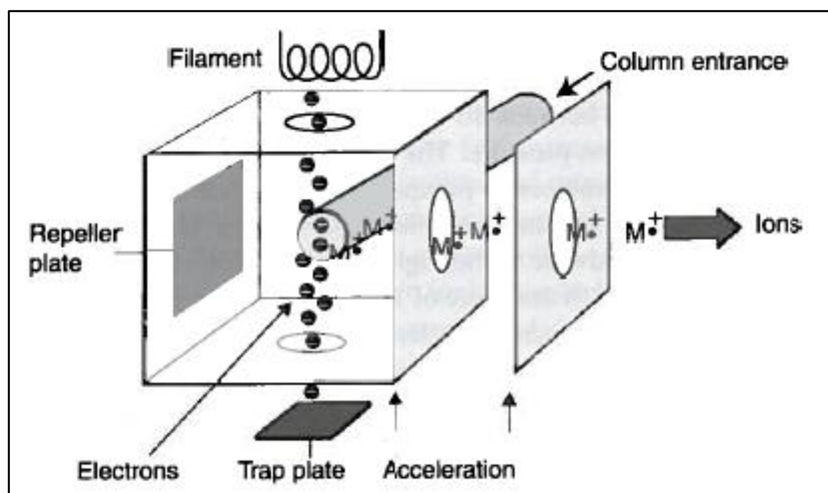

Figure 7- Ionization source (13)

The analyzer consists of four parallel cylindrical rods, quadropole, given direct (U) and alternating current (V_0) in radio frequent range. The voltage supply is connected to adjacent rods, making the ions spinning in a cylindrical pattern. Large ions will spin in smaller circles, whereas smaller ions spin in larger circles. Keeping the U/V_0 ratio and frequency constant, ions with different mass to charge (m/z) ratio will move towards the detector one by one. If only alternating voltage is applied all ions hit the detector, called scan mode. Applying varying direct voltage, only ions with specific m/z ratio will reach the detector, called single ion monitoring (SIM). SIM mode is much more sensitive and foremost used for target analysis. On the contrary scan mode is used for profiling and dealing with unknown metabolites. Scan mode will be used for identification of metabolites in the following experiments (12, 13).

Detection in MS is based on the conversion dynode and electron multiplier. One ion hitting the conversion dynode, releases secondary ions which hits an electron multiplier and a cascade of electrons is released and more than 10^5 electrons generate a current. The current it further amplified and measured by either an analog or digital converter (13). A mass spectrum shows the abundance of each ion mass of an ionized and fragmented analyte as a function of its mass to charge ratio.

1.3 Building a Deconvolution Reporting Software (DRS) Library

Each metabolite is fragmented in a specific way and process a unique mass spectrum when GC-MS is applied. A DRS library holds a set of mass spectrum for selected metabolites. Compounds or peaks in chromatograms can be identified if the mass spectrum matched a mass spectrum in an established DRS library. The ion with the larges abundance is called the target ion (TI), and the following three ions called qualifier ion 1, 2, and 3 (Q1, Q2 and Q3). If the ratio between TI and qualifier ions matches the ratio in the library and the retention time is correct the presence of a certain metabolite is verified. Several components can appear in the same peak, but have slightly different retention times. Each component is identified by their unique spectra. This is illustrated in Figure 8. In addition to qualifiers, the whole spectra for each metabolite must be evaluated as a whole.

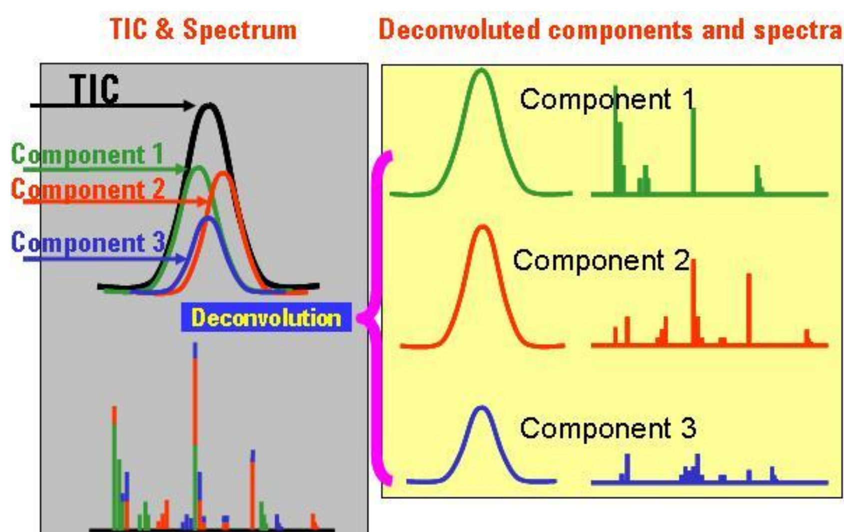

Figure 8- Illustration of identification of several components in one peak in a chromatogram by applying deconvolution (14)

When building a DRS library, metabolites to be included are MCF-derivatizated and analyzed by GC-MS. The data programs involved in the production of a DRS library are AMDIS and ChemStation. Each chromatogram is analyzed in AMDIS to define its retention time and recording of electron impact ionization mass spectrum (EI spectrum). The spectra are evaluated and TI, Q1, Q2 and Q3 selected. To program ChemStation part of a library, mass to charge ratio for TI, Q1, Q2, and Q3, and the ratio of qualifiers to TI are written. So are the name of each compound, its retention time, a certain CAS number, file name, company ID and uncertainties in percentage. ChemStation displays TI, Q1, Q2 and Q3, whereas AMDIS shows the whole spectra. An example of how data are displayed after applying a library is shown in Figure 9.

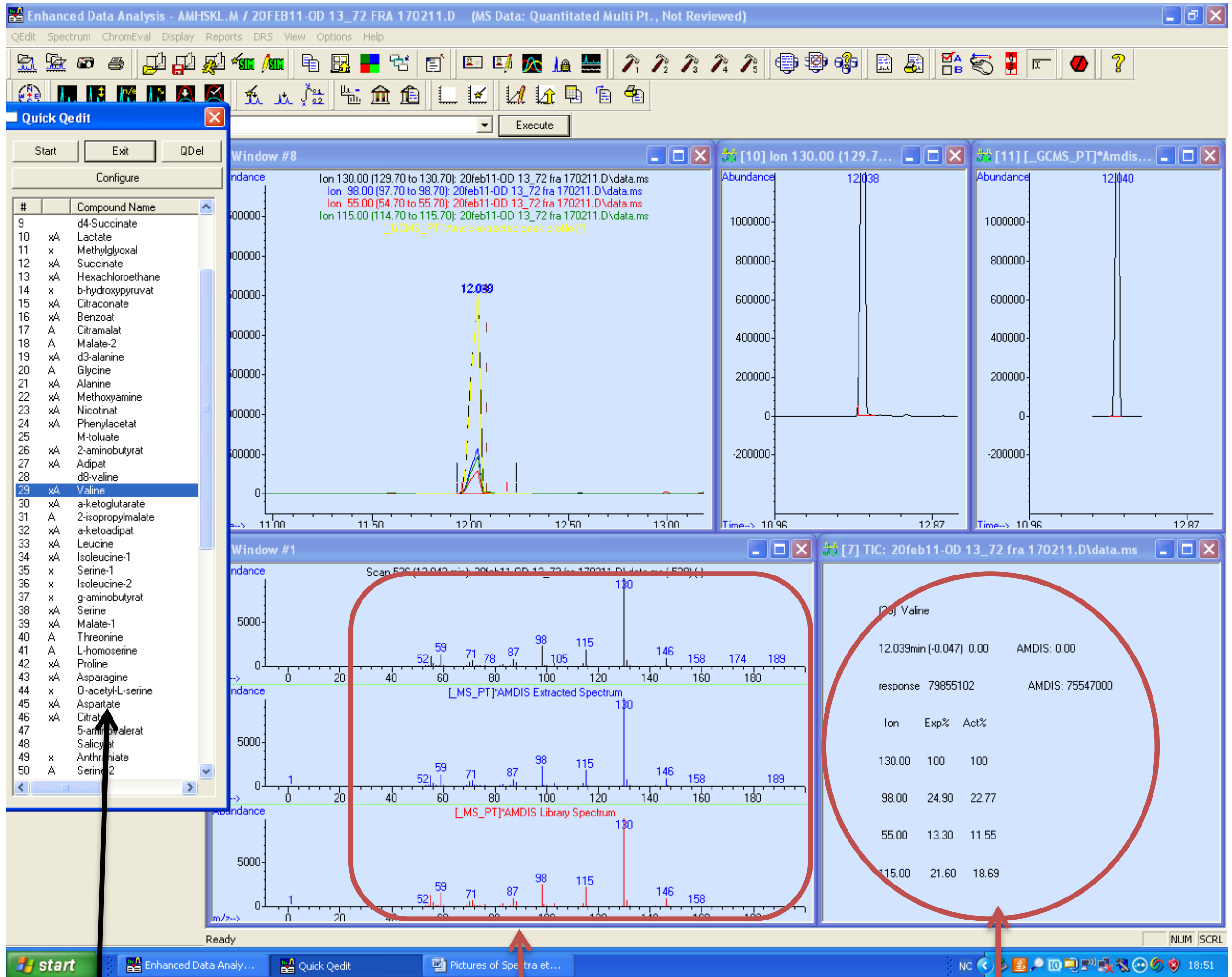

Figure 9 An example of a result screen after DRS are applied on a metabolic extract

Showing metabolites in library

Upper: Raw spectra from sample
Middle: Extracted spectra from sample
Lower: Library spectra

Showing relative abundance of target ion and qualifiers in the library, Exp%, and what is found in the analyzed extract, Act%.

1.4 Stress Agent 5-Fluorouracil

Anticancer drug promote cell damage of cancer cells. Such drugs can either inhibit biosynthetic processes or be incorporated into macromolecules or both. The fluoropyrimidine 5-fluorouracil (5-FU) is used in treatment of colorectal cancer for more than 40 years and represents both mechanisms above. It is a uracil analog, where the hydrogen atom at the fifth carbon is exchanged with a fluorine atom, Figure 10, and enters the cell in the same way as uracil (15). Fluorine does not alter the overall shape of the nucleic acid base, but changes the chemical properties so the compound does not function in cell metabolism. Nucleic acid synthesis is therefore blocked (16).

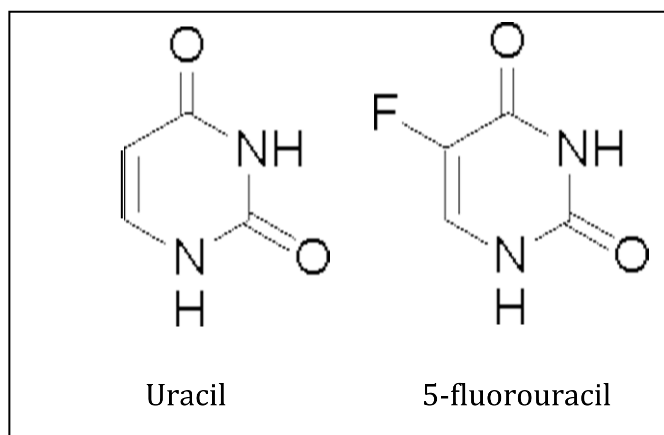

Figure 10- Structures of uracil and 5-fluorouracil

Intracellular, 5-FU is converted to active metabolites such as FdUMP, FdUTP and FUTP, which disrupt the RNA synthesis and thymidylate synthase (TS) activity. TS converts dUMP to dTMP by reduced folate (CH_2THF) as methyl donor, shown in Figure 11. This represents the single synthesis of dTMP, which is necessary for DNA replication and repair. FdUMP bind to the nucleotide binding side on the enzyme. TS, FdUMP and CH_2THF form a stabile ternary complex, blocking the binding of dUMP and thereby inhibiting the synthesis of dTMP (17, 18).

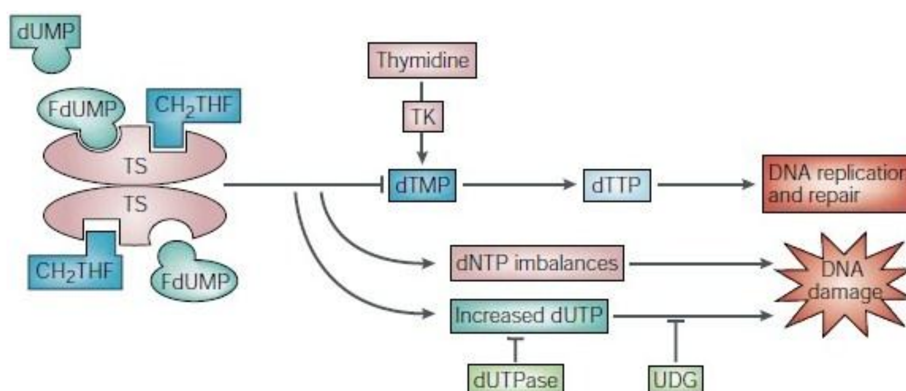

Figure 11-Mechanism of thymidylate synthase (TS) inhibition by 5-fluorouracil (15)

The inhibited synthesis of dTMP leads to depletion of dTTP which again causes changes in the production of other deoxynucleotides, through feedback mechanisms. Imbalances in deoxynucleotide pools are thought to disrupt DNA synthesis and DNA repair leading to lethal DNA damage (19, 20), shown in Figure 12.

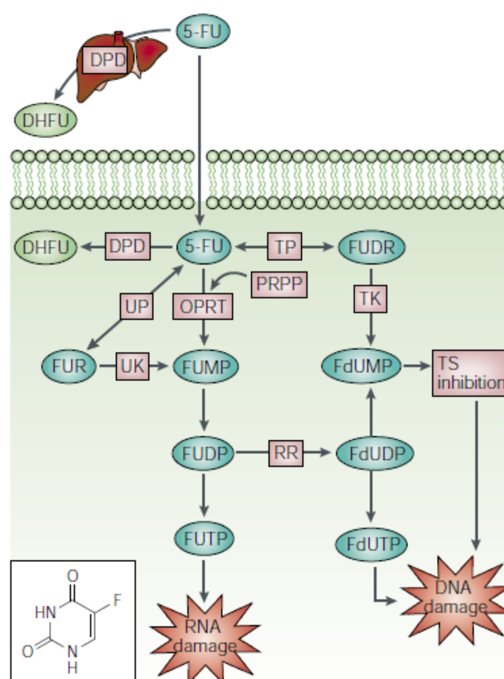

Figure 12-Metabolism of 5-fluorouracil (15)

Normal RNA processing and function are disrupted by incorporation of FUTP, leading to loss of reproductive characteristics shown in human colon and breast cancer cell lines. 5-FU incorporation can result in toxicity to RNA at several levels, and disrupt many aspects of RNA prossessing. This can lead to profound effects on cellular metabolism and viability.

Different schedules been applied to determine optimum dose and mode of administration. However, treatment of 5-FU alone gives relative low response rate, 10-15%. Modulations of 5-FU has successfully increased its anticancer activity by reducing the degradation of 5-FU, increasing the 5-FU activation and increasing the thymidylate synthase (TS) binding activity of FdUMP. Leucovorin are used to increase the intracellular levels of CH_2THF which ensure optimal binding of FdUMP to TS, and enhances the stability of the ternary complex. To diminish the degradation of 5-FU, several strategies has been developed to inhibit the enzyme DPD which degrades 5-FU to DHFU. Methotrexate (MTX) is the last modulator strategy being described. MTX inhibit synthesis of purine enhancing the levels of PRPP, which is a cofactor required to convert 5-FU to FUMP. Giving MTX before 5-FU as shown to increase the anticancer activity of 5-FU. Response rate changed from 10 to 19% and overall survival changed from 10,7 to 9,1 month going from singly 5-FU treatment to combination of MTX and 5-FU (21).

1.5 Aim of Study

Cytostatic agents used in cancer therapy affect healthy cells as well as the targeted cancer cells. By the use of model organism, illustrating healthy cells, earlier research have studied the effect of cytostatic agents on protein levels. This project aims to develop sampling and analytical protocols for the study of metabolic changes of single intracellular metabolites. Metabolites showing diverging affect by DNA damaging agents might be a target for making the treatment less harmful for healthy cells or the cytostatic agent more effective.

For intracellular metabolite analysis, it is important neither to overestimate nor underestimate its concentrations. Intracellular metabolite concentration will be underestimated if leakage occurs under sample preparation. The first aim of this project was therefore to evaluate the extent of leakage of intracellular metabolites in two sampling methods by the use of shaking flask cultivations. The two methods were evaluated in respect of glycolytic intermediates by using enzyme assays. Enzyme assays were optimized in respect of volumes of standards and enzymes added to reach end point within 2-3 minutes.

The second aim was to up-scale preliminary experiments to fermenter size of 1L, add stress agents and analyze metabolic changes before stress exposure, at the time of exposure and a time series after stress exposure. For identification of peaks in chromatograms, a DRS library with 83 metabolites was made prior to the experiments. Since this project was one of the first experiments studying single metabolites, the focus was to get an overview of metabolic changes caused by stress agents.

2 MATERIALS AND METHODS

2.1 Shaking Flask Cultivation

2.1.1 Mineral Media

Mineral media used for preliminary studies in shaking flask, was adopted from Verduyn et al (1992). Stock solutions are presented in Appendix I- Stock Solutions. Cultivation concentration of glucose, nitrogen, phosphate and magnesium is shown below.

Mineral	g/L
Glucose	5,0
$(NH_4)_2SO_4$	5,0
KH_2PO_4	3,0
$MgSO_4$	0,5

2.1.2 Cultivations

Initially, *S.cerevisiae* CEN pk (CEN pk) and *S.cerevisiae* BY 4743 (BY 4743) were provided by Per Bruheim. Freeze stocks of each strain were made by growing 100μL of each strain in 0,5 X YSPG media for 24 hours at 30°C and 220 rpm in Infors HT Minitron incubator shaker. 0,5 X YSPG media consists of 5g/L Yeast Extract and 20g/L Soyton Pepton and will be referred to as rich media onwards. 45mL culture from each rich media culture were transferred to two separate sterile 50mL tubes, and centrifuged for 2 minutes at 5000 rpm at room temperature. Approximately 25mL of supernatants were decanted, followed by addition of 5mL sterile glycerol and the tubes vigorously shaken to re-suspend the pellets. 1mL of re-suspended pellets was allocated in 25 separate ampoules. All ampoules were marked properly, and stored at -80°C.

For cultivation in rich media, 100μL freeze stock was used as inoculum. If not stated otherwise, 500μL culture grown in rich media for 24 hours was used as inoculum for mineral media cultivations. All cultures were incubated at 30°C and 220rpm in Infors HT Minitron incubator shaker.

Whenever needed, pH was measured after calibration with buffer 4 and 7 by Radiometer Copenhagen pH, Standard pH meter.

2.1.3 Sampling

In this project, two quenching protocols were tested in preliminary experiments, for comparing which method giving least leakage of intracellular metabolites into extracellular media. The quenching protocols are described in detail below. Extraction

by freeze-thaw in liquid nitrogen and concentration by lyophilization was used for both quenching methods. OD was measured at the time of sampling.

Filtration prior to quenching

5 mL of culture were filtrated on a 0,8μm Milipore filter placed on a vacuum filtration set up, and washed with 2 x 5mL 2,63 % NaCl. The filter was transferred to 50mL tubes holding 25 mL 60 % methanol pre-cooled to -40°C, given a short and vigorous shake and placed in -40°C cryostat. The filtrates were collected soon after and placed in cryostat. Filtrates and tubes with filter were treated equally onwards. If several samples were taken at the same time, all were filtrated and placed in cryostat before they were given a quick vortex and placed in liquid nitrogen for 5 minutes for extraction. Thereafter, the samples were thawed in cryostat for approximately 15 minutes. From the point of vortexing, the procedure was repeated two more times. After the last thawing, filter pieces were removed, and the tubes centrifuged for 5 minutes at 8000 rpm and -9°C in a pre-cooled centrifuge. The volume of supernatants transferred to empty tubes pre-cooled to -40⁰C were adjusted to obtain the desirable amount of replica. Equal volume of water as supernatants was added to the tubes to lower the alcohol content to make them freeze at -80°C prior to lyophilization.

Direct quenching followed by cold centrifugation

5mL culture was transferred to a tube holding 25 mL pure methanol pre-cooled to -45°C, followed by centrifugation at 4000 g for 5 minutes and -20°C in a pre-cooled centrifuge. 5mL of supernatants were transferred to an empty pre-cooled tube, before the remaining supernatants were decanted to a waste beaker. Tubes with pellets were placed in the -45°C cryostat, while 5mL of distilled water pre-cooled to ~0°C were added to tubes with 5mL supernatants. 2mL chloroform pre-cooled to -20°C, was added to the water-supernatants mixture and the tube quickly vortexed. The tubes were centrifuged for 5 minutes, 5000 g and -20°C. 5mL of the methanol phase were transferred to sterile 15mL tubes and another 5mL of pre-cooled distilled water was added. The tube was stored at -80°C until freeze-drying took place.

Prior to extraction, the pellets were resuspended in 12,5mL 50% methanol pre-cooled to -45°C, quickly vortexed and placed in liquid nitrogen. After 3-5 minutes the tube was thawed in ice bath for 3-5 minutes. The process from vortexing was repeated two more times. After extraction the tubes were centrifuged at 5000 g for 5 minutes at -20°C. The supernatant was decanted to pre-cooled empty tubes in cryostat and the pellets resuspended in 12,5mL 50% methanol, given a quick vortex and centrifuged again under the previous conditions. The supernatant was decanted into the tube already holding the first supernatant. 5mL chloroform, pre-cooled to -20°C, was added and the tube quickly vortexed before another centrifugation. 8mL of the methanol phase was transferred to sterile tubes followed by addition of 8 mL MiliQ water pre-cooled to ~0°C. The tube was stored at -80°C until freeze-drying took place.

2.1.4 Analysis

Enzyme assays were used to analyze G-6-P, F-6-P, DHAP, F-1,6-BP and pyruvate. Concentrations of stock solutions, volumes added and final concentrations of TEA buffer, KCl, $MgCl_2$, NADH and $NADP^+$ for each reaction was adapted from De Koning and Van Dam (1992). Final volume was completed to 1mL by MiliQ water. The use of NADH and $NADP^+$ in the respective reactions was measured by decrease and increase in OD at 340 nm, using Ultraspec 200 UV/Visible Spectrophotometer, and are proportional with concentrations. According to Beer-Lambert law, the valid measuring range were 0,1-0,8.

Prior to measuring in metabolic extracts, standards were used to stipulate the delta OD for a certain concentration of metabolites. Delta OD in metabolic extracts was compared to results from assays with standards, and the concentration in extracts estimated. The amounts of enzyme added were optimized to reach end point within 2-3 minutes reaction time.

<u>Glucose-6-Phosphate and fructose-6-phosphate:</u>
100 µL 0,5M TEA buffer, 10 µL 0,5M $MgCl_2$ and 40µL 10 mM $NADP^+$ were added to each reaction. 10mM G-6-P and 10mM F-6-P standards were added in varying volumes and OD measured prior to addition of varying volumes of G-6-P dehydrogenase. At end point, G-6-P isomerase was added in varying volumes to measure F-6-P. Either the volume of enzyme or standards were changed at the time. Delta OD was measured for each trial, and the optimal amounts of G-6-P and F-6-Pstandards and G-6-P dehydrogenase and G-6-P isomerase were found.

<u>Dihydroxyacetone phosphate (DHAP) and fructose-1,6-biphosphate:</u>
100µL 0,5M TEA buffer and 15µL 10mM NADH were added to each reaction. 10mM DHAP standard and 10mM F-1,6-BP standard were added in varying volumes and OD measured prior to addition of G-3-P dehydrogenase/triose phosphate isomerase mix. At end point, F-1,6-BP aldolase was added in varying volumes. Either the volume of enzyme or standards were changed at the time. Delta OD was measured for each trial, and the optimal amounts of DHAP and F-1,6-P standards and G-3-P dehydrogenase and F-1,6-BP aldolase were found.

<u>Pyruvate:</u>
100µL 0,5M TEA buffer, 100µL 1M KCl, 20µL 0,5M $MgCl_2$ and 15µL 10mM NADH were added to each reaction. 0,1M and 10mM pyruvate standard were added in varying volumes and OD measured prior to addition of lactate dehydrogenase in varying volumes. Either enzyme or standard volumes were changed at the time. Delta OD was measured for each trial, and the optimal amounts of pyruvate standard and lactate dehydrogenase was found.

2.1.5 Dry Weight Measurements and Correlation Curve of Gram Dry Weight Per Liter and OD

For dry weight measurements of a suspension, liquid is transferred to dried and pre-weighted aluminums scales, and incubated in Termaks incubator holding 110°C overnight. The scales are weighted the day after, and the change in weight being the dry weight of the suspension. For estimation of dry weights in cultures, a correlation curve showing OD in relation to gram dry weight per liter was made for each strain. 40mL, 20mL, 10mL, 5mL and 2,5mL culture were transferred to separate sterile 50 mL tubes, and volumes completed to 40mL using 1,5 % NaCl (aq). OD was measured in each tube, prior to centrifugation for 5 minutes at 6000 rpm at room temperature. The supernatants were decanted and pellets resuspended in 20mL 1,5 % NaCl (aq), and centrifuged under same conditions. Supernatants were decanted, and the pellets re-suspended in the smallest volume possible using MiliQ water. Re-suspended pellets were transferred to dry and pre-weighted aluminums scale and dry weight measured as described above.

2.2 Fermenter Cultivation

The fermenter part of this project is performed in cooperation with master student Simon Rey. Mineral media, cultivation condition and sampling protocols will therefore be similar in both reports, whereas implementation of analytical protocol is performed individually.

2.2.1 Mineral Media

The glucose concentration in mineral media from preliminary studies was increased, from 5 to 80g/L, to reach stationary phase at a desirable OD. The other minerals were added in the equal amounts as in preliminary studies, except doubling of vitamin concentration. Stock solutions are presented in Appendix I- Stock Solutions. Concentration of glucose, nitrogen, phosphate and magnesium are shown below.

Mineral	g/L
Glucose	80
$(NH_4)_2SO_4$	5,0
KH_2PO_4	3,0
$MgSO_4$	0,5

2.2.2 Cultivation

The following fermenter cultivations were done in 1L New Brunswick fermenter with working volume of 700mL. The fermenter was programmed to hold temperature at 30°C, pH at 5 and dissolved oxygen (DO) at 40 % by auto adjusting agitation. Regular calibration of pH probe, O_2 probe, O_2 saturation and CO_2 saturation were performed

prior to inoculation. The inoculum was prepared by transferring a desired volume of culture grown in rich media into sterile 50mL tube and centrifuge for 5 minutes at 5000 rpm at room temperature. The supernatant was decanted and the pellets re-suspended in approximately 5mL of mineral media from the fermenter, and added to the fermenter for inoculation. OD was measured in rich media and the volume used for inoculation in the fermenter adjusted so the culture had a certain OD at a wanted time of sampling.

2.2.3 Sampling

5mL culture was sampled from reference cultivation and culture exposed to osmotic stress, whereas 10mL were sampled from cultures exposed to 0,1mM and 0,5mM 5-fluorouracil. The sampling volume was transferred to 0,8 µm Milipore filter placed on a filtration setup and washed two times with volumes of 2,63% NaCl equal the sampling volume. The filter was placed in a tube containing 7mL 75 % ethanol pre-heated to 95°C and 10µL 10mM d3-alanine internal standard added before 3 minutes incubation. Thereafter the tube was placed in -40°C cryostat until centrifuged for 5 minutes and 5000 rpm at room temperature. The filter was removed before centrifugation. 5mL supernatant were transferred to sterile 15 mL tube and the solvent evaporated at 60°C using SAVANT SPD2010 SpeedVac Concentrator from Thermo Electron Corporation. If not evaporation was done directly, the tubes was held in cryostat. After evaporation and before analysis, the tube was stored at -80°C.
Internal standard were added to normalize metabolic results to internal standard and thereby calculate metabolic recoveries.

2.2.4 GC-MS Analysis

For analysis of metabolite extracts gas chromatography system Agilent Technologies 7890 A GC System and mass spectrometer system Agilent Technologies 5975 inert Mass Selective Detector (GC-MS SQ) was used, shown in Figure 13. Metabolite extracts were derivatized using methyl chloroformate (MCF) and N-methyl-N-trimethylsilytrifluoroacetamide (MSTFA). Derivatization protocols are described below.

Figure 13-Picture of the GC-MS SQ used in this project

2.2.4.1 MCF Derivatization
Following the method of Villas-Bôas et al (2003), metabolite extracts were dissolved in 380µL 1M NaOH, and the entire volume transferred to silylated tubes. 20µL d5-glutamate was added as internal standard, prior to addition of 333 µL methanol and 67 µL pyridine and the tube quickly vortexed. 40µL MCF was added and the tube vortexed for 30 seconds, before an additional 40µL MCF and vortexed for another 30 seconds. 400µL chloroform was added, and vortexed for 10 seconds before 400 µL $NaHCO_3$ was added to the tubes and a final vortex for 10 seconds. The chloroform phase was transferred to new silylated tubes. Three spoons of dried Na_2SO_4 were added to remove residual water (22). The chloroform was thereafter transferred to marked vials with inserts and caps were placed on. The samples were analysed immediately using the SKLMCFRTL#2_mod method on GC-MS SQ. Specifications of method are not included in this report.

2.2.4.2 MSTFA derivatization
This derivatization protocol was adopted from Kind et al (2009) for applying the commercially available Agilent Fiehn Library to identify metabolites not identified by AMHSKL or SKLMCFD2 library. 5mL 40g/L fresh O-methylhydroxylamine hydrochloric acid in dried pyridine was prepared daily. Metabolite extracts were dissolved in 300 µL O-methylhydroxylamine hydrochlorine in pyridine, 100µL of dissolved metabolite extracts were transferred to vials with inserts and vortexed for 30 seconds. The vials were incubated at 30°C for 90 minutes on a shaker. 100µL MSTFA with 1% trimethylchlorosilane (TMCS) was added and vials incubated on a shaker for another 30 minutes at 37°C (23). Vials were covered with caps and samples analysed immediately using the method SKLFMLDR on GC-MS SQ. Specifications of method are not included in this report.

3 RESULTS

3.1 Shaking Flask Cultivations

3.1.1 Characterization of Growth

Under batch fermentations nutrients will eventually be exhausted and stop growth. This is called the limiting nutrient. Growth curves of batch cultures give a characteristic sigmoid shape, where exponential phase is represented in the linear area when plotted in semi-log scale. Batch growth profiles were characterized for both strains prior to exposure of stress agent. CEN pk was grown in mineral media, whereas BY 47473 was grown in mineral media with addition of leucine, histidine, methionine and uracil due to auxotrophy. Three glucose concentrations were tested for the amount of biomass obtained, data not shown. 5g/L glucose resulted in enough biomass for sampling, and this concentration was used onwards. To investigate how pH in mineral media effects growth, both strains were grown in one mineral media in which pH was adjusted to 5 and another mineral media which was not pH adjusted. Growth profile in both mineral media is shown in Figure 14-A for CEN pk and Figure 14-B for BY 4743. The same dry weight yields are reached for culture grown in pH adjusted mineral media and culture grown in not pH adjusted mineral media. Based on these results pH will not be adjusted during the rest of the study. Metabolome sampling in exponential phase much be done before OD 3, where stationary phase appears.

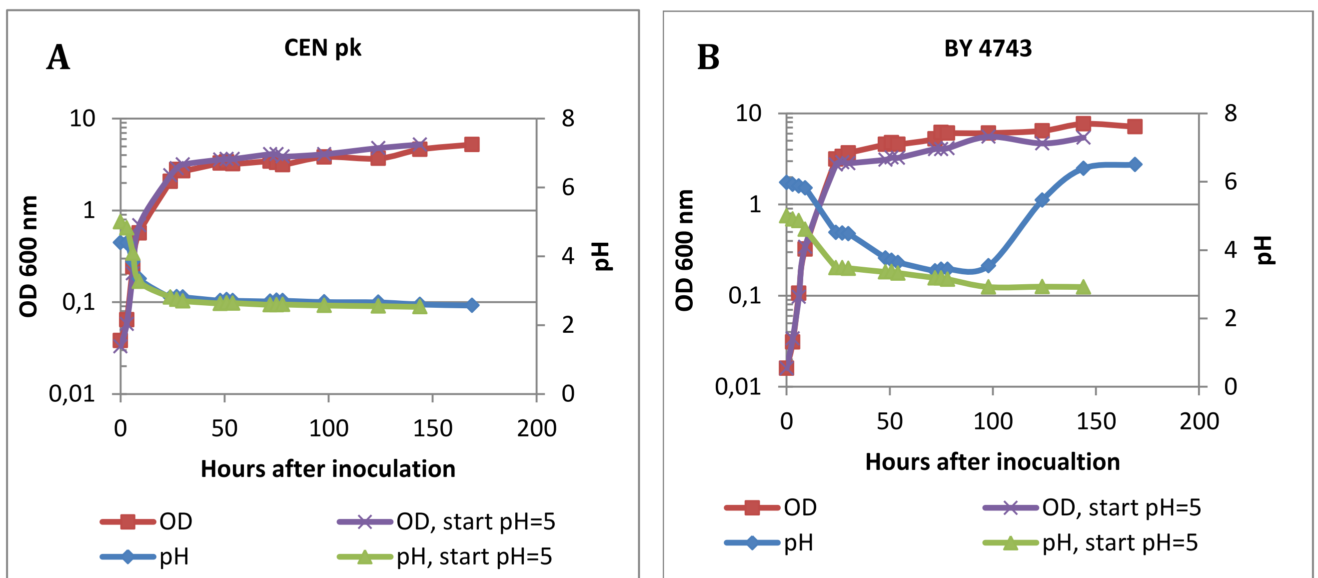

Figure 14-pH and OD measurements for cultivations with pH in mineral media adjusted to 5 and pH not adjusted for A) CEN pk and B) BY 4743

3.1.2 Correlation Curve of Gram Dry Weight Per Liter and OD

A correlation curve, for each strain, showing grams dry weight per liter (g DW/L) in relation to OD was made to estimate amount of dry weight sampled from the fermenter or shaking flask and analyzed. Correlation curves are shown in Figure 15. Raw data are presented in Appendix II- Raw Data From Correlation Curve of Gram Dry Weight Per Liter and OD.

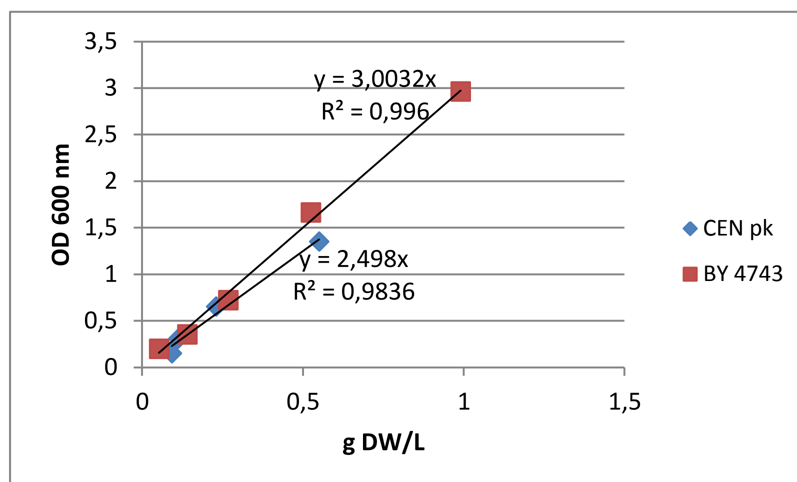

Figure 15- Correlation curve of gram dry weight per liter and OD for CEN pk and BY 4743, its raw data can be seen in Appendix II.

For BY 4743, OD measured divided by factor 3 gives gram dry- weight per liter.
For CEN, OD measured divided by factor 2,5 gives gram dry-weight per liter.

3.1.3 Optimization of Enzyme Assay

Enzyme assays for quantification of G-6-P, F-6-P, DHAP, F-1,6-BP and pyruvate were established to enable quick quantitative analyses of metabolite extracts. Delta OD from extracts was compared with delta OD from enzyme assay with standards and the concentrations in extracts estimated. Concentrations of stock solutions, volumes added and final concentrations of TEA buffer, KCl, $MgCl_2$, NADH and $NADP^+$ for each reaction was adapted from De Koning and Van Dam (1992). Assays were optimized in respect of volumes of standards and enzymes added to reach end point within 2-3 minutes and OD measuring range between 0,1 and 0,8. Substrate must be the limiting reactant for estimation of its concentration. When increasing the amount of substrate, a linear graph is seen when change in OD is plotted against volume of substrate added. For all the reactions below, increased volume of substrate resulted in equal increase in delta OD. A summary of the results from optimization is described below, whereas raw data and graphic presentation for all reactions are presented in Appendix III- Raw Data From Enzyme Assay Optimization.

<u>G-6-P and F-6-P</u>

The optimized volumes were 1μL for both G-6-P dehydrogenase and G-6-P isomerase, and 5μL of both 10mM G-6-P and 10mM F-6-P. 100μL 0,5mM TEA buffer, 10μL 0,5M

$MgCl_2$ and 40µL 10mM $NADP^+$ were added to each reaction. Both 5 and 10µL standard was tried out using 1 and 2µL enzyme for both reactions. Using 1 and 2µL enzyme gave the same time for reaching end point. For economic reasons, 1µL enzyme was selected.

DHAP and F-1,6-BP

The optimized volumes were 1µL of both G-3-P dehydrogenase/triose isomerase mix and F-1,6-BP aldolase, and 2µL of both 10mM DHAP and 10mM F-1,6-BP. 100µL 0,5M TEA buffer and 15µL 10mM NADH were added to each reaction. Delta OD for F-1,6-BP is generally higher than DHAP.

Pyruvate

The optimized volumes were 3µL lactate dehydrogenase and 3µL 10 mM pyruvate standard. 100µL 0,5mM TEA buffer, 100µL 1M KCl and 20µL 0,5mM$MgCl_2$ were added to each reaction. Using 10µL standard the final OD exceeded the measurement range, and was therefore excluded. Using 5 and 3µL standard, time taken to reach end point was the same, and 3µL chosen to be used for reference.

3.1.4 Evaluation of Intracellular Leakage in Metabolic Extracts Using Enzyme Assays

For evaluation of intracellular leakage in the two sampling methods, optimized enzyme assays were used for analysis of glycolytic intermediates in intra- and extra cellular metabolic extracts. Extracts were re-suspended in MiliQ water prior to analysis, and standards were added to each cuvette after end point was reached as a positive control for each assay.

Metabolite extracts, from direct quenching followed by cold centrifugation were dissolved in 2mL MiliQ water, except extracellular extracts from CEN pk which was dissolved in 1mL. Three parallels with 100 and 200µL extracts were analyzed. Delta OD was measured for each reaction and compared with delta OD from reference assays. Delta OD of samples showed 0,001 and 0,002, whereas addition of standards gave expected results confirming functionality of the assay. Delta OD of 0,001 and 0,002 indicates no metabolite detection by the use of direct quenching.

Extracts from filtration method were dissolved in 600µL MiliQ water and three parallels were only analyzed for pyruvate, due to same results as for centrifugation samples. For CEN pk 100µL, 200µL and 300µL were analyzed in parallel 1 ,2 and 3 respectively. For BY 4743, 200µL were analyzed in each parallel. Delta OD ranged from 0,003-0,007 for, whereas delta OD after addition of standards was coherent with reference assays. As for the direct quenching method, no metabolites were detected.

20mL culture was sampled from another BY 4743 cultivation, and not allocated in replica, giving the same results as above. Addition of standards in extracellular extract from BY 4743, showed diverging delta OD compared to results from optimization. All results are shown in Table 6, Table 7 and Table 8 in Appendix IV - Measuring Intra- and Extracellular Metabolites in Metabolite Extracts Using Enzyme Assays Enzyme assay as analytical method was not sensitive enough to be used further. Therefore, enzyme assays will be replaced by GC-MS SQ as analytical method. Moreover, other metabolic groups, as amino acids, citric acids cycle intermediates and fatty acids, will be analyzed by GC-MS SQ.

Two replica of the same extract were dissolved in 30% methanol and 50µL G-6-P and 50 µL F-6-P added. The tubes were freeze-dried in 17 and 21 hours respectively to investigate for any loss of metabolites under this process. 30% methanol was also freeze dried over the same time periods for estimation of background noise. Standards were added at end point as a positive control for assay. Extracts with additional G-6-P and F-6-P showed expected results. As far as this experiment shows, there is no loss of G-6-P and F-6-P during the process of freeze-drying in metabolic extracts. There are reasons to believe that this accounts for other phosphorylated glycolytic intermediates. Only methanol did not show any sign of neither G-6-P nor F-6-P, and addition of standards after end point showed expected results. In conclusion, only methanol gave no background noise and no loss of G-6-P or F-6-P were revealed under the freeze-dry process.

3.2 Creation of GC-MS DRS Library

A GC-MS DRS library holds a set of metabolites, each with a specific mass to charge ratio spectrum at a certain retention time. By GC-MS analysis, each metabolite will appear after a certain retention time and in different amounts resulting in peaks in chromatograms. Peaks in metabolite extracts can be identified by comparing mass to charge ratio spectrum with a library. An original library, SKLMCFD2, consisted of 38 known and named metabolites and 142 unknown and temporary named metabolites identified in *Pseudomonas fluorescens* and *Streptomyces coelicolor* extracts. For extending the already existing library, a new library with 43 other metabolites were made as described in the introduction, and named AMHMCFN2. The latter library was combined with known and named metabolites from SKLMCFD2, creating a library consisting of 83 known metabolites, named AMHSKL. Metabolites in the different libraries are listed in Table 1. The ChemStation part of the library was typed manually, and the final document showed in Appendix V- ChemStation Part of the New MCF-DRS Library.

Table 1- List of metabolites in different DRS libraries

Library			
AMHMCFN2	SKLMCFD2	SKLMCFD2	AMHSKL
Metabolites	**Metabolites**	**Metabolites**	**Metabolites**
Methylvalerat	6.3001 min, _FFI_1_2	OH-Proline	Methylvalerat
Malanoic acid	6.4634 min, _FFI_1_2	Methionine	Tetrachloroethane
3-methyl-2-oxovalerate	NNp-40	17.3142 min, _FFI_1_2	Malanoic acid
Methylglyoxal	6.8533 min, _FFI_1_2	17.3287 min, _FFI_1_2	Pyruvate
b-hydroxypyruvat	Tetrachloroethane	17.4890 min 31OKT08MCF_MUCA_FFI_1	3-methyl-2-oxovalerate
Citraconate	Pyruvate	BMAA	Pentachloroethane
Benzoat	7.3277 min, _FFI_1_2	17.5086 min, _FFI_1_2	Fumarate-1
Citramalat	7.5110 min, _FFI_1_2	17.8611 min 31OKT08MCF_MUCA_FFI_1	Fumarate-2
Methoxyamine	NNp-42	Cysteine	d4-Succinate
Nicotinat	7.7532 min, _FFI_1_2	18.0564 min, _FFI_1_2	Lactate
Phenylacetat	Pentachloroethane	18.0797 min, _FFI_1_2	Methylglyoxal
M-toluate	7.7977 min, _FFI_1_2	18.1575 min, _FFI_1_2	Succinate
2-aminobutyrat	NNp-7	Isocitrate	Hexachloroethane
Adipat	8.2387 min, _FFI_1_2	Phenylalanine	b-hydroxypyruvat
2-isopropylmalate	Fumarate-1	NNp-14	Citraconate
a-ketoadipat	Fumarate-2	18.8185 min, _FFI_1_2	Benzoat
g-aminobutyrat	d4-Succinate	18.9540 min, _FFI_1_2	Citramalat
Serine	Lactate	18.9862 min, _FFI_1_2	Malate-2
Threonine	Succinate	19.1640 min, _FFI_1_2	d3-alanine
L-homoserine	9.2608 min, _FFI_1_2	Cadaverine	Glycine
O-acetyl-L-serine	Hexachloroethane	19.4640 min, _FFI_1_2	Alanine
5-aminovalerat	9.3386 min, _FFI_1_2	19.4929 min, _FFI_1_2	Methoxyamine
Salicylat	9.4319 min, _FFI_1_2	?? NNp-16	Nicotinat
Anthraniate	9.7085 min, _FFI_1_2	19.7495 min, _FFI_1_2	Phenylacetat
N-acetyl-L-glutamat	Malate-2	? NNp-34	M-toluate
Hydroxyproline	d3-alanine	20.1294 min, _FFI_1_2	2-aminobutyrat
Methionine	Glycine	20.1942 min 31OKT08MCF_MUCA_FFI_1	Adipat
b-methylamino-L-alanine	Alanine	20.2261 min, _FFI_1_2	d8-valine
Putresine	10.2843 min 31OKT08MCF_MUCA	20.2727 min, _FFI_1_2	Valine
Phenylpyruvat	10.3854 min 31OKT08MCF_MUCA	20.3183 min, _FFI_1_2	a-ketoglutarate
Hippurat	11.0983 min, _FFI_1_2	NNp-18	2-isopropylmalate
Threo-b-hydroxyasparta	11.1450 min, _FFI_1_2	Ornithine	a-ketoadipat
4-imidazoleacrylate	11.1550 min, _FFI_1_2	20.5427 min, _FFI_1_2	Leucine
Allantoin	11.5361 min, _FFI_1_2	NNp-20	Isoleucine-1
2,4-diaminobutyrate	?? NNp-26	20.6560 min, _FFI_1_2	Serine-1
4-aminobenzoat	NNp-19	20.7963 min 31OKT08MCF_MUCA_FFI_1	Isoleucine-2
Cadaverine	11.7261 min, _FFI_1_2	20.8616 min, _FFI_1_2	g-aminobutyrat
Oxaloacetat	11.8027 min, _FFI_1_2	20.9093 min, _FFI_1_2	Serine
Histamine	d8-valine	21.0404 min, _FFI_1_2	Malate-1
p-coumarate	Valine	21.1618 min 31OKT08MCF_MUCA_FFI_1	Threonine
N-glycyl-proline	a-ketoglutarate	21.1926 min, _FFI_1_2	L-homoserine
Ferulate	? NNp-43	21.3182 min, _FFI_1_2	Proline
2,6-deaminopimelat	12.5648 min, _FFI_1_2	21.3615 min, _FFI_1_2	Asparagine
Serotonine	12.8815 min, _FFI_1_2	21.4915 min, _FFI_1_2	O-acetyl-L-serine

Library			
AMHMCFN2	SKLMCFD2	SKLMCFD2	AMHSKL
Metabolites	**Metabolites**	**Metabolites**	**Metabolites**
	12.9984 min 31OKT08MCF_MUCA	21.5026 min, _FFI_1_2	Aspartate
	13.0448 min, _FFI_1_2	NNp-21	Citrate
	13.0526 min, _FFI_1_2	Lysine	5-aminovalerat
	Leucine	21.6340 min 31OKT08MCF_MUCA_FFI_1	Salicylat
	13.2925 min, _FFI_1_2	21.7081 min, _FFI_1_2	Anthraniate
	Isoleucine-1	21.8203 min, _FFI_1_2	Serine-2
	13.3981 min, _FFI_1_2	21.9514 min, _FFI_1_2	Glutamine
	13.4103 min, _FFI_1_2	21.9806 min 31OKT08MCF_MUCA_FFI_1	d5-glutamate
	Serine-1	Histidine	Glutamate
	Isoleucine-2	22.2836 min, _FFI_1_2	N-acetyl-L-glutamat
	13.4869 min, _FFI_1_2	NNp-25	OH-Proline
	Threonine	22.5036 min, _FFI_1_2	Hydroxyproline
	NNp-9	22.5969 min, _FFI_1_2	Methionine
	Malate-1	22.6913 min, _FFI_1_2	b-methylamino-L-alanine
	Proline	NNp-38	Cysteine
	13.9680 min, _FFI_1_2	? NNp-45	Isocitrate
	Asparagine	Tyrosine	Phenylalanine
	14.0249 min 31OKT08MCF_MUCA	23.7168 min, _FFI_1_2	Putresine
	Oxaloacetate	23.8793 min 31OKT08MCF_MUCA_FFI_1	Phenylpyruvat
	14.0883 min 31OKT08MCF_MUCA	24.0070 min 31OKT08MCF_MUCA_FFI_1	Hippurat
	14.1835 min, _FFI_1_2	NNp-37	Threo-b-hydroxyaspartat
	14.4846 min, _FFI_1_2	24.1211 min, _FFI_1_2	4-imidazoleacrylate
	14.5604 min 31OKT08MCF_MUCA	24.2434 min, _FFI_1_2	Allantoin
	?? NNp-10	24.3025 min 31OKT08MCF_MUCA_FFI_1	2,4-diaminobutyrate
	NNp-44	24.3825 min 31OKT08MCF_MUCA_FFI_1	4-aminobenzoat
	14.8901 min, _FFI_1_2	24.4803 min 31OKT08MCF_MUCA_FFI_1	Cadaverine
	Aspartate	24.5492 min 31OKT08MCF_MUCA_FFI_1	Oxaloacetat
	Citrate	24.6247 min 31OKT08MCF_MUCA_FFI_1	Histamine
	?? NNp-33	24.9410 min, _FFI_1_2	p-coumarate
	15.2212 min, _FFI_1_2	Tryptophan	Ornithine
	15.3067 min, _FFI_1_2	25.2132 min, _FFI_1_2	N-glycyl-proline
	15.3678 min, _FFI_1_2	25.6921 min, _FFI_1_2	Lysine
	15.3759 min 31OKT08MCF_MUCA	25.9298 min, _FFI_1_2	Histidine
	15.4678 min, _FFI_1_2	25.9590 min 31OKT08MCF_MUCA_FFI_1	Ferulate
	15.7266 min, _FFI_1_2	25.9720 min, _FFI_1_2	Tyrosine
	NNp-1	26.0023 min 31OKT08MCF_MUCA_FFI_1	2,6-deaminopimelat
	Serine-2	26.0820 min, _FFI_1_2	Tryptophan
	Glutamine	26.1068 min 31OKT08MCF_MUCA_FFI_1	Serotonine
	NNp-12	26.2834 min 31OKT08MCF_MUCA_FFI_1	
	? NNp-11	26.4086 min, _FFI_1_2	
	16.1211 min, _FFI_1_2	26.6522 min 31OKT08MCF_MUCA_FFI_1	
	NNp-15	26.7633 min 31OKT08MCF_MUCA_FFI_1	
	NNp-13	26.8189 min 31OKT08MCF_MUCA_FFI_1	
	16.4666 min, _FFI_1_2	26.9300 min 31OKT08MCF_MUCA_FFI_1	
	d5-glutamate	27.1077 min 31OKT08MCF_MUCA_FFI_1	
	Glutamate	28.3995 min, _FFI_1_2	
		32.5171 min 31OKT08MCF_MUCA_FFI_1	

3.3 Fermenter Cultivations

Preliminary experiments in shaking flasks were performed for establishing sampling protocols to be used for sampling from fermentation cultivations. Fermenter cultivations have the advantage of controlled pH and dissolved oxygen can be controlled and kept at a constant level. By applying the same mineral media in fermenter cultivations as in shaking flask cultivations, stationary phase appeared at approximately OD 2. To ensure enough cell material for metabolite analysis, it was desirable to sample around OD 8. For the purpose of this project, it was desirable to sample during exponential phase. Several cultivations were grown to adjust mineral media so OD 8 appeared in exponential phase of the growth profile. The mineral media was only adjusted in respect of the CEN pk strain. Optimization of mineral media for BY 47473 would include changes in the amounts of amino acids and uracil, and was not carried out due to time limitations. Therefore, CEN pk is the only strain to be used onwards. Growth profile obtained by applying the final media to be used in fermentation cultivations and growth profile for cultivations with mineral media adopted from shaking flasks are shown in Figure 16. The major differences in mineral media was the glucose concentration of 80g/L used in fermentation cultivations compared to 5g/L in shaking flask cultivations.

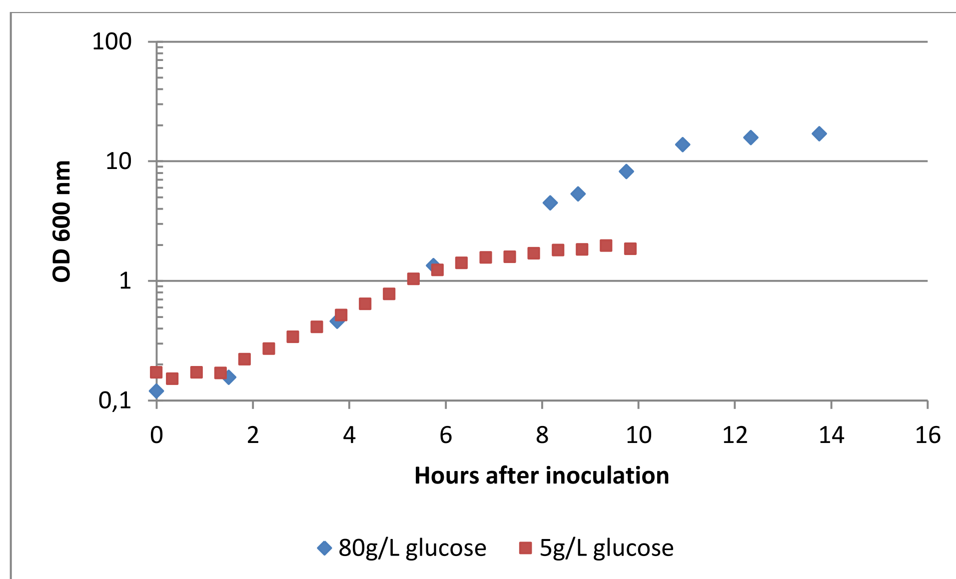

Figure 16- Growth profile for CEN pk in fermenter with mineral media with 5 and 80 g/L glucose

Yield dry weight per gram glucose was calculated to 0,160 in the experiment with 5g/L glucose and 0,085 in the experiment with 80g/L glucose. The growth rate, μ_{max}, was calculated to 0,41 h^{-1} and 0,49 h^{-1} for the experiment with 5g/L glucose and 80g/L glucose respectively. This is slower than one duplication per hour, 0,69 h^{-1}. Calculations for yield and growth rate are shown in Appendix VI- Calculations of Yield and Growth Rate. GC-MS were used for metabolite analysis for all fermentation cultivations, as enzymatic assays proved to be less sensitive for shaking flask experiments, refer to

subchapter 3.1.4. Moreover, amino acids, citric acid cycle intermediates and fatty acids are analyzed by GC-MS compared with glycolytic intermediates when applying enzyme assays.

3.3.1 General metabolite analysis

An example of chromatogram from the analysis by GC-MS is shown in Figure 17. When applying a GC-MS DRS library for metabolite analysis, the given number of hit in the library is shown as number of targets, blue T in red circle in Figure 17. Not all targets appear as large peaks. 47 targets are shown in the figure below, but only 15 relatively large peaks are observed. Relatively large peaks identified with the AMHSKL library are given letters A-H. Extracts were reanalyzed using the SKLMCFD2 library, and five additional metabolites were identified, named X-1 to X-5. Name of each abbreviated metabolite is given below.

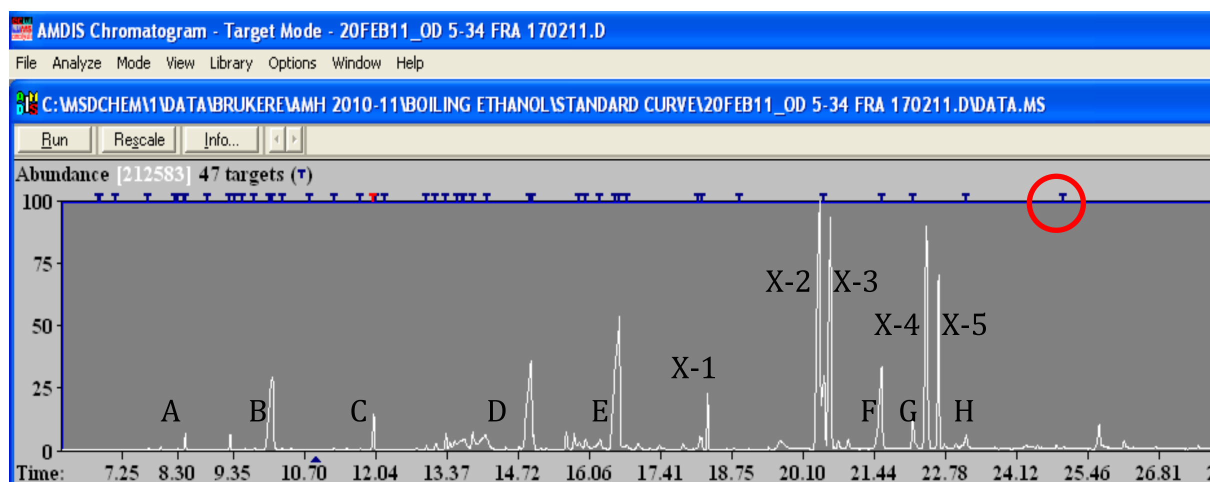

Figure 17-Example of AMDIS spectra of metabolite extract from fermenter cultivation. Peak with letters and X-1 to X-5 indicates identified metabolites. The red circle shows the symbol indicating a hit in the library applied.

A: Succinate
B: Alanine
C: Valine
D: Aspartate
E: Glutamat
X-1: NNp-14
X-2: NNp-18
X-3: NNp-20
F: Lysin
G: Histidine
X-4: NNp-25
X-5: 22.6913min_FFI_1_2
H: Tyrosine

The purpose of this scientific research is to obtain an overview of metabolic changes in *S.cerevisiae* caused by stress exposure. Seven amino acids, succinate and metabolite X-1 to X-2 will be studied in more detail, being the most abundant metabolites in the above chromatogram. Study of other metabolites would be more time consuming. The number and type of metabolites studied should also be coherent with the aim of study.

Results from GC-MS analysis were displayed as area under peaks, integration. The base line for integration was set automatically, manually inspected and adjusted if needed. Differences from automatic and manual base line did appear, emphasizing the need for manual inspection. The differences between automatic and manual baseline are more pronounced for low abundant metabolites. The importance of correct base line setting must be seen in correlation with the aim of study. For the purpose of this semi-quantitative study it is of less importance, but attention should be paid in experiments with other purposes.

3.3.2 Metabolome Analysis of Stress Responses

Batch cultivations of *S.cerevisiae* were used during this scientific research, meaning that the cells grow until one nutrient is limiting its growth. One cultivation was grown and not exposed to stress to characterize how metabolite generates under normal conditions, referred to as reference cultivation. Three following cultivations were exposed to osmotic stress, 0,1mM 5-fluorouracil and 0,5mM 5-fluorouracil.

CO_2 in outgas of the fermenter reflect respiration of cells and was measured every second in all cultures. For simplicity, every second minute of respiration is displayed in graphs showing progression of respiration and OD. OD measurements were taken at the time of inoculation and thereafter occasionally to monitor growth, as well as at the time of sampling. Response values from chromatographic analysis were normalized to the corresponding OD at the time of sampling. Neither of the internal standards was found under analysis, and calculation of losses and recoveries during sampling preparation was therefore not carried out. In graphs showing progression of respiration and OD, a vertical black line indicates the point of nutrient limitation. This line is repeated in graphs showing metabolic changes as well as respiration in the time period of sampling. A light brown vertical line indicates the point of stress exposure in the latter graphs, and the time scale in is limited to the time period of sampling.

The aim of this project is to study changes in metabolite levels before time of stress exposure, at the point of exposure and in a time series after stress exposure. Results from the semi-quantitative GC-MS method are therefore presented as log2 diagrams. In log2 diagrams a doubling of response values from chromatographic analysis are displayed as factor 1, and a fourfold increase is displayed as factor 2 and so on. In the case of halving and fourfold decrease, factor of -1 and -2 is displayed. In all experiments, the basis of log2 calculations is the first sample in the sample series. Succinate, seven amino acids and five other metabolites were studied in detail. Interestingly succinate showed diverging results compared to the other metabolites. Therefore, three additional citric acid cycle intermediates, citrate, malate and fumarate, were also studied in detail. For each experiment preformed, graphs with metabolic changes are displayed with equal scaling for comparison.

3.3.2.1 Reference cultivation

To examine metabolic levels during exponential phase, a cultivation was grown without stress agent exposure, referred to as reference cultivation. Three samples were taken during exponential phase (see arrows in Figure 18) and metabolites analyzed. The progression of respiration and OD in reference cultivation is shown in Figure 18. A disturbance in respiration can be seen approximately 10 hours after inoculation, caused by removal of heating belt for varies reasons. However, it is seen that respiration is reestablished after restoration of temperature control, emphasizing its temperature dependence. The second drop in respiration is caused by limitation of carbon source. Changes in metabolites levels in relation to respiration are seen in
Figure 19, next page.

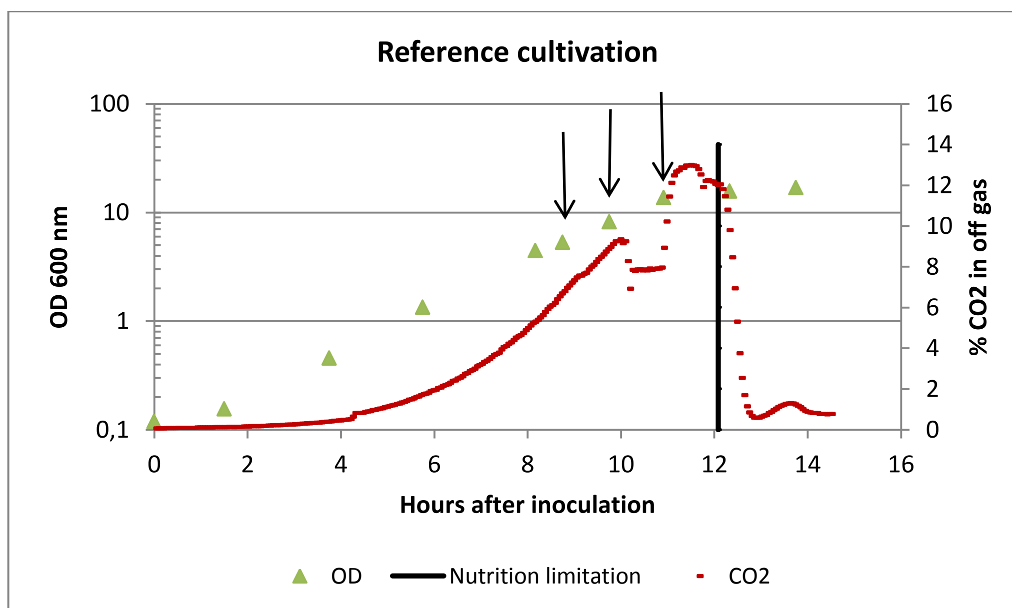

Figure 18- Progression of respiration and OD in reference cultivation. Arrows shows the point of sampling

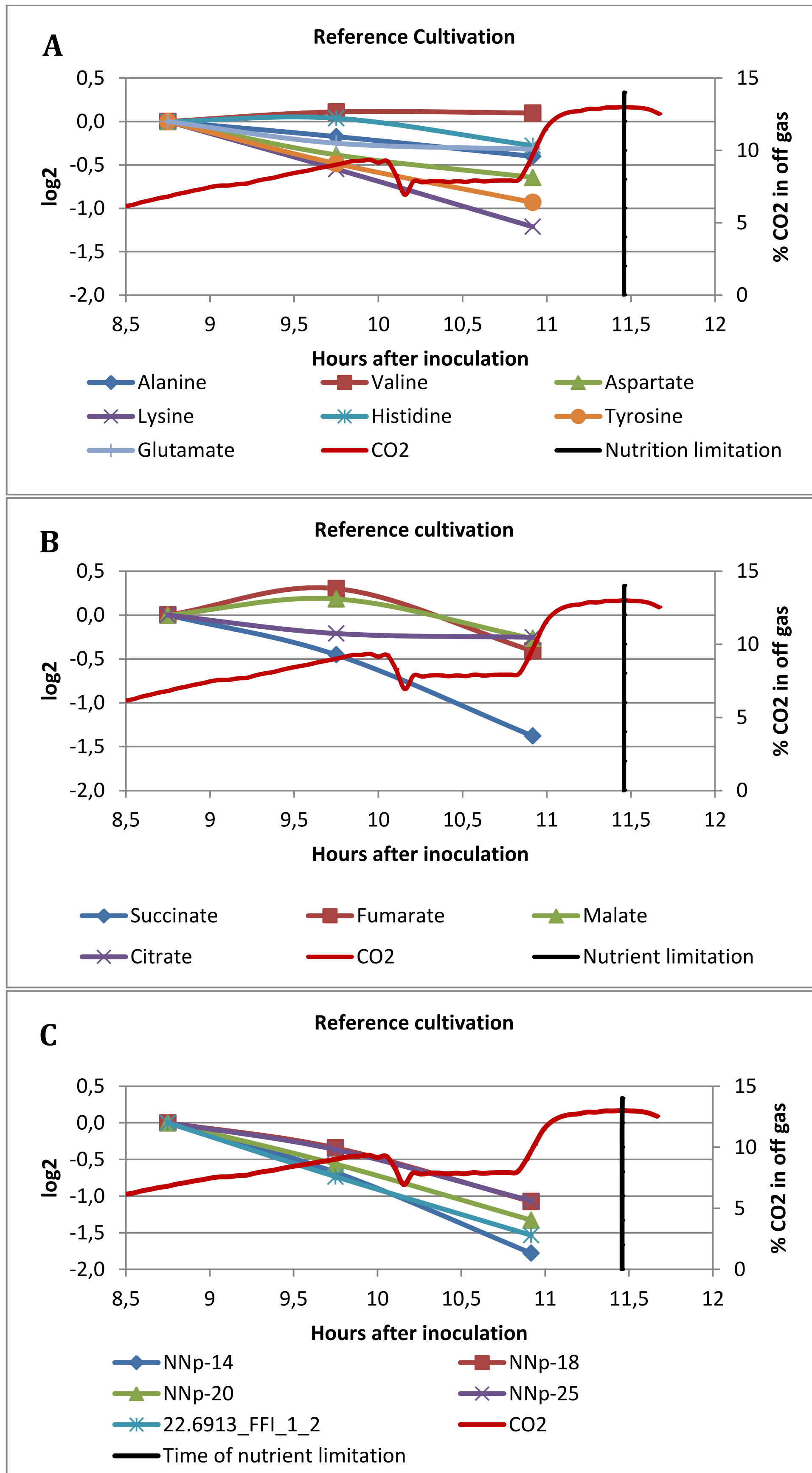

Figure 19- Changes in metabolite levels at three different OD states shown as log2, and respiration in the time interval of sampling for A) amino acids, B) citric acid cycle intermediates and C)NNp-14, NNp-18, NNp-20, NNp-25 and 22.6913min_FFI_1_2

A general reduction in metabolite level is observed. An exception is the minor increase of valine from OD 5,34 to OD 13,72 in in Figure 19-A. Histidine stands out with a small increase before showing the smallest reduction of all metabolites together with glutamate. Lysine shows the largest degrease of all amino acids. Citric acid cycle intermediates in Figure 19-B show diverging changes of metabolic levels. The level of succinate is diminished by three-fold whereas citrate is slightly decreased. Malate and fumarate shows an increase before a decrease in the same extent as citrate. Metabolites in Figure 19-C, show the greatest reduction in metabolite levels. These metabolites show the same pattern of changes as the amino acids studied, but to a larger degree. Additional samples in stationary phase are needed for comparing metabolic changes in exponential and stationary phase. However, highest concentration of metabolites is expected to appear in exponential phase.

Increasing concentration of metabolites could be expected in exponential phase. Each result is normalized to OD and shows decreased levels as exponential phase proceeds. The results contradict with the expected, but might reflect the *in vivo* situation. How the metabolome is affected by the disturbance in temperature is not known. To investigate this in more detail, cultivation can be set up without disturbing the temperature and taking samples approximately at the same stage in growth.

However, to get a wider understanding of the total growth, additional groups of metabolites could be studied using other analytical methods. LC-MS can be applied for the study of glycolytic intermediates and nucleotides.

3.3.2.2 Osmotic stress

One culture was exposed to 1M NaCl to evaluate how cells respond to osmotic stress. At the time of stress exposure, respiration drops instantly, shown in Figure 20. Respiration is gradually improved, but does never reach the level prior to stress exposure. The second drop in respiration is slow and probably not caused by nutrient limitation, which appears as a fast and sudden drop. It is reasonable to believe that the cells are affected by the osmotic stress and down regulates its metabolism, resulting in decreased respiration. Maximum OD reached in this experiment was 13,48 compared to 16,92 in reference cultivation, indicating that the cells are affected when osmotic stress is applied.

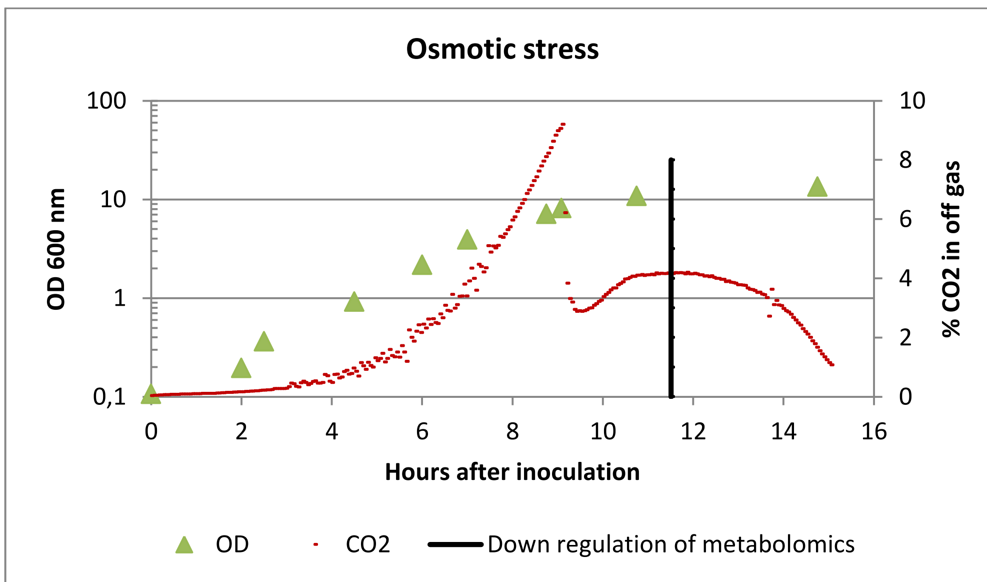

Figure 20- Progression of respiration and OD in culture exposed to osmotic stress

Figure 21 show changes in metabolites at the time of stress exposure, 5 minutes after and 60 minutes after stress exposure, as well as respiration in the time period of sampling. No sample was taken prior to stress exposure, which is a drawback in this experiment.

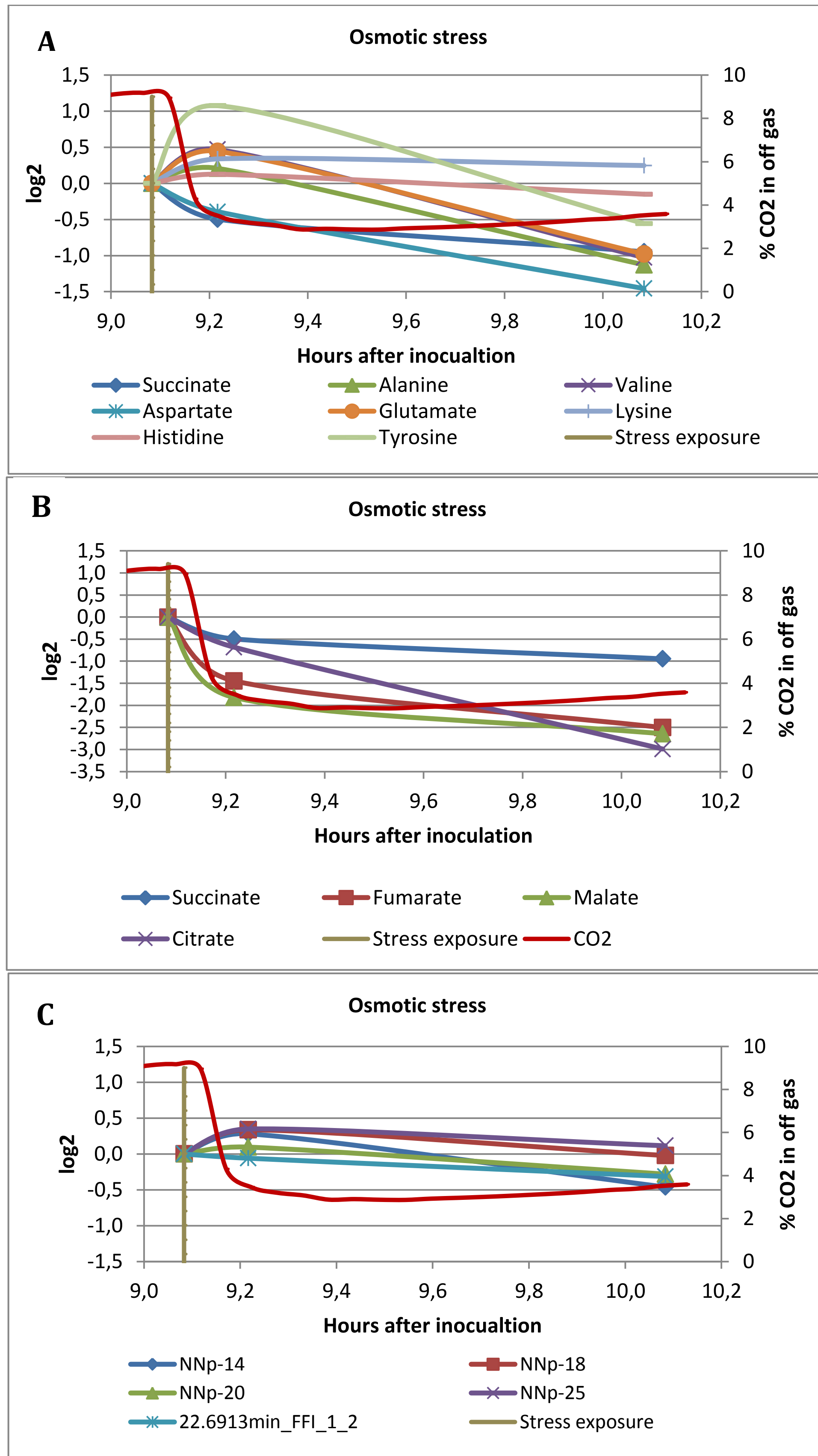

Figure 21- Changes in metabolite levels at the time of stress exposure, 5 minutes and 60 minutes after exposure shown as log2, and the respiration in the time interval of sampling for A) amino acids and succinate, B) citric acid cycle intermediates and C) NNp-14, NNp-18, NNp-20, NNp-25 and 22,6913MIN_FFI_1_2

A general trend for most metabolites is a decreased level from the first sampling to sampling 60 minutes after stress exposure. Two exceptions are levels of lysine and NNp-25, which slightly increases. In comparison, the level of valine increased in reference cultivation, whereas other metabolites decreased. Tyrosine shows the same pattern as in reference cultivation by a small increase prior to a decrease. Glutamate, tyrosine and valine show increased levels when respiration drops and decreased levels when respiration slightly improves. Levels of histidine and valine are relative stable compared to the other metabolites. The level of aspartate falls directly whereas all the other metabolite levels increases before decreasing. Noticeably, this pattern is not found in reference cultivation.

Levels of citric acid cycle intermediates show a larger decreased than the amino acids studied. Changes of malate and fumarate show equal pattern, and succinate is the least affected citric acid cycle intermediate.

Regarding NNp-14, NNp-18, NNp-20, NNp-25 and 22.6913min_FFI_1_2, the same trend is seen as for amino acids. All metabolites show an overall decreased levels, with the exceptions of NNp-25. A small increase can be seen before reduction appears, with the exception of 22.6913min_FFI_1_2. The larges decrease in this experiment is seen as factor -0,5, whereas reference cultivation reveal a decrease of factor -2. The metabolites seem to be more stable under stress exposure than in reference cultivation.

3.3.2.3 0,1mM 5-fluorouracil

After exposure of 0,1mM 5-fluorpuracil, respiration drops instantly. A second drop is seen after improved respiration. This is most probably caused by nutrient limitations. The improved respiration shows the cells capacity to adapt to environmental changes. Progression of respiration and OD in the culture is presented in Figure 22.

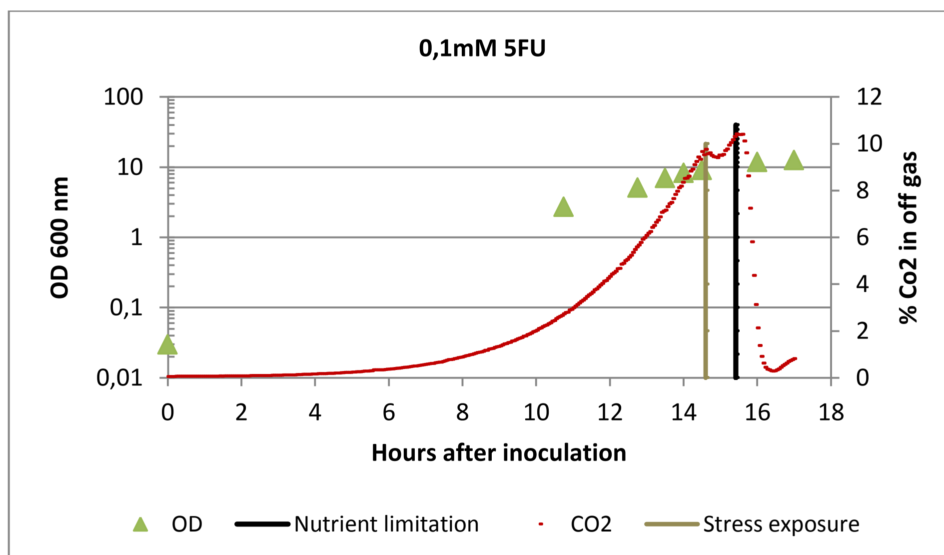

Figure 22- Progression of respiration and OD for a culture exposed to 0,1mM 5FU. Light brown line indicates the point of stress exposure and black line indicates point of nutrient limitation.

For metabolome analysis, one sample was taken before stress exposure, one at the time of exposure followed by 5 additional each with 6 minutes time interval. Changes of metabolite levels in the sampling period are presented in Figure 23.

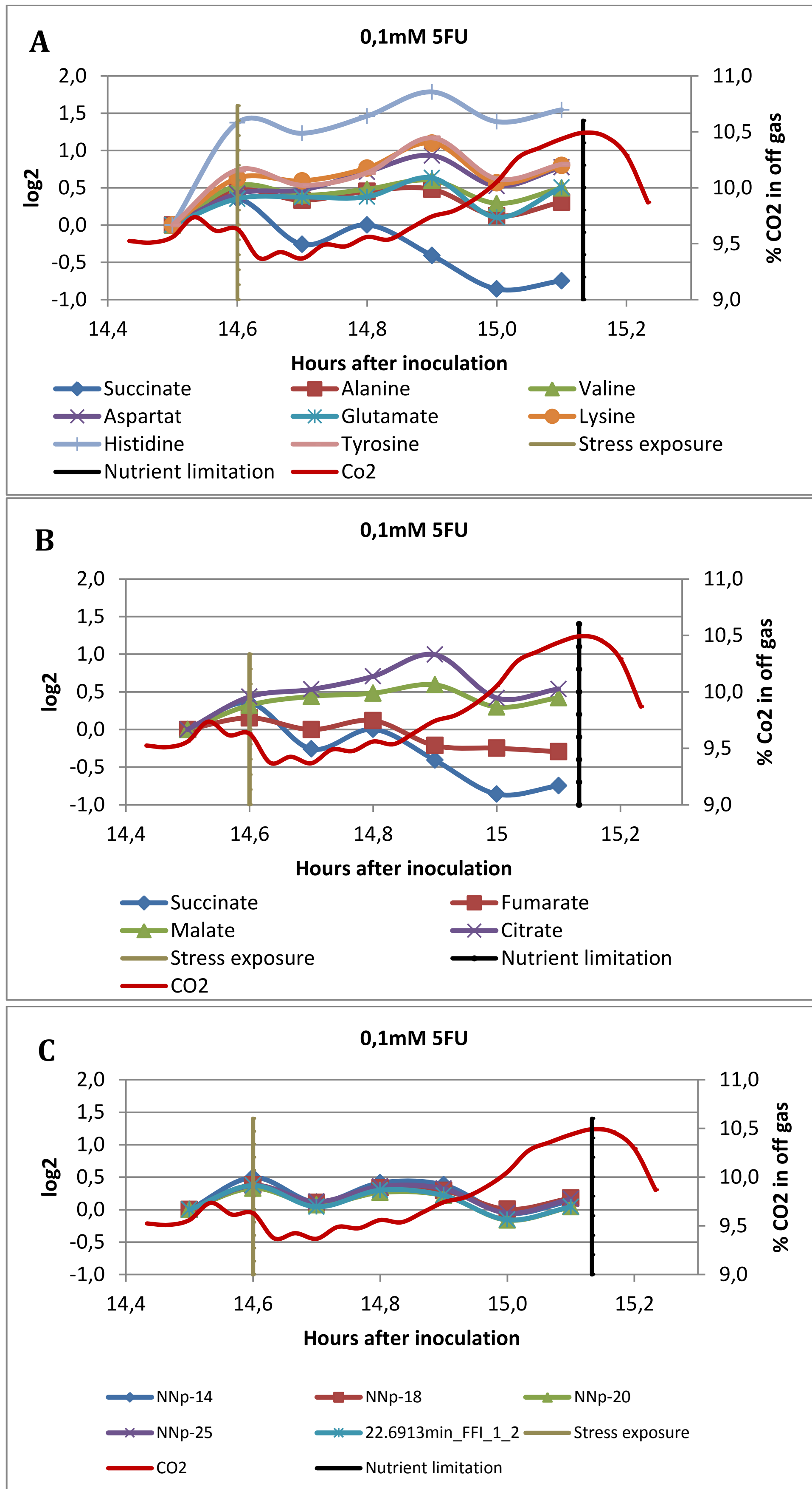

Figure 23 -Changes in metabolite levels shown as log2 for the sample before stress exposure, at the time of exposure and the 5 following with 6 minutes intervals for A) succinate and amino acids B) citric acid cycle intermediates and C) NNp-14, NNp-18, NNp-20, NNp-25 and 22.6913min_FFI_1_2.

All amino acids show the same pattern of changes, but to different extent. Again, histidine show larger increase in the metabolite level than the other amino acids. In reference cultivation and the experiment with osmotic stress, histidine show only minor changes. Succinate diverges from amino acids studied by a large decreased level. In comparison, malate and citrate increases whereas the level of fumarate is relative stable. As respiration increases before stress exposure, so does the level of all metabolites. Right after stress exposure both respiration and metabolites fluctuates. Trends in metabolic changes indicate a possible improvement as respiration improves before the point of nutrient limitation.

NNp-14, NNp-18, NNp-20, NNP-25 and 22.6913min_FFI_1_2 shows equal metabolic changes. Increased levels are seen from sampling before stress exposure to the point of exposure. These metabolites are stable in comparison with amino acids and citric acid cycle intermediates.

3.3.2.4 0,5mM 5-fluorouracil

Progression of OD and respiration in culture exposed to 0,5mM 5-fluorouracil is shown in Figure 24. A sudden drop in respiration occurs at the time of stress exposure, and is not reestablished before nutrient limitation occurs. OD is no longer improving after point of stress exposure. It is difficult to estimate what causes the halt of OD because point of stress exposure and nutrient limitation occur right after another.

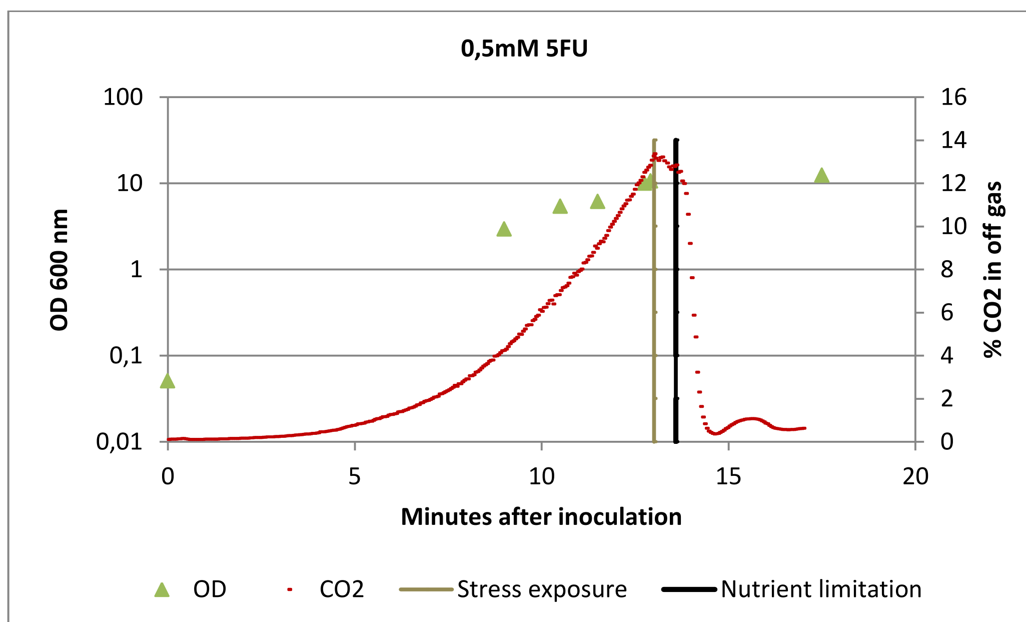

Figure 24- Progression of respiration and OD in a culture exposed to 0,5mM 5FU.

Two samples were taken before stress exposure, one at the time of exposure and 10 samples after stress exposure, each with 6 minutes time interval. The five last samples are taken after point of nutrient limitation where metabolic changes no longer are caused by stress exposure alone. Metabolic changes after point of stress exposure and nutrient limitations reflect changes caused by stress exposure. Figure 25 and Figure 26 show metabolic changes and respiration in the time period of sampling.

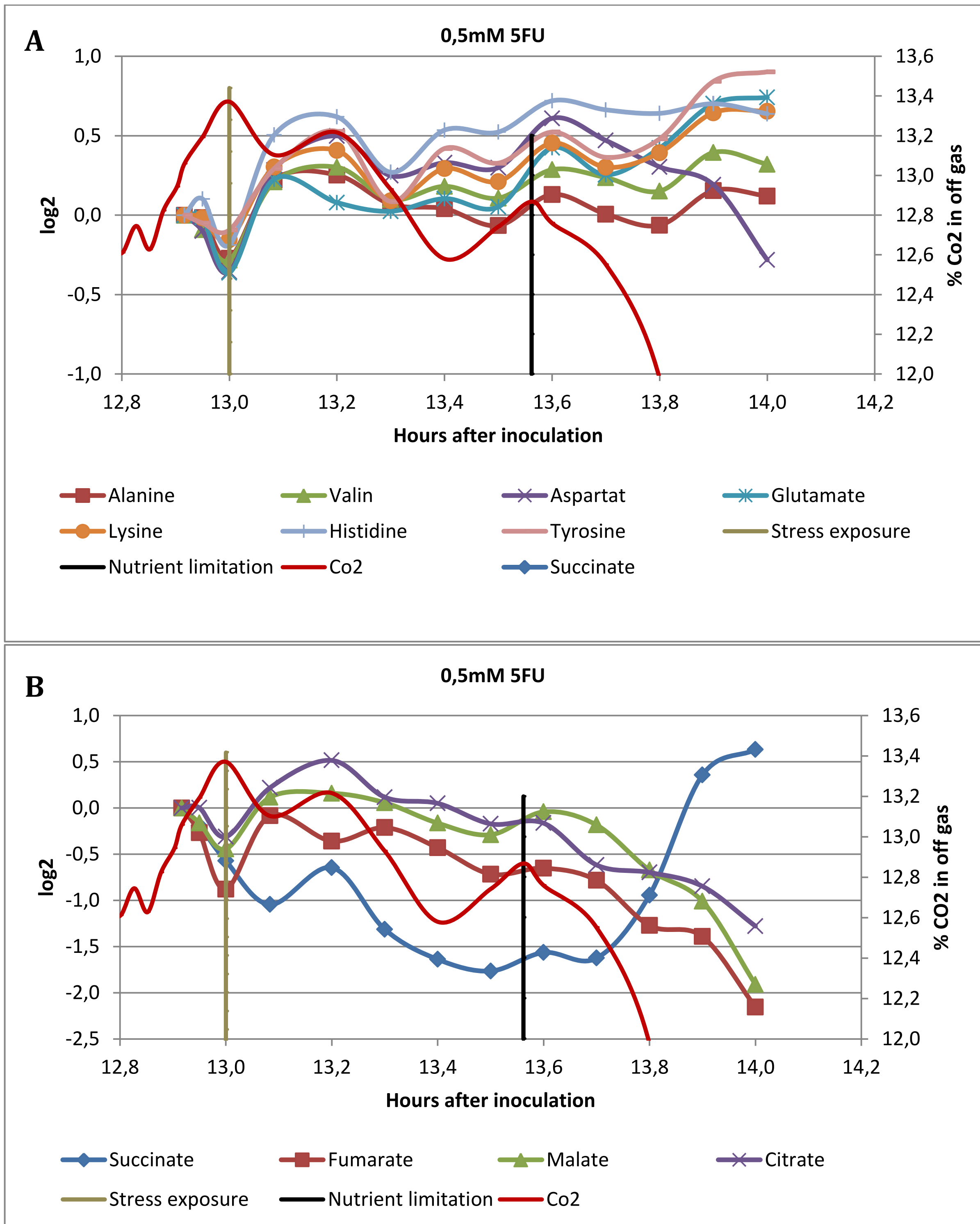

Figure 25- Changes in metabolite levels shown as log2 for samples before stress exposure, at the time of exposure and 10 following samples with 6 minutes intervals for A) amino acids and B) citric acid cycle intermediates.

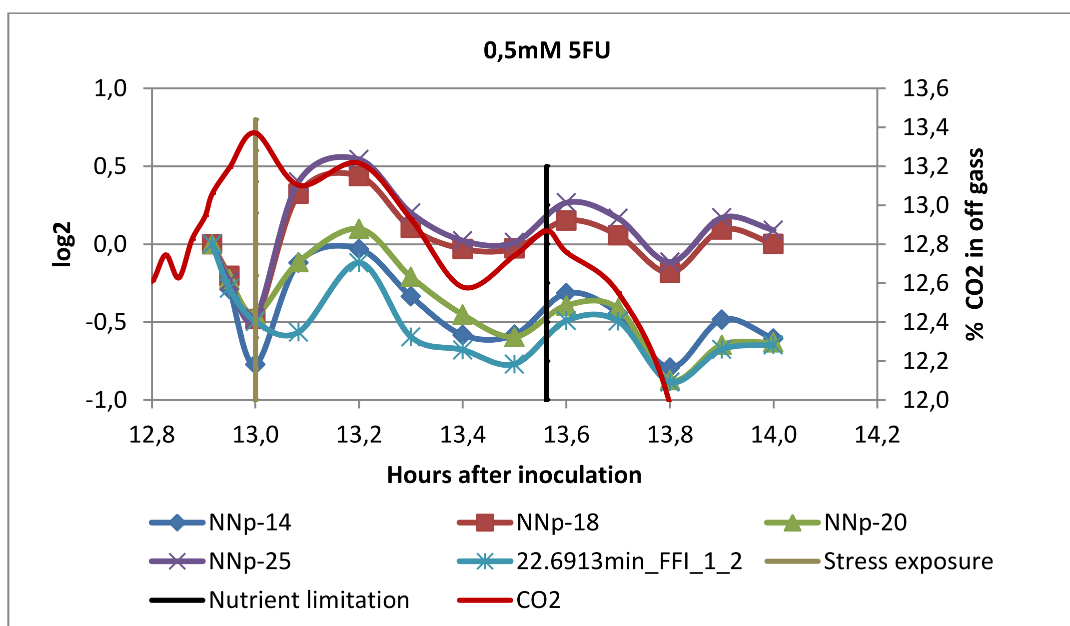

Figure 26- Changes in metabolites levels shown as log2 for samples before stress exposure, at the time of exposure and 10 following samples with 6 minutes intervals for NNp-14, NNp-18, NNp-20, NNp-25 and 22.6913min_FFI_1_2.

Before stress exposure, the level of all metabolites decreases. In comparison metabolic changes in the experiment with 0,1mM 5-fluorourail showed increased levels in the same time period. All metabolites show increased levels after stress exposure followed by decrease to varying extents. In Figure 25-A, histidine differs from the other amino acids by a larger increased level after stress exposure. The other amino acids show the same pattern of changes after point of stress exposure and before nutrient limitation. After nutrient limitation, asparate decreases, histidine stabilizes and the remaining amino acids are still varying. No specific correlation between respiration and change in metabolite levels are seen in this experiment.

Whereas the level of amino acids increases in the time period of sampling, citric acid cycle intermediates decrease. Citric acid cycle intermediates are more influenced by stress exposure than amino acids. After point of nutrient limitation, the level of succinate improves drastically, whereas the other citric acid cycle intermediates still declines.

Metabolites in Figure 26 show the same pattern of changes. NNp-14, NNp-20 and 22.6913min_FFI_1_2 reveals larger metabolic changes than NNp-18 and NNp-25. Metabolites in Figure 21-C, Figure 23-C and Figure 26 are more stable in experiments with stress agent compared to reference cultivation. Comparing the two concentrations of 5-fluorouracil, 0,1mM gives largest metabolic changes. The exception is succinate, where exposure to 0,5mM gives largest changes. Metabolic changes for single metabolites for the two concentrations are presented in Figure 33 and Figure 34 in Appendix VII- Metabolic Changes for Single Metabolites.

3.4 Identification of Unknown Peaks in GC-MS Chromatogram

Five peaks being unidentified using the library AMHSKL, were identified as NNp-14, NNp-18, NNp-20, NNp-25 and 22.6913min_FFI_1_2 when reanalyzes by the SKLMCFD2 library. Four of the compounds were further identified by names by applying the public NIST library. The results are listed below.

Name in SKLMCFD2	→	Name in NIST library
NNp-14	→	Tetradecanoic acid
NNp-18	→	Palmitoleic acid
NNp-20	→	Palitic acid
22.6913min_FFI_1_2	→	Stearic acid

3.5 Applying MSTFA Derivatization and Fiehn Library

For identification of additional metabolites in metabolite extracts, MSTFA derivatization (23) and commercially available Fiehn library was applied on samples from the experiment of 0,1mM 5-fluorouracil. Four additional metabolites were found, being porphine, glutamine, mannitol and ergosterol. However, they were only identified for qualitative reasons and not studied in detail.

4 DISCUSSION

4.1 Sampling and Extraction

In metabolomics, the ultimate goal is to develop a protocol where intact cells are separated from the cell culture liquid while preserving the intracellular conditions at time of sampling. In this project, two quenching protocols were tested in preliminary experiments, for comparing which giving least leakage of intracellular metabolites into extracellular media. The first method tested was filtration followed by quenching of cells in 60% methanol. The second, direct quenching in pure methanol followed by separation of cells and media by centrifugation. Unfortunately, comparisons of the two methods failed due to too low amount of cell material analyzed for the analytical method used in this introductory part of the master project. Both protocols could have been repeated with an increased amount of cell material. However, it was decided to apply filtration as separation technique onwards. Direct quenching was rejected as a method due to location of equipment. Incubation in quenching solution would be prolonged due to transport of equipment from 3rd floor to ground floor for centrifugation.

When sampling from fermentation cultivations, quenching and extraction was carried out in one step using 75% boiling ethanol after separation of cells and media by filtration. The latter is a modification of the optimal quenching and extraction method proposed by Canelas et al (2008), Villas-Bôas et al (2005) and Canelas et al (2009). They proposed direct quenching in pure methanol, followed by immediate cold centrifugation and extraction in 75% boiling ethanol. The modified protocol was less time consuming and easier to perform compared to method of filtration or centrifugation prior to extraction in liquid nitrogen. However, it is not possible for a more rapid immediate quenching because extraction and quenching do occur in the same step. The modified protocol was not evaluated for uncertainties, metabolic recoveries or if it favored groups of metabolites. This is a drawback and should be studied further, by using internal standards for estimation of metabolic recoveries and analysis of parallels for each sample taken.

Filtration prior to quenching is only valid for study of metabolites with long turn over times, such as amino acids. Metabolite extracts from fermentation cultivations were analyzed in respect of amino acids, selected citric acids cycle intermediates and fatty acids, making the use of filtration prior to quenching a valid method.

Lyophilization for metabolite concentration was used when developing quenching protocols using shaking flask cultivations, but replaced by solvent evaporation under reduced pressure and ambient temperature (SpeedVac) when sampling from fermenter. Villas-Bôas et al (2005) presented a diverse recovery pattern depending on the class of

metabolites using lyophilization. In this project, no losses of glucose-6-phosphate were observed applying the same concentration method. Based on results from Villas-Bôas et al (2005) studying several groups of metabolites, solvent evaporation were used as concentration method onwards.

4.2 Analytical Methods

Enzyme assays were used to analyze glycolytic intermediates in metabolic extracts from shake flask cultivations, and GC-MS were used for analyzes of seven amino acids, four citric acid cycle intermediates and five fatty acids in metabolic extracts from fermentation cultivations.

Internal standards were added right after filtration (d3-alanine) and at the beginning of derivatization (d5-glutamate). None of the standards were seen in chromatograms, and losses and recoveries of metabolites during sample preparation would therefore not be calculated. One possible explanation is that something was wrong with the solutions used. The absent detection of internal standards should have been detected during early analytical run. However, samples were collected and analyzed at the end of the experimental part of the master project.

It was prioritized to study an overview of the metabolic profile of stress exposed *S. cerevisiae.* Even though the DRS library applied included 83 metabolites, only the 11 most abundant metabolites were studied in detail. The fatty acids studied were identified, as tetradecanoic acid, palmitoleic acid, palitic acid and stearic acid, by the public NIST library. Applying the commercially available Fiehn library, porphine, glutamine, mannitol and ergosterol were additionally identified. Still, groups of metabolites are not covered revealing that several analytical methods are needed for metabolic profiling. Fermentation part of this project was done in cooperation with master student Simon Rey, who applied a third derivatization protocol for the study of amino acids. Refer to his report for results on the same samples analyzed in this report. LC-MS is one example of another analytical method which can be applied for analysis of phosphorylated glycolytic intermediates and nucleotides (24).

Study of nucleotide pool will improve the relevance of this project seen in relation of the type of stress agent applied. Changes in nucleotide pool will reflect the direct/primary consequence of 5-fluorouracil, whereas changes in amino acids, citric acid cycle intermediates and fatty acids are affected indirectly/secondary.

4.3 Growth and Experimental Design

More samples are recommended to be taken and analyzed in both exponential and stationary phase of a reference cultivation, for better understanding of metabolic activity under normal conditions. This will give a basis of evaluating metabolic activity seen after stress exposure. The time between stress exposure and the point of nutrient limitation must be prolonged compared to what was done in this project. Either stress can be applied earlier in exponential phase or more of the limiting nutrient can be added making stationary phase appear later, or both of the above.

OD was used as a measure of cell growth. Anyhow, there is not a direct proportionality between OD and dry weight, but it is a fast and cheap measurement of cell culture progression. Results from GC-MS analysis were normalized to the measured OD at the time of sampling. It is believed that it is more correct to normalize to OD compared to not doing so.

4.4 Stress Agent Exposure - Metabolome Analysis

Only metabolic changes appearing after stress exposure and before nutrient limitations are caused by the stress agents. Stress agents in this project were 1M NaCl, 0,1mM 5-fluorouracil and 0,5mM 5-fluorouracil. To observe metabolic responses over a longer time period, it is recommended to apply stress agents earlier, which will lead to a prolonged time between point of stress exposure and nutrient limitation. Observations of metabolic activity in exponential phase of reproducible reference cultivation are needed as a basis for evaluating metabolic changes appearing after stress exposure.

Sampling prior to stress exposure reflect metabolic status under normal conditions, and give a basis for evaluation of changes occurring after stress exposure. However, no samples were taken prior to application of osmotic stress, which should have been performed.

Groups of metabolites tend to be affected in the same way. Citric acid cycle intermediates is most affected, followed by amino acids and fatty acids. The fatty acids are more stable in stress situation compared to non-stress situations. Compared to amino acids and citric acid cycle intermediates only minor metabolic changes are observed for fatty acids.

Citric acid cycle intermediates tend to be more influenced by stress exposure than amino acids. When respiration falls quickly, succinate level increases. Succinate is most influenced and citrate is least influenced in reference cultivation and in the experiments of both 0,1mM and 0,5mM 5-fluorouracil. These two intermediates are located early and

late in the citric acid cycle. Other citric acid cycle intermediates, as acetyl-CoA, iso-citrate, α-ketoglutarate, succinyl-CoA and oxaloacetate, can be studied further for a more complete picture of how the cycle is affected as a whole. DRS library used in this project includes other citric acid cycle intermediates and only a more detailed study of the analyzed samples is needed. Moreover, the additional intermediates probably show low abundance compared to those studied in detail.

4.5 Concluding Remarks

For comparative metabolomics, neutral extraction method covering an extensive specter of metabolites is preferred. For a narrow focus on specific metabolite groups, use of selected extraction methods might be of preference, as for example acidic or alkaline solutions. Therefore, the choice of extraction method must be coherent with the aim of study. For further study on this project, acidic extraction can be used for extraction of nucleotides (6). For a more complete metabolic profiling, including other groups of metabolites, LC-MS/MS should be applied. This analytical method will cover groups, such glycolytic intermediates and nucleotides, which can not be analyzed by GC-MS, due to their volatility.

This research aimed to evaluate metabolic changes caused by the cytostatic agent 5-fluorourail. 5-fluorouracil is used as cancer treatment, and in a wider perspective it is desirable to study how mammalian cells will respond to the same exposure. Genetic variations can be studied by using several strains and mutations. DNA repair systems in humans are genetically dependent and the use of several strains will improve the relevance of the study. However, only one effector must be changed at the time, and experimental design must be developed for one strain before moving to the next level in research.

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ABBREVIATIONS

5-FU: 5-fluorouracil
DHAP: dihydroxyacetonephosphate
DHFU: dihydrofluorouracil
DNA: deoxyribonucleic acid
DPD: dihydropyrimidine dehydrogenase
DRS: deconvolution reporting software
dTMP: deoxythymidine monophosphate or 5’-thymidylate
dTTP: deoxythymidine triphosphate
dUMP: deoxyuridine monophosphate
F-6-P: fructose-6-phosphate
F-1,6-BP: fructose-1,6-biphosphate
FdUMP: 5-fluoro-2’-deoxyuridine 5’-monophosphate
FdUTP: 5-fluoro-2’-deoxyuridine 5’-triphosphate
FUMP: 5-fluoro uridine 5’-monophosphate or 5-fluoro-uridylate
FUTP: 5-fluorouridinetriphosphate
GC-MS: gas chromatography-mass spectrometer
G-6-P: glucose-6-phosphate
g: gram
h^{-1}: per hour
L: liter
LDH: lactate dehydrogenase
m/z: mass to charge ratio
MCF: methyl chloroformate
mg: milligram
mM: milimolar
mL: milliliter
MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide
MTX: methotrexate
NAD^+: nicotinamide adenine dinucleotide
NADH: reduced form of NAD^+
$NADP^+$: nicotinamide adenine dinucleotide phosphate
NADPH: reduced form of $NADP^+$
nm: nanometer
OD: Optical density
PRPP: phosphoribosyl pyrophosphate
Q1: qualifier 1
Q2: qualifier 2
Q3: qualifier 3
RNA: ribonucleic acid

rpm: rounds per minutes
TI: target ion
TS: thymidylate synthase
µL: microliter
µm: micrometer

APPENDIX I- STOCK SOLUTIONS

The mineral media for cultivations was based on Verduyn et al (1992). Mineral media consists of salt solutions, trace elements, vitamins, magnesium and glucose, which are all added from stock solutions. The applied stock solutions are described in detail below.

Salt solution

5 g/L $(NH_4)_2SO_4$ (Merck, LOT A801717705)
3 g/L KH_2PO_4 (Merck, LOT A672173 637)

The salts were dissolved in MiliQ water, and 90 mL allocated in separate shaking flasks. If the media were to include histidine, methionine, leucine and uracil, these were dissolved along with the salts in the following concentrations.

0,933 g/L Histidine (Sigma Chemical Company, LOT 57HO386)
0,121 g/L Methionine (Sigma Chemical Company, LOT 82F-0529)
0,327 g/L Leucine (Sigma Chemical Company, LOT 11KO899)
0,094 g/L Uracil (Sigma Chemical Company)

Trace element stock solution for shaking flasks cultivations

0,30g $FeSO_4$*7H_2O (Merck, LOT 1173391)
0,45g $ZnSO_4$*7H_2O (Merck)
0,228 g $CaCl_2$ (Merck, LOT F356083 802)
0,08g $MnCl_2$ *2H_2O (Merck, LOT 9657938)
0,03g $CoCl_2$ *6H_2O (Reagenzien Merck, LOT 8489035)
0,4g Na_2MoO_4 *2H_2O
1 g H_3BO_3
0,03g $CuSO_4$ *5H_2O (Acros Organics, LOT A014205301)
0,01g KI (Merck, LOT B158343 234)
1,3g EDTA (Sigma Chemical Company, LOT 61F-0318)

The trace elements were dissolved in 1000 mL of MiliQ water, and the pH adjusted to 4 by 2M NaOH and 0,1M KOH before sterile filtration.

Trace element stock solution for fermenter cultivations

1,5g $FeSO_4$*7H_2O (Merck, LOT 1173391)
2,28g $ZnSO_4$*7H_2O (Merck)
1,13g $CaCl_2$ (Merck Pro Analysis, LOT F356083 802)
0,5g $MnCl_2$ *2H_2O (Merck, LOT A852327 823)
0,15g $CoCl_2$ *6H_2O (Reagenzien Merck, LOT 1 025 39 0100)
0,15g $CuSO_4$ *5H_2O (Acros Organics, LOT A014205301)
0,2g Na_2MoO_4 *2H_2O
0,5g H_3BO_3
0,05g KI (Merck, LOT B158343 234)

7,5g EDTA (Sigma Chemical Company, LOT 61F-0318)

Trace elements were dissolved in 500 mL of MiliQ water one by one holding the pH at 6. Prior to addition of EDTA, the solution was gently heated. Finally, pH was adjusted to 4,0 by 6M and 1M NaOH before sterile filtration.

<u>Vitamin stock solution</u>

26,8 mg d-biotin was dissolved in 10 mL 0,1 M NaOH, 400 mL distilled water was added and pH adjusted to 6,5. The given amounts of vitamins below was added and the pH adjusted to 6,5.

0,500 g	Ca-Pantothenat	(Merck, LOT 251637 art: 2316)
0,500 g	Thiamin-HCl	(ACROS Organics, LOT A016611101)
0,500 g	Pyridoxin-HCl	(Sigma, LOT 76F-0367,No. P-9755)
0,500 g	Nicotinic acid	(Sigma, LOT 51C-1670, No.N-4126)
0,100 g	p-aminobenzoic acid	(Sigma)

12g m-inositol (Sigma, No. I-5125) was added, and pH again adjusted to 6,50. MiliQ water was added to complete the volume to 500 mL, before sterile filtration and cold storage.

<u>Glucose stock solution for shaking flask cultivations</u>

100 g glucose (AnalaR Normapur, LOT 07E070003)

500 mL MiliQ water

<u>Glucose stock solution for fermenter cultivations</u>

56 g glucose (AnalaR Normapur, LOT 07E070003)

100 mL MiliQ water

<u>$MgSO_4$ stock solution</u>

25 g $MgSO_4$ (Merck Pro analysi, LOT 0076819)

500 mL MiliQ water

APPENDIX II- RAW DATA FROM CORRELATION CURVE OF GRAM DRY WEIGHT PER LITER AND OD

For calculation of gram dry weight per liter and its correlated OD, different volumes of culture were transferred to sterile 50mL tubes. Volumes were completed to 40mL and OD measured. Each tube were centrifuged, the supernatants decanted and pellets re-suspended in a small volume distilled water. Each suspension was transferred to pre-weighted scales and dried over- night. The scales were weighted again and the change in weight being the dry weight. Calculations were done, and a correlation curve of gram dry weight per liter and OD was made. Raw data are shown in Table 2.

Table 2- Raw data from dry weight measurements and correlation curve calculations

	CEN					BY 4743				
Volume culture	**40 mL**	**20 mL**	**10 mL**	**5 mL**	**2.5 mL**	**40 mL**	**20 mL**	**10 mL**	**5 mL**	**2.5 mL**
OD	2,37	1,35	0,652	0,307	0,149	2,96	1,66	0,718	0,35	0,196
Before (g)	2,1709	2,1677	2,1524	2,1527	2,1574	2,155	2,1585	2,1671	2,1606	2,1584
After (g)	2,2192	2,1897	2,1616	2,1572	2,1611	2,1946	2,1795	2,1778	2,1662	2,1605
Dry weight	0,0483	0,022	0,0092	0,0045	0,0037	0,0396	0,021	0,0107	0,0056	0,0021
g DW/L	1,2075	0,55	0,23	0,1125	0,0925	0,99	0,525	0,2675	0,14	0,0525

APPENDIX III- RAW DATA FROM ENZYME ASSAY OPTIMIZATION

Each enzyme assay used in this project was optimized in respect of the amount of standards and enzyme added to reach endpoint within 2-3 minutes and measuring range from OD 0,1 to 0,8. The amount of standards and enzymes were change to find a suitable combination of the amounts added. OD measurements from each trail, reaction progression and the linear effect of changing amount of standards for each enzyme assay are shown below. The blue shaded columns indicate the optimized amounts of standards and enzymes added in each assay.

G-6-P and F-6-P

The optimized volumes were 5μL G-6-P and F-6-P standards, and 1μL G-6-P dehydrogenase and G-6-P isomerase. Raw data for each trial of optimization is presented in Table 3.

Table 3-OD measurements from optimization of G-6-P and F-6-P enzyme assay

G-6-P dehydrogenase	5μL	5μL	3μl	3μL	2μL	2μL	1μL	1μL
G-6-P isomerase	5μL	5μL	3μL	3μL	2μL	2μL	1μL	1μL
10mM G-6-P Stndard	5μL	10μL	5μL	10μL	5μL	10μL	5μL	10μL
10mM F-6-P Standard	5μL	10μL	5μL	10μL	5μL	10μL	5μL	10μL
0,5 minutes	0,277	0,53	0,26	0,509	0,267	0,512	0,259	0,461
1 minute	0,284	0,558	0,274	0,546	0,281	0,542	0,268	0,504
1,5 minutes	0,286	0,567	0,278	0,565	0,286	0,55	0,272	0,525
2 minutes	0,288	0,573	0,282	0,569	0,289	0,559	0,274	0,536
2,5 minutes	0,288	0,575	0,282	0,568	0,289	0,562	0,274	0,54
3 minutes	0,287	0,576	0,282	0,572	0,29	0,564	0,275	0,544
3,5 minutes	0,287	0,576	0,282	0,574	0,29	0,565	0,275	0,546
4 minutes	0,287	0,576	0,283	0,574	0,29	0,566	0,275	0,547
5 minutes		0,576	0,283	0,571				
Delta OD	0,287	0,576	0,283	0,571	0,29	0,566	0,275	0,547
G-6-P isomerase added								
0,5 minutes	0,2	0,35	0,18	0,274	0,198	0,315	0,21	0,215
1 minute	0,239	0,435	0,23	0,383	0,255	0,411	0,23	0,346
1,5 minutes	0,25	0,47	0,248	0,436	0,275	0,459	0,248	0,402
2 minutes	0,26	0,488	0,259	0,461	0,283	0,483	0,257	0,445
2,5 minutes	0,263	0,5	0,262	0,476	0,288	0,495	0,259	0,466
3 minutes	0,263	0,506	0,264	0,483	0,29	0,503	0,261	0,48
3,5 minutes	0,263	0,509	0,265	0,486	0,29	0,507	0,262	0,487
4 minutes	0,263	0,512	0,265	0,487	0,29	0,511	0,262	0,494
5 minutes	0,263	0,513	0,266	0,494	0,291	0,514	0,263	0,5
6 minutes		0,515	0,266	0,498		0,515	0,264	0,504
7 minutes						0,52		
Delta OD	0,263	0,515	0,266	0,498	0,291	0,515	0,264	0,504

A graphic presentation of the reaction progression for G-6-P and F-6-P using 5µL and 10µL standard and 1µL and 2µL enzyme are shown below.

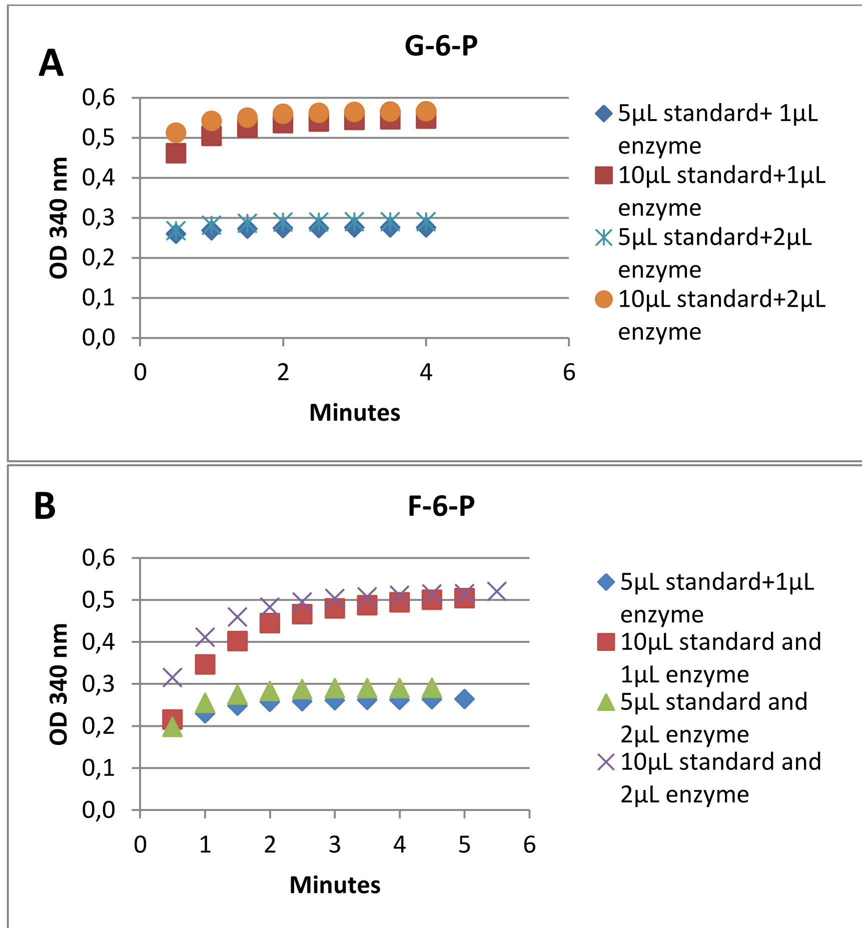

Figure 27- Progression of enzyme reaction of A) G-6-P using G-6-P dehydrogenase and 10mM G-6-P standard and B) F-6-P using G-6-P isomerase and 10mM F-6-P

Substrate as the limiting reactant is verified by the linear correlation of delta OD and increased amount of G-6-P and F-6-P standard added, Figure 28.

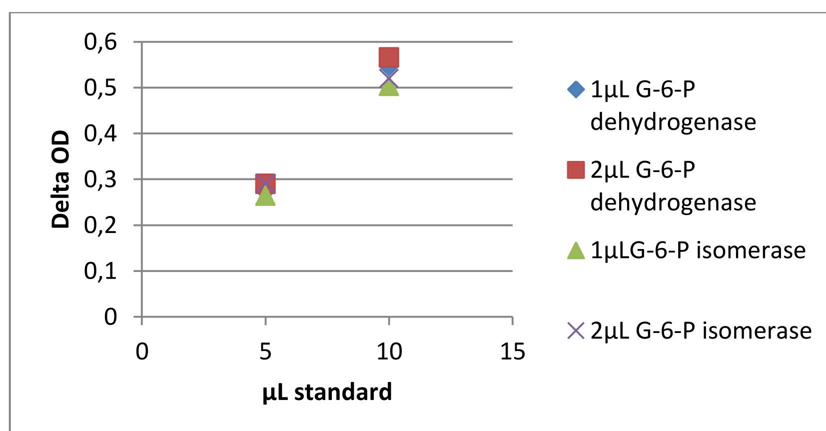

Figure 28- Changes in delta OD as a result of varying the volumes of G-6-P and F-6-P standard added, using the enzymes G-6-P dehydrogenase and G-6-P isomerase in the respective reactions.

DHAP and F-1,6-BP

The optimized volumes were 2μL DHAP and F-1,6-BP standards and 1μL G-3-P dehydrogenase and F-1,6-BP aldolase. Raw data for each trial of optimization is shown in Table 4.

Table 4-OD measurements from optimization of DHAP and F-1,6-BP enzyme assay

G-3-P dehydrogenase	2μL	2μL	2μL	2μL	2μL	1μL	1μL	1μL
F-1,6-BP aldolase	2μL	2μL	2μL	2μL	2μL	1μL	1μL	1μL
10mM DHAP Standard	5 μL	2μL	1μL	2μL	3μL	1μL	2μL	3μL
10mM F-1,6-BP Standard	6 μL	2μL	1μL	2μL	3μL	1μL	2μL	3μL
Before	0,65	0,638	0,712	0,757	0,704	0,682	0,752	0,713
0,5 minutes	0,411	0,54	0,669	0,662	0,574	0,647	0,675	0,59
1 minute	0,408	0,537	0,667	0,662	0,566	0,64	0,667	0,582
1,5 minutes	0,408	0,534	0,668	0,661	0,566	0,64	0,665	0,58
2 minutes	0,408	0,533	0,668	0,661	0,564	0,64	0,655	0,579
2,5 minutes	0,407	0,534	0,668	0,661	0,564	0,64	0,655	0,579
3 minutes	0,407	0,534	0,668	0,661	0,564	0,64	0,655	0,579
3,5 minutes								
Delta OD	0,243	0,104	0,044	0,096	0,14	0,042	0,097	0,134
F-1,6-BP aldolase added								
Before	0,407	0,534	0,668	0,661	0,564	0,637	0,655	0,579
0,5 minutes	0,06	0,213	0,54	0,402	0,266	0,541	0,507	0,35
1 minute	0,059	0,192	0,532	0,381	0,195	0,516	0,444	0,25
1,5 minutes	0,059	0,187	0,53	0,377	0,176	0,506	0,414	0,198
2 minutes	0,059	0,188	0,53	0,379	0,171	0,502	0,395	0,193
2,5 minutes		0,187	0,53	0,378	0,17	0,501	0,387	0,192
3 minutes		0,185	0,53	0,379	0,17	0,5	0,385	0,192
3,5 minutes		0,183					0,383	
4 minutes		0,183					0,381	
Delta OD	0,348	0,351	0,138	0,282	0,394	0,137	0,274	0,387

A graphic presentation of the reaction progression for DHAP and F-1,6-BP using 1, 2 and 3µL standard and 1µL and 2µL enzyme are given below.

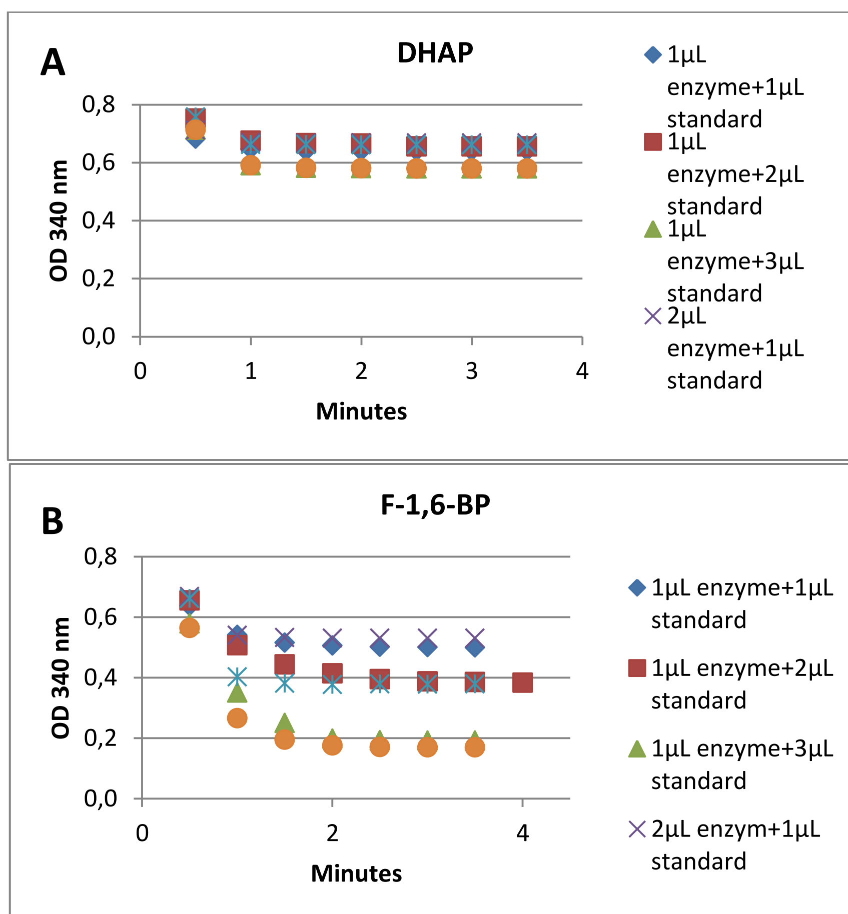

Figure 29-Progression of enzyme reaction of A) DHAP using G-3-P dehydrogenase and 10mM DHAP standard and B) F-1,6-BP using F-1,6-BP aldolase and 10mM F-1,6-BP standard

Substrate as the limiting reactant is verified by the linear correlation of increased delta OD and increased amount of DHAP and F-1,6-BP standard added, Figure 30.

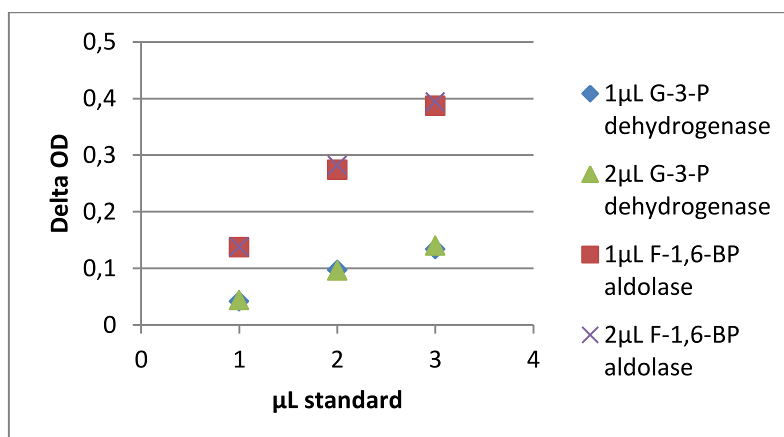

Figure 30-Changes in delta OD as a result of varying the amount of DHAP and F-1,6-BP standard added, using G-3-P dehydrogenase and F-1,6-BP aldolase in the respective reactions.

Pyruvate

Optimized volumes were 3µL pyruvate and 3µL lactate dehydrogenase. Raw data from each trial of optimization is shown in Table 5.

Table 5- OD measurements from optimization of pyruvate enzyme assay

Lactate dehydrogenase	5 µL	5µL	5µL	2µL	3µL	4µL	3µL	3µL	3µL	3µL
0,1M Pyruvate Standard	1 µL	2 µL	5 µL	1µL	1µL	1µL				
10 mM Pyruvate Standard							3µL	5µL	7µL	10µL
Before	0,775	0,874	0,85	0,659	0,787	0,744	0,686	0,766	0,661	0,678
0,5 minutes	0,278	0,092	0,089	0,28	0,29	0,184	0,536	0,532	0,328	0,245
1 minute	0,236	0,087	0,09	0,204	0,252	0,135	0,532	0,508	0,306	0,191
1,5 minutes	0,232	0,086	0,091	0,178	0,232	0,126	0,52	0,504	0,298	0,169
2 minutes	0,228	0,086	0,089	0,159	0,219	0,116	0,52	0,503	0,297	0,157
2,5 minutes	0,23	0,086	0,089	0,15	0,223	0,111	0,52	0,502	0,297	0,151
3 minutes	0,23	0,087	0,089	0,147	0,228	0,111	0,52	0,502	0,296	0,15
3,5 minutes	0,23			0,146	0,226	0,113	0,52	0,502	0,296	0,148
4 minutes	0,23			0,144	0,225	0,111	0,52	0,502	0,296	0,147
Delta OD	0,545	0,787	0,761	0,515	0,562	0,633	0,166	0,264	0,365	0,531

A graphic presentation of the reaction progression for pyruvate using 3, 5, 7 and 10µL standard and 3µL lactate dehydrogenase are given below.

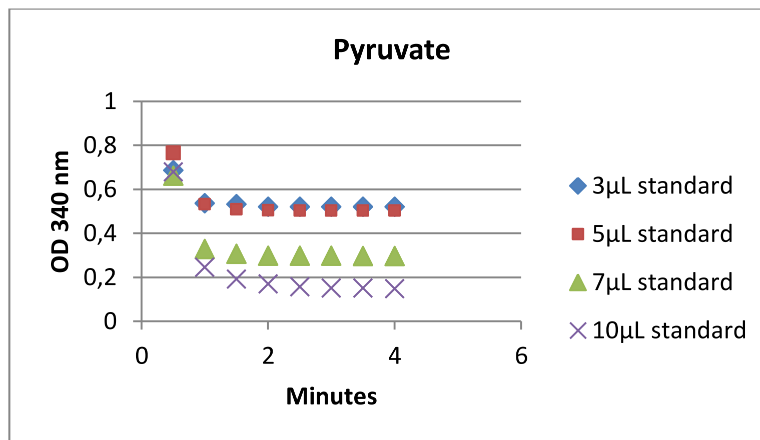

Figure 31- Progression of enzyme reaction of 10mM pyruvate using 3µL lactate dehydrogenase

Substrate as the limiting reactant is verified by the linear correlation of delta OD and increased amount of standard added, Figure 32.

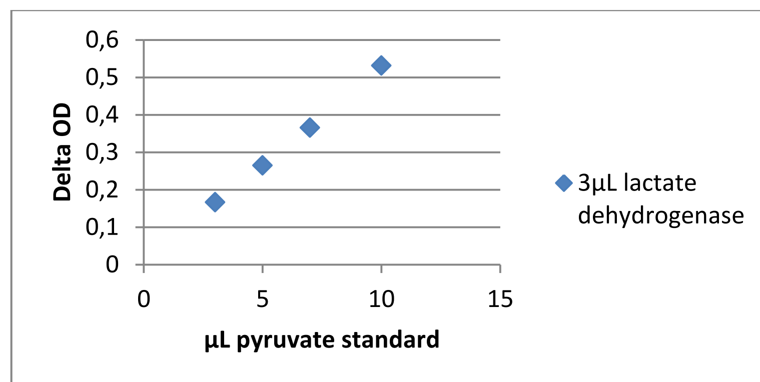

Figure 32-Change of delta OD as a result of varying the volume of pyruvate standard added using lactate dehydrogenase

APPENDIX IV - MEASURING INTRA- AND EXTRACELLULAR METABOLITES IN METABOLITE EXTRACTS USING ENZYME ASSAYS

Enzyme assays were used for analysis of glycolytic intermediates in intra-and extracellular extracts from the two sampling methods. Delta OD values from the extracts could be compared to delta OD from assays with standards and concentration in extracts calculated. Delta OD in three parallels for all extracts for both strains and both sampling method are show below. Delta OD of 0,000 indicates no metabolites in extracts. Moreover, addition of standards gave expected delta OD proving the functionality of each assay.

Table 6- Delta OD values from analyses of G-6-P, F-6-P, DHAP, F-1,6-P and pyruvate and standards in extracts from CEN pk and BY 4743 cultures, applying direct quenching

		G-6-P				F-6-P			
	Parallel	1	2	3	average	1	2	3	average
CEN pk intracellulært	**sample**	0	0	0	0,000	0	0	0	0,000
	standard	0,276	0,251	0,251	0,259	0,298	0,264	0,264	0,275
CEN pk extracellulært	**sample**	0	0,001	0	0,000	0	0,002	0	0,001
	standard	0,317	0,277	0,286	0,293	0,31	0,291	0,276	0,292
BY 4743, intracellulært	**sample**	0	0	0	0,000	0	0,002	0	0,001
	standard	0,254	0,256	0,275	0,262	0,245	0,253	0,26	0,253
BY 4743, extracellulært	**sample**	0	0,001	0	0,000	0	0	0	0,000
	standard	0,276	0,28	0,279	0,278	0,249	0,25	0,243	0,247
		DHAP				**F-1,6-BP**			
	Parallel	1	2	3	average	1	2	3	average
CEN pk intracellulært	**sample**	0,002	0,001	0,001	0,001	0,002	0,001	0,001	0,001
	standard	0,06	0,062	0,065	0,062	0,229	0,315	0,289	0,278
CEN pk extracellulært	**sample**	0,001			0,001	0,003			0,003
	standard	0,065			0,065	0,313			0,313
BY 4743, intracellulært	**sample**	0	0,001	0,001	0,001	0,001	0,001	0,001	0,001
	standard	0,078	0,099	0,094	0,090	0,299	0,27	0,271	0,280
BY 4743, extracellulært	**sample**	0	0,002	0,001	0,001	0	0,001	0	0,000
	standard	0,066	0,069	0,063	0,066	0,298	0,272	0,282	0,284
		Pyruvate							
	Parallel	1	2	3	average				
CEN pk intracellulært	**sample**	0,015	0,016	0,015	**0,015**				
	standard	0,36	0,359	0,359	**0,359**				
CEN pk extracellulært	**sample**	0,014	0,016	0,017	**0,016**				
	standard	0,364	0,365	0,369	**0,366**				
BY 4743, intracellulært	**sample**	0,016	0,015	0,015	**0,015**				
	standard	0,37	0,362	0,361	**0,364**				
BY 4743, extracellulært	**sample**	0,015	0,015	0,015	**0,015**				
	standard	0,364	0,366	0,366	**0,365**				

Pyruvate was the first and only metabolite analyzed in extracts from the filtration protocol. Because the same results was seen as extracts from direct quenching, no other metabolites were analyzed. Results from filtration extracts are seen in Table 7.

Table 7- Delta OD values from analyses of pyruvate and standard for CEN pk and BY 4743 using filtration prior to quenching

		Pyruvate			
	Parallel	1	2	3	average
CEN pk intracellulært	sample	0,004	0,003	0,004	0,004
	standard	0,367	0,356	0,362	0,362
CEN pk extracellulært	sample	0,002	0,006	0,012	0,007
	standard	0,367	0,371	0,377	0,372
BY 4743, intracellulært	sample	0,004	0,002	0,004	0,003
	standard	0,362	0,375	0,378	0,372
BY 4743, extracellulært	sample	0,008	0,004	0,006	0,006
	standard	0,357	0,379	0,388	0,375

No metabolites were detected when the culture was allocated in replica as in the above experiments. To increase the biomass analyzed in each extract, samples from a third culture was not allocated in replica. No metabolites were detected despite increased amount of biomass analyzed. Furthermore, addition of standards did no show expected delta OD in this experiment, and the functionality of assays could not be confirmed.

Table 8- Delta OD values from analysis of metabolite extracts from BY4743 not allocated in replica after filtration and quenching. Addition of standards did not give the expected results, marked with red shading.

		G-6-P				F-6-P			
	Parallel	1	2	3	average	1	2	3	average
BY 4743, intracellulært	sample	-0,001	0,002	0,001	0,001	-0	-0,002	0	-0,001
	standard	0,287	0,284	0,306	0,292	0,27	0,275	0,282	0,276
BY 4743, extracellulært	sample	-0,001	0,001	0	0,000	0,001	-0,003	-0,001	-0,001
	standard	0,109	0,138	0,08	0,109	0,109	0,074	0,114	0,099

		DHAP				F-1,6-BP			
	Parallel	1	2	3	average	1	2	3	average
BY 4743, intracellulært	sample	-0,003	0,003	0,005	0,002	0,005	-0,001	0,001	0,002
	standard	0,21	0,105	0,089	0,135	0,277	0,312	0,268	0,286
BY 4743, extracellulært	sample	0,004	0,004	-0,002	0,002	0,002	0,003	0,002	0,002
	standard	0,008	0,015	0,088	0,037	0,011	0,023	0,068	0,034

		Pyruvate			
	Parallel	1	2	3	average
BY 4743, intracellulært	sample	0,007	0,006	0,003	0,005
	standard	0,374	0,376	0,37	0,373
BY 4743, extracellulært	sample	0,021	0,021	0,017	0,020
	standard	0,377	0,39	0,37	0,379

APPENDIX V- CHEMSTATION PART OF THE NEW MCF-DRS LIBRARY

In the process of building a Deconvolution Reporting Software library, mass to charge ratio for TI, Q1, Q2 and Q3 were manually plotted for each metabolite. So were names for each metabolite, each qualifier ratio to target ion, a certain CAS number, retention times, company ID, file name and uncertainties in percentage. The library composed in this project includes 38 known compounds from an already existing library and 43 new compounds. The manual typed information for each metabolites included in the new library are shown in

Table 9 below.

Table 9- Complete information for each metabolite included in the new DRS library sorted by retention times

open	name	cas	mol form	mol wt	RT	open	company ID	file name	target ion	q1	q1 ratio	q1 %un	q2	q2 ratio	q2 %un	q3	q3 ratio	q3 %un	unc tp: 0=
	Methylvalerat	1010		500	6,267		cmp10	C:\msdchem\1\DAT	74	87	71,9	20	99	36,8	20	88	24,8	20	1
	Tetrachloroethane	630206	C2H2Cl4	167,85	6,8569		cmpd005	C:\msdchem\1\DAT	83	85	66,1	20	131	13,6	20	168	12	20	1
	Malanoic acid	1008		500	6,949		cmp08	C:\msdchem\1\DAT	101	74	41,1	20	69	8,1	20	102	4,4	20	1
	Pyruvate	127173	C3H4O3	88,06	7,1534		cmpd006	C:\msdchem\1\DAT	89	117	40,6	20	85	14,3	20	57	16,2	20	1
	3-methyl-2-oxovalerate	1033		500	7,527		cmp33	C:\msdchem\1\DAT	85	144	6,8	20	86	5,5	20	84	5,36	20	1
	Pentachloroethane	100000049		500	7,7721		cmpd011	C:\msdchem\1\DAT	167	117	79,8	20	165	76,2	20	130	28,2	20	1
	Fumarate-1	1110178	C4H404	116,07	8,32		cmpd015	C:\msdchem\1\DAT	113	85	9,8	20	59	15,5	20	82	2,2	20	1
	Fumarate-2	2110178	C4H404	116,07	8,3644		cmpd016	C:\msdchem\1\DAT	113	85	49,4	20	59	20,8	20	82	5,3	20	1
	d4-Succinate	14493426	C4H2D4O4	122,12	8,442		cmpd017	C:\msdchem\1\DAT	119	117	15,2	20	91	14,9	20	58	33,2	20	1
	Lactate	50215	C3H6O3	500	8,4847		cmpd018	C:\msdchem\1\DAT	103	59	174,5	20	131	8,2	20	87	6,1	20	1
	Methylglyoxal	1031		500	8,484		cmp31	C:\msdchem\1\DAT	103	131	9	20	130	8	20	87	6,2	20	1
	Succinate	110156	C4H6O4	118,09	8,4976		cmpd019	C:\msdchem\1\DAT	115	87	18,1	20	114	28,4	20	55	42,4	20	1
	Hexachloroethane	67721	C2Cl6	236,7	9,3275		cmpd021	C:\msdchem\1\DAT	201	117	86	20	166	45,7	20	203	63,9	20	1
	b-hydroxypyruvat	1043		500	9,347		cmp43	C:\msdchem\1\DAT	201	117	76,4	20	119	73,8	20	199	65,1	20	1
	Citraconate	1044		500	9,526		cmp44	C:\msdchem\1\DAT	127	99	22,6	20	99	22,6	20	126	9,4	20	1
	Benzoat	1002		500	9,637		cmp02	C:\msdchem\1\DAT	105	77	59	20	136	47,5	20	106	8,9	20	1
	Citramalat	1021		500	9,95		cmp21	C:\msdchem\1\DAT	117	85	61,2	20	75	10,5	20	101	5,3	20	1
	Malate-2	26915157	C4H6O5	134,09	9,9752		cmpd025	C:\msdchem\1\DAT	103	71	74	20	61	32,7	20	59	19	20	1
	d3-alanine	63546270	C3H4D3NO2	500	10,042		cmpd026	C:\msdchem\1\DAT	105	61	19	20	73	9,7	20	59	16,3	20	1
	Glycine	56406	C2H5NO2	500	10,064		cmpd027	C:\msdchem\1\DAT	88	147	5,6	20	56	9,6	20	116	2,7	20	1
	Alanine	56417	C3H7NO2	89,09	10,075		cmpd028	C:\msdchem\1\DAT	102	58	17,8	20	70	9,5	20	56	5,2	20	1
	Methoxyamine	1029		500	10,183		cmp29	C:\msdchem\1\DAT	74	119	96,1	20	132	25,9	20	104	22	20	1
	Nicotinat	1036		500	10,382		cmp36	C:\msdchem\1\DAT	106	78	74,1	20	137	71,6	20	136	34,9	20	1
	Phenylacetat	1014		500	10,948		cmp14	C:\msdchem\1\DAT	91	150	53,9	20	65	15,9	20	92	100	20	1
	M-toluate	1035		500	11,437		cmp35	C:\msdchem\1\DAT	119	91	58,4	20	150	54,2	20	120	10,8	20	1
	2-aminobutyrat	1016		500	11,582		cmp16	C:\msdchem\1\DAT	116	84	8,2	20	117	7,5	20	146	6,5	20	1
	Adipat	1045		500	11,96		cmp45	C:\msdchem\1\DAT	114	111	76,1	20	143	75	20	101	70,8	20	1
	d8-valine	35045728	C5H3D8NO2	125,2	11,997		cmpd039	C:\msdchem\1\DAT	138	106	18,7	20	116	9,9	20	147	6,7	20	1
	Valine	72184	C5H11NO2	117,15	12,086		cmpd040	C:\msdchem\1\DAT	130	98	24,9	20	55	13,3	20	115	21,6	20	1
	a-ketoglutarate	328507	C5H6O5	146,11	12,097		cmpd041	C:\msdchem\1\DAT	115	87	11,2	20	130	3,3	20	55	43,2	20	1
	2-isopropylmalate	1025		500	12,425		cmp25	C:\msdchem\1\DAT	145	101	36,5	20	113	32,8	20	127	20	20	1
	a-ketoadipat	1027		500	13,236		cmp27	C:\msdchem\1\DAT	129	101	63,7	20	157	13,2	20	97	12,4	20	1
	Leucine	61905	C6H13NO2	131,17	13,275		cmpd048	C:\msdchem\1\DAT	144	88	72,2	20	102	21,1	20	128	5,7	20	1

	Isoleucine-1	1443798	C6H13NO2	131,17	13,341		cmpd050	C:\msdchem\1\DAT	144	88	51,8	20	69	17,6	20	76	8,9	20	1
	Serine-1	156451	C3H7NO3	105,09	13,419		cmpd053	C:\msdchem\1\DAT	115	146	81,6	20	102	14,9	20	59	68,5	20	1
	Isoleucine-2	2443798	C6H13NO2	131,17	13,442		cmpd054	C:\msdchem\1\DAT	144	88	51,1	20	69	17,2	20	76	8,8	20	1
	g-aminobutyrat	1009		500	13,562		cmp09	C:\msdchem\1\DAT	102	88	66,5	20	112	43,1	20	144	23,1	20	1
	Serine	1022		500	13,692		cmp22	C:\msdchem\1\DAT	89	131	71,2	20	127	52,1	20	99	31,9	20	1
	Malate-1	16915157	C4H6O5	134,09	13,775		cmpd058	C:\msdchem\1\DAT	75	117	74,2	20	113	56,4	20	101	10,1	20	1
	Threonine	1041		500	13,893		cmp41	C:\msdchem\1\DAT	115	147	23,7	20	100	20,9	20	87	15,1	20	1
	L-homoserine	1006		500	13,904		cmp06	C:\msdchem\1\DAT	115	100	87	20	101	58,7	20	128	13,9	20	1
	Proline	147853	C5H9NO2	115,13	13,941		cmpd059	C:\msdchem\1\DAT	128	82	11,9	20	59	8,5	20	187	3,7	20	1
	Asparagine	70473	C4H8N2O3	132,12	14,019		cmpd061	C:\msdchem\1\DAT	127	56	31,3	20	146	10,1	20	83	8,9	20	1
	O-acetyl-L-serine	1012		500	14,492		cmp12	C:\msdchem\1\DAT	105	161	80,2	20	77	75,1	20	117	63,5	20	1
	Aspartate	56848	C4H7NO4	133,1	14,963		cmpd071	C:\msdchem\1\DAT	160	128	42,9	20	118	24,9	20	86	22,2	20	1
	Citrate	77929	C6H8O7	192,12	15,019		cmpd072	C:\msdchem\1\DAT	143	101	57,9	20	175	13,2	20	69	6,9	20	1
	5-aminovalerat	1032		500	15,214		cmp32	C:\msdchem\1\DAT	88	157	41,9	20	101	40	20	130	33	20	1
	Salicylat	1039		500	15,802		cmp39	C:\msdchem\1\DAT	135	121	40,7	20	92	40,4	20	165	34,2	20	1
	Anthraniate	1018		500	15,815		cmp18	C:\msdchem\1\DAT	146	177	53	20	90	30,2	20	118	4,6	20	1
	Serine-2	256451	C3H7NO3	105,09	15,863		cmpd081	C:\msdchem\1\DAT	100	176	14,2	20	159	7,7	20	127	7,3	20	1
	Glutamine	56859	C5H10N2O3	500	15,897		cmpd082	C:\msdchem\1\DAT	141	109	36,5	20	56	20,4	20	82	11,8	20	1
	d5-glutamate	100000013	C5H4D5NO4	152,16	16,517		cmpd089	C:\msdchem\1\DAT	179	118	73,4	20	147	59,2	20	207	7,9	20	1
	Glutamate	56860	C5H9NO4	147,13	16,585		cmpd090	C:\msdchem\1\DAT	114	174	96,5	20	142	86,1	20	82	25,5	20	1
	N-acetyl-L-glutamat	1037		500	16,661		cmp37	C:\msdchem\1\DAT	116	84	93,6	20	158	92,9	20	144	48	20	1
	OH-Proline	51354	C5H9NO3	131,13	16,719		cmpd091	C:\msdchem\1\DAT	144	126	12,3	20	82	12,4	20	68	8	20	1
	Hydroxyproline	1017		500	16,891		cmp17	C:\msdchem\1\DAT	144	126	13,6	20	82	12,3	20	145	7,7	20	1
	Methionine	1030		500	16,948		cmp30	C:\msdchem\1\DAT	115	147	97,1	20	114	41	20	128	33,2	20	1
	b-methylamino-L-alanine	1040		500	17,713		cmp40	C:\msdchem\1\DAT	102	157	10,3	20	173	9,3	20	189	8,1	20	1
	Cysteine	52904	C3H7NO2S	121,16	17,926		cmpd099	C:\msdchem\1\DAT	192	176	82,8	20	132	48,4	20	146	43,8	20	1
	Isocitrate	320774	C6H8O7	192,12	18,163		cmpd103	C:\msdchem\1\DAT	129	157	46,6	20	201	34,4	20	101	2,1	20	1
	Phenylalanine	63912	C9H11NO2	165,19	18,262		cmpd104	C:\msdchem\1\DAT	162	91	64	20	146	51,6	20	178	27,6	20	1
	Putresine	1005		500	18,369		cmp05	C:\msdchem\1\DAT	88	128	34	20	172	20,9	20	117	14,1	20	1
	Phenylpyruvat	1011		500	18,395		cmp11	C:\msdchem\1\DAT	118	119	99	20	90	89	20	192	81,7	20	1
	Hippurat	1013		500	14,511		cmp13	C:\msdchem\1\DAT	105	134	35,1	20	193	18,1	20	161	16,3	20	1
	Threo-b-hydroxyaspartat	1026		500	18,591		cmp26	C:\msdchem\1\DAT	202	146	61,9	20	234	37,9	20	126	29,2	20	1
	4-imidazoleacrylate	1028		500	19,191		cmp28	C:\msdchem\1\DAT	179	210	97	20	107	45,3	20	152	41,9	20	1
	Allantoin	1003		500	19,195		cmp03	C:\msdchem\1\DAT	161	129	38,6	20	146	8,5	20	204	7,9	20	1
	2,4-diaminobutyrate	1034		500	19,28		cmp34	C:\msdchem\1\DAT	114	147	56,2	20	115	45,1	20	88	44,8	20	1
	4-aminobenzoat	1001		500	19,558		cmp01	C:\msdchem\1\DAT	178	209	92,9	20	146	57,8	20	134	17,4	20	1
	Cadaverine	1019		500	19,58		cmp19	C:\msdchem\1\DAT	88	143	19,1	20	101	12,7	20	187	8,1	20	1
	Oxaloacetat	1038		500	19,936		cmp38	C:\msdchem\1\DAT	185	143	71,3	20	153	68,8	20	125	36,6	20	1
	Histamine	1046		500	20,047		cmp46	C:\msdchem\1\DAT	152	140	66,2	20	81	53	20	107	12,6	20	1
	p-coumarate	1023		500	20,302		cmp23	C:\msdchem\1\DAT	161	236	62,2	20	133	29,6	20	205	16,4	20	1
	Ornithine	70268	C5H12N2O2	132,16	20,485		cmpd122	C:\msdchem\1\DAT	128	88	14	20	230	11,2	20	198	8,1	20	1
	N-glycyl-proline	1015		500	21,525		cmp15	C:\msdchem\1\DAT	128	88	32,8	20	212	23	20	153	15,1	20	1
	Lysine	56871	C6H14N2O2	146,19	21,585		cmpd137	C:\msdchem\1\DAT	142	88	22,7	20	153	14,2	20	244	8,3	20	1
	Histidine	71001	C6H9N3O2	500	22,174		cmpd143	C:\msdchem\1\DAT	210	226	57,3	20	194	63,7	20	139	90,1	20	1
	Ferulate	1020		500	22,254		cmp20	C:\msdchem\1\DAT	266	222	71,4	20	191	63,5	20	207	50,7	20	1
	Tyrosine	60184	C9H11NO3	181,19	23,25		cmpd151	C:\msdchem\1\DAT	121	236	83,1	20	165	56,1	20	161	20,7	20	1
	2,6-deaminopimelat	1004		500	23,757		cmp04	C:\msdchem\1\DAT	200	140	69,7	20	168	13,1	20	201	12,3	20	1
	Tryptophan	73223	C11H12N2O2	204,23	25,062		cmpd164	C:\msdchem\1\DAT	130	276	9,4	20	77	4,6	20	103	4,4	20	1
	Serotonine	1024		500	26,901		cmp24	C:\msdchem\1\DAT	218	231	82,9	20	306	45,2	20	159	39,2	20	1

APPENDIX VI- CALCULATIONS OF YIELD AND GROWTH RATE

Glucose concentrations in preliminary studies were 5g/L. Applying the same glucose concentration in fermenter cultivation, OD in exponential phase were too low for sampling. Glucose concentration was therefore adjusted to 80g/L, resulting in late exponential phase appearing around OD 8. This was believed to give enough biomass for metabolite analysis. Yield biomass per gram glucose (Y) and growth rate (μ_{max}) were calculated for 5g/L and 80g/L glucose in fermenter cultivation, showed in Table 10. Complete calculations for 5g/L are shown below.

Calculation of yield and growth rate for cultivation with 5g/L glucose

YIELD:

Max OD = 1,968

g DW/L corr. factor = 2,5

g DW/L = $\frac{1{,}968}{2{,}5}$ = $\underline{0{,}787}$

$Y_{5g/l\ glu} = \frac{0{,}787\ g\ DW/L}{5\ g\ glucoe/L}$

$\mathbf{Y_{5g/L\ glu} = 0{,}157\ g\ DW/\ g\ glucose}$

GROWTH RATE:

$\mu_{max} = \frac{lnX2 - lnX1}{\Delta time}$

$\mu_{max} = \frac{ln0{,}773 - ln0{,}339}{4{,}83 - 2{,}83}$

$\mathbf{\mu_{max} = 0{,}412\ h^{-1}}$

Table 10- Calculated yield and growth rate for the two glucose concentrations

Glucose concentration (g/L)	Yield (g DW/g glucose)	Growth rate-μ_{max} (h^{-1})
5	0,157	0,412
80	0,085	0,490

APPENDIX VII- METABOLIC CHANGES FOR SINGLE METABOLITES

In this report metabolic changes from experiments with 0,1mM and 0,5mM 5-fluorouracil are presented separately. However, it is also interesting to study how different concentrations affect each and every metabolite. Figure 33 and Figure 34 presents metabolic changes caused by different concentrations of 5-fluorouracil for single metabolites during sampling interval. Point of nutrient limitation is not shown.

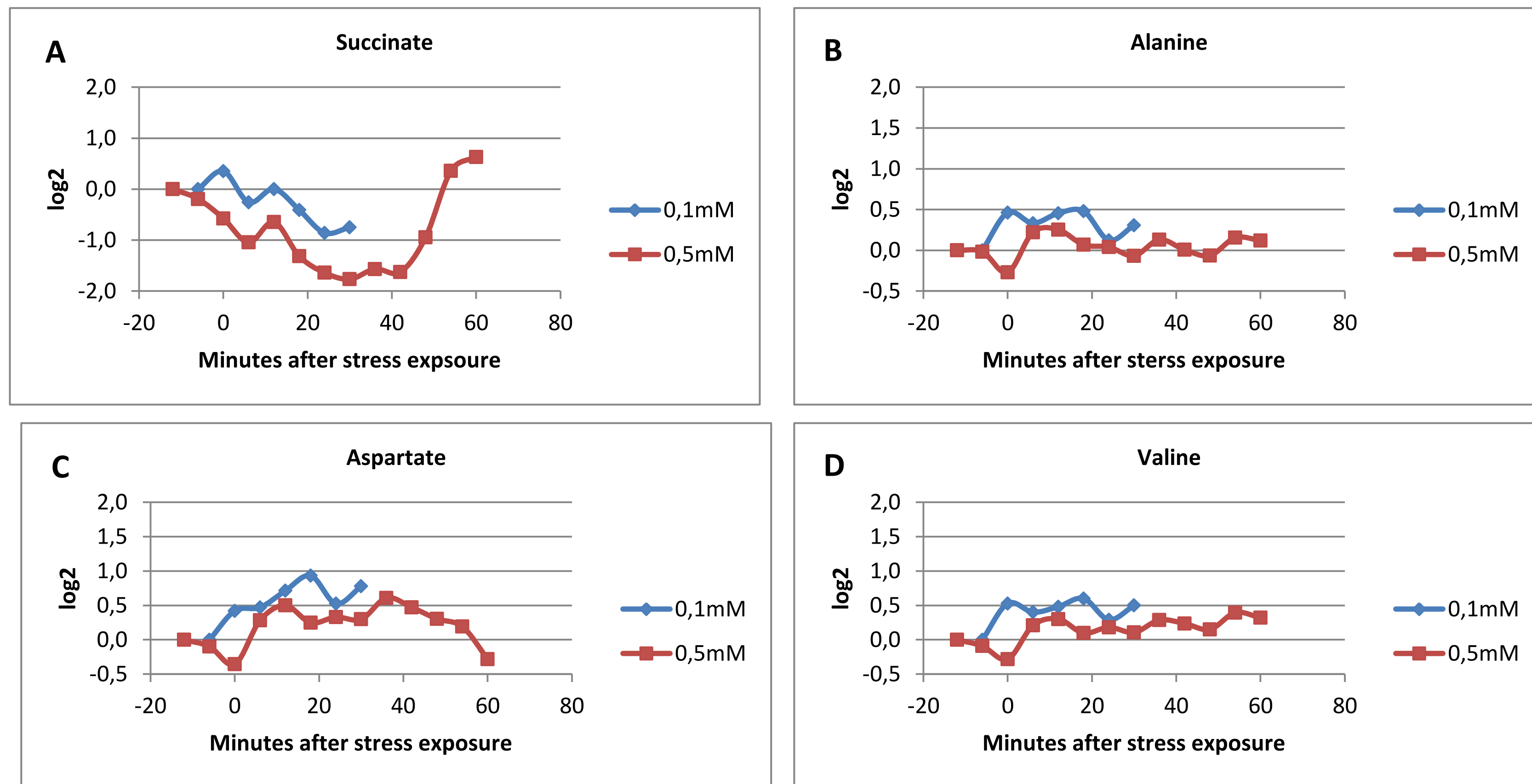

Figure 33- Metabolic changes before, at the time and after stress exposure by 0,1mM and 0,5mM 5-fluorouracil for A) Succinate, B) Alanine, C) Aspartate and D) Valine

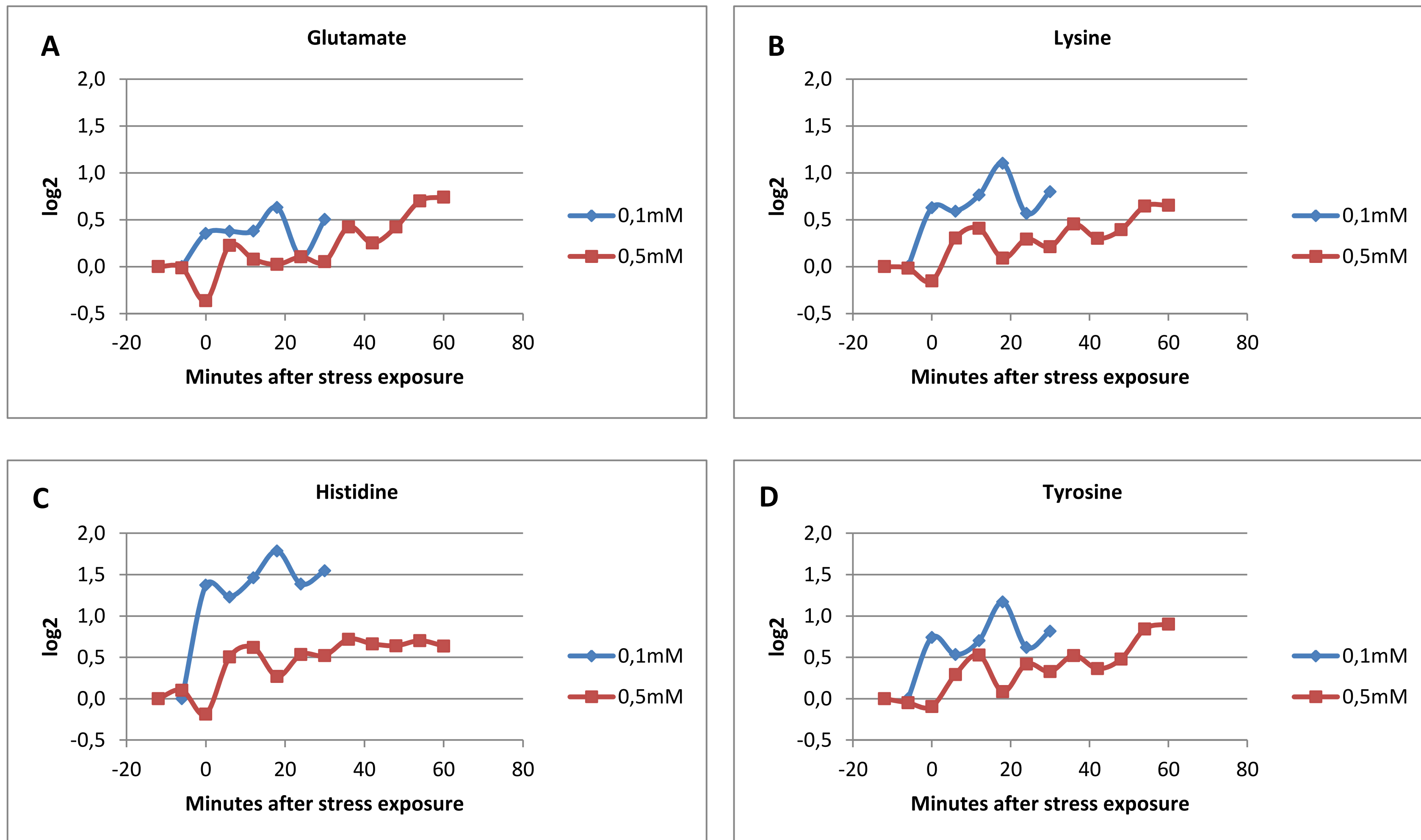

Figure 34- Metabolic change before, at the time and after stress exposure of 0,1mM and 0,5mM 5-fluorouracil for A) Glutamate, B) Lysine, C) Histidine and D) Tyrosine