A Study of Gene Expression of *Saccharomyces cerevisiae* in Oscillating Continuous Cultures Using DNA Microarray Technology

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Erklärung

Ich versichere, dass ich diese Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen durchgeführt habe. Diese Arbeit wurde nicht als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet.

Hannover, Juli 2008

Ahmed Abd Allah Khalil Ahmed

Dedication

I dedicate this work to my family:

My Lovely Mother
My Great Father
My Dear Brother
My Beloved Wife
&
My Beautiful Daughter.

I am always thankful to God for the presence of this family in my life. They usually support me with endless love and moral support.

Ahmed Abd Allah Khalil Ahmed

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Abstract

The yeast *Saccharomyces cerevisiae* is often considered the most ideal eukaryotic microorganism for biological studies. The ease of genetic manipulation and cultivation of yeast allows its use for conveniently analyzing and functionally dissecting gene products from other eukaryotes. The present study aimed to develop different types of microarrays for the whole genome expression and specific low denisty oligonucleotides microarrays to follow up the differential gene expression and regulation for chemostat-cultivated *S. cerevisiae* (H620) cells during especially G1 and S-phase events. The cells were cultivated for this aim continuously on the level of 2L bioreactor and the whole proces was optimized successfully to the level of 10L. The cells exhibited autonomous oscillations with periodic oxido-reductive metabolism, when grown aerobically in the continuous culture using glucose as the main carbon and energy source. The total RNA was isolated from the cells to be used as a starting material in the cDNA hybridization protocol of the microarrays. RNA was purified using hot phenol technique and enzymatic lysis method of RNeasy Midi Kit. Both methods were fast, suitable and reproducible, but the Midi kit was the protcol of choice in further preparations of the required RNA for the microarray hybridizations because of the higher yield and purity of its product. The relationship between total RNA inside the cells and the corresponding cell cycle phases was studied. During the 2L cultivation, the highest yield of RNA was in the mean time of S-phase (9.4 µg/µl), whereas the lowest value (3.4 µg/µl) during G2/M. During the 10L cultivation, RNA concentrations were also at their maximum levels at S-phase peaks; moreover, a doubling in RNA concentration had seen once from 5.5µg/µl during G1 to 10.5µg/µl during the same cycle. A prescreeing step was of great important for the whole gene expression during G1 and S-phase using the commerial yeast whole genome chip (MWG PAN yeast arrayII). The results of this screeing showed 532 reproducible genes, of which 130 genes were up regulated and 402 genes were down regulated. 90 genes, concerned with essential regulatory events during *S. cerevisiae* cell cycle, were selected among these reproducible genes to be used in further production of specific low denisity oligonucleotides microarrays. Another 50 genes, chosen by department of microbiology, Helmholtz center, Lepizig, concerned mainly with glucose metabolism, were added to the cell cycle regulatory genes to sum up 140 genes. This catalog of genes was used successfully to monitor the gene expression and regulation of 3 sequential cycles of cells collected from 10 L bioreactor. The cellular regulatory functions of yeast like that of cell wall biogensis, DNA and RNA synthesis, spindle pole body duplication and protein kinases were monitored easily in correlation with the metaolic status of cells inside the reactor using this catalog of genes.

Zusammenfassung

Die Bäckerhefe *S. cerevisiae* hat als Modellorganismus für Genexpressionsanalysen mit DNA-Chips mehrere Vorteile: Sie verbindet als einzelliger Eukaryont eine hohe Ähnlichkeit zu Säugerzellen mit prokaryontischer Handlichkeit: Hefen besitzen eine kurze Generationszeit (90 min auf Vollmedien mit Glukose als Energiequelle), sind ungiftig und auf preiswerten Medien auch in großen Mengen (kg-Maßstab) mit geringem apparativem Aufwand leicht anziehbar.

In der vorliegenden Arbeit soll ausgehend von Genexpressionsanalysen synchroner Hefekulturen auf kommerziellen whole genome arrays ein zellzyklusspezifischer low density Hefechip entwickelt werden. Hierzu wurden die Hefezellen zunächst kontinuierlich in einem 2 L Bioreaktor kultiviert. Der vollständige Prozess wurde anschließend erfolgreich auf ein 10 L Niveau upgescalt. Die Synchronisation von Hefezellen im Bioreaktor ermöglicht die Präsenz vieler Zellen in der gleichen Phase, die in idealer Weise für Genexpressionsanalysen mit DNA-Chips benutzt werden können, da ausreichende RNA Mengen (100 µg pro Chiphybridisierung, Qiagen RNAeasy Midi Kit) isolierbar sind. Das Prinzip eines Array-Experiments besteht darin, alle auf dem Array befindlichen Genproben simultan mit einer Nukleinsäureprobe zu hybridisieren. Dazu wird in erster Linie fluoreszenzmarkierte cDNA eingesetzt. Entscheidend ist hierbei, dass die durch invertierte reverse Transkriptase von mRNA aus (Hefe-) Zellen gewonnene cDNA im Idealfall alle dort spezifisch exprimierten Gene umfasst. Das parallele Hybridisieren einer Nukleinsäureprobe mit einer Vielzahl von komplementären Genproben auf einem DNA-Array führt zu einem charakteristischen Muster mmit entsprechender Hybridisierungsintensität. Während der 2 L-Kultiverung lag die höchste RNA Konzentration in der S-Phase vor, die niedrigste während der G2/M Phase. In der 10 L-Kultiverung waren die RNA-Konzentrationen während der S-Phasen maximal. Das Vorscreening mit einem kommerziellen whole genome array (MWG PAN yeast array II) zeigte 532 reproduzierbare Gene, von denen 130 Gene hoch- und 402 Gene runter-reguliert wurden. Hiervon wurden 90 zellzyklusspezifische Gene zur Produktion des low-density arrays ausgewählt. Weitere 50 Gene, die hauptsächlich den Glukosemetabolismus betreffen, wurden durch Abteilung von Frau PD Susann Müller, UFZ Leipzig hinzugefügt. Dieser Chip, der zusammen 140 Gene umfasst, wurde im TCI mit einem Affymetrix 427 Arrayer selbst hergestellt und für weitergehende Analysen der synchronen Kulturen sowohl im 2 L als auch im 10 L Reaktor genutzt. Nach Clustern der Genen zeigt sich, dass folgende biologische Prozesse zellzyklusabhängig sind: Zellwandsnythese, DNA und RNS-Synthese, Spindelpfostenkörperverdopplung und Proteinkinasen.

Key words: Yeast, *Saccharomyces cerevisiae*, oscillation, continuous cultivation, Gene expression, RNA, DNA, Microarray

Schlagwörter: Hefe, *Saccharomyces cerevisiae*, Kultiverung, Geneexpression, DNA, RNA, Microarray

Contents

1. Introduction

The yeast *Saccharomyces cerevisiae* has long been used as a model organism for studying genetic and biochemical processes because the genetic manipulation of yeast is very easy and cheap, whereas such manipulation, even when possible in mammalian systems, is neither easy nor cheap. *Saccharomyces cerevisiae* is a unicellular microbial eukaryote with a genome of 12 Mb which encodes approximately 6000 proteins.

The first complete DNA sequence of an eukaryotic genome, that of the yeast *Saccharomyces cerevisiae* was released in electronic form more than ten years ago. This achievement was followed in 1998 by the comprehensive identification of 800 cell cycle regulated genes using microarray hybridization technology. That is to say, in the post-genomic era, yeast was at the forefront in functional genomics.

Even though *Saccharomyces cerevisiae* is among the best-studied experimental organisms, 60% of its genes still have no experimentally determined function (Botstein *et al.*, 1997). Many yeast genes have homologues in human DNA, especially those involved in cell cycle control, meiosis and DNA repair. Studying the yeast phenotypes of mutants in these genes leads to new knowledge of human genes important in cancer, aging and other diseases.

The microarray approach was used successfully to follow the global changes in gene expression during the natural transition of yeast cells from fermentative metabolism to respiration, known as diauxic shift. Despite decades of study on the metabolic pathways involved, the microarray genomic expression revealed many previously unrecognized features of the metabolic transition (DeRisi *et al.*, 1997). Nearly 30% of the yeast genome was significantly altered in expression as the available glucose in the medium was depleted and subsets of genes displayed distinct temporal patterns of transcript varaiation.

The microarray technology studies of genomic expression have presented a wealth of information about yeast biology. Characterization of transcript fluctuation during the yeast cell cycle not only identified the cell cycle regulated genes but also painted a more complete picture of the biological processes that occur during each cell cycle phase (Spellman *et al.*, 1998).

Aim of the work

The main objectives of this research study were directed to the following items :

- Synchronous continuous cultivation of *S. cerevisiae* under well defined conditions on the level of 2 L bioreactor and optimization of this cultivation process to the level of 10 L.
- Setting up fast, reproducible and economic method for the purification of *S. cerevisiae* RNA in order to be easily used in further investigations of yeast genome expression assays using the microarray technology.
- Monitoring of total RNA level of *S. cerevisiae* during the different phases of the cell cycle, in order to find a correlation between RNA concentration inside the yeast cell and its corresponding cell cycle phase.
- Production of different types of microarrays for the whole genome expression and specific low denisty microarrays to follow up the gene expression and regulation especially during G1 and S-phase which are important periods in *S. cerevisiae* cell cycle progression.

2. Theoretical Background

2.1 Yeast characteristics

The yeast might be viewed to be one of the most important eukaryotic microorganisms used in biotechnology. It has served mankind for several thousands of years in making bread and alcoholic beverages. Many enzymes and biological compounds useful in biochemical research have been produced from yeast cells. In the mid-thirties of the 20th century, yeast has been introduced as an experimental system for molecular biology and has since received increasing attention. The elegance of yeast genetics and the ease of manipulation of yeast, and finally the technical breakthrough of yeast transformation to be used in reverse genetics, have substantially contributed to the enormous growth in yeast molecular biology. This success is also due to the fact, which was not anticipated then a couple of years ago, that the extent to which basic biological structures and processes have been conserved throughout eukaryotic life is remarkable. Yeast is a tiny form of fungi or plant-like microorganism (visible only under a microscope, as shown in Figure 2.1) that exists in or on all living matter i.e. water, soil, plants, air, etc. A common example of yeast is the bloom we can observe on grapes. As a living organism yeast needs sugars, water and warmth to stay alive. In addition, albumen or nitrogenous materials are also necessary for yeast to thrive.

There are two forms in which yeast cells can survive and grow, haploid and diploid. The haploid cells undergo a simple life cycle of mitosis and growth, and under conditions of high stress will generally simply die. The diploid cells (the preferential form of yeast) similarly undergo a simple life cycle of mitosis and growth, but under conditions of stress can undergo sporulation, entering meiosis and producing a variety of haploid spores, which can go on to mate (conjugate), reforming the diploid.

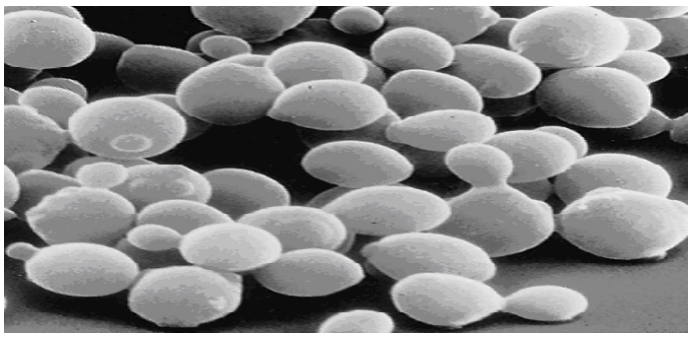


Figure (2.1): Budding Yeast cells (taken from Calzone *et al*., 2004- http://mpf.biol.vt.edu)

There are hundreds of different species of yeast identified in nature, but the genus and species most commonly used for baking is *Saccharomyces cereviae*. The scientific name *Saccharomyces cerevisiae*, means a mold which ferments the sugar in cereal to produce alcohol and carbon dioxide. The ultimate reaction of importance in this process is the conversion of simple sugars to ethyl alcohol and carbon dioxide.

Simple Sugar → Ethyl Alcohol + Carbon Dioxide

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

2.2 Metabolism in Yeast

Yeast derives its chemical energy in form of ATP, from the break down of organic compounds. Knowledge of different metabolic mechanisms is not only valuable in the understanding of principles of regulation but also of great importance in biotechnology, if new metabolic capabilities have to be exploited.

It is well established that yeast employs sugars as the main carbon and hence energy source but, sometimes it can utilize non-conventional carbon sources (Table 1). With regard to nitrogen metabolism, it is capable of assimilating simple nitrogenous sources to synthesize amino acids and proteins. Phosphorus and sulphur metabolism as well as aspects of metabolism of other inorganic compounds have been studied predominantly in the yeast, *Saccharomyces cerevisiae*.

Table (1): Nutrients for growth of *S. cerevisiae* cells

Substrates	Intermediates	Enzymes	Products
Saccharose		Invertase	Glucose + Fructose
Maltose		Maltase	Glucose
Melibiose		Melibiase	Glucose + Galactose
Glucose			Products of gylcolysis
Ethanol	Acetaldehyde, Acetyl- CoA & oxaloacetate	Alcohol Dehydrogenase	Glucose by gluconeogenesis
Lactate	pyruvate	Lactate dehydrogenase	Glucose by gluconeogenesis
Glycerol	Glycerol-3-phosphate & Dihydroxyacetonephosphate		Glucose by gluconeogenesis
Amino Aacids			
Glutamate			
Ammonium			

2.2.1 Carbohydrate Metabolism in yeast

Glucose is the major source for energy production in *S.cerevisiae* and in turn glycolysis is the general pathway for the conversion of glucose to pyruvate, whereby the production of energy in ATP form is coupled to generation of intermediates and reducing power in form of NADH for biosynthesis pathways. In presence of oxygen, pyruvate enters the mitochondrial matrix where it is decarboxylated to acetyl CoA. This reaction links the glycolsis pathway to the citric acid cycle, in which the complete oxidation of acetyl CoA takes place to give two molecules of CO_2 and reductive equivalents in form of NADH and $FADH_2$. However, the citric acid cycle is an amphibolic pathway, since it combines both catabolic and anabolic functions. The intermediate compounds of this cycle are precursors for the synthesis of amino acids and nucleotides.

During alcoholic fermentaion of sugars, yeast reoxidzes NADH to NAD in two step reaction from pyruvate, which is first decarboxylated by pyruvate decarboxylase followed by the reduction of acetaldehyde, catalysed by alcohol dehydrogenase (ADH) (Murray *et al*., 1993).

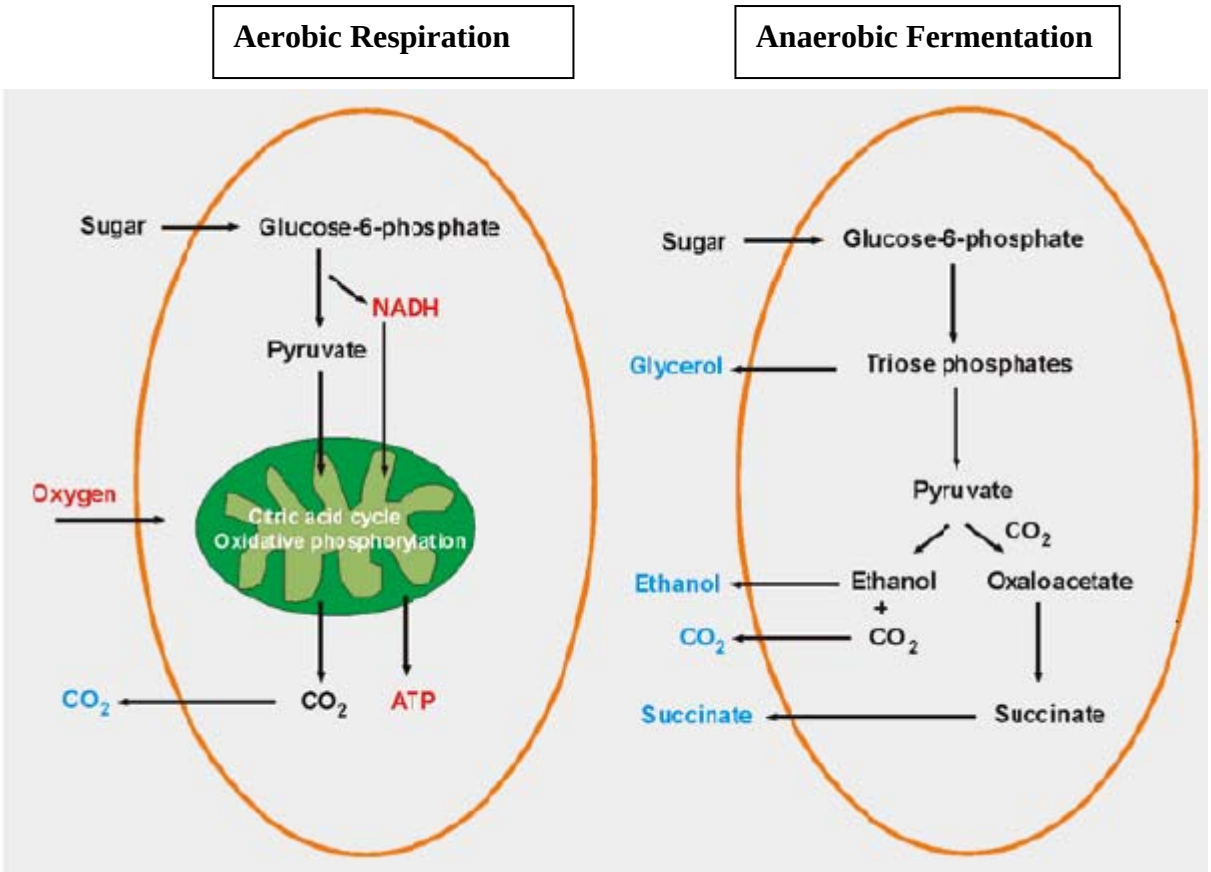


Figure (2.2): Metabolism in yeast under aerobic and anaerobic conditions (taken from Walker, 1997)

An alternative mode of glucose oxidation is the hexose phosphate pathway, also known as the pentose phosphate cycle, which provides the yeast cell with pentose sugars and cytosolic NADPH, necessary for the biosynthesis of fatty acids and amino acids. The first step is the dehydrogenation of glucose-6-phosphate to 6-phosphogluconolactone and the generation of one mole of NADPH (by glucose -6-phosphate dehydrogenase), then 6-phosphogluconate is decarboxylated by the phosphogluconate dehydrogenase to give ribulose 5 phosphate and a second mole of NADPH. The major function of this pathway is the production of ribose sugars which serve in the biosynthesis of nucleic acid precursors and nucleotide coenzymes (Berg *et al*., 2007).

The redox carriers NAD^+ and FAD^+, which become reduced during the break down of sugars to NADH and $FADH_2$, are reoxidized in the respiratory (electron transport) chain in the inner mitochondrial membrane. The energy released during the transfer of electrons is coupled to the process of oxidative phosphorylation, which is affected by ATPsynthase, an enzyme complex which is also located in the inner mitochondrial membrane and designed to synthesize ATP from ADP and inorganic phosphate (Murray *et al*., 1993).

These biochemical pathways in yeast are regulated at various levels:

(i) Enzyme sythesis - induction, repression and derepression of gene expression;

(ii) Enzyme activity- allosteric activation, inhibition, or interconversion of isoenzymes;

(iii) Cellular compartmentalization – localization of particular pathways to the cytosol, mitochondria, peroxisomes or the vacuole;

(iv) Transport mechanisms – internalization, secretion, trafficking of compounds between the various cellular compartments.

S. cerevisiae may thrive on a variety of carbon sources but glucose is the preferred one. When it is present, the enzymes required for utilization of alternative carbon sources are synthesized at low rates or not at all. This is known as catabolite repression, therefore when *S. cerevisiae* is grown aerobically on high concentration of glucose, it will repress the transcription of genes encoding enzymes required for the utilization of alternative carbon sources. Fermentation will account for the bulk of glucose consumption. In batch cultures, when the level of glucose declines, cells become gradually derepressed, resulting in the

induction of respiratory enzyme synthesis. This in turn results in oxidative consumption of ethanol; when cells enter a second phase of growth know as the diauxic shift (Juana, 1998).

Yeast can survive on different types of hexoses, pentoses, disaccharides, alcohols, hydrocarbons, fatty acids and organic acids. It should be also remembered that free glucose is rare in natural environments or natural products used to feed yeast cells. The growth of yeast on non carbohydrate substrates as sole carbon sources necessitates the synthesis of sugars, especially the glucose, required for macromolecules and complex polysaccharides biosynthesis. Like in other organisms, gluconeogenesis, the conversion of pryuvate to glucose is dependent on ATP as an energy donor and NADH as a reducing power.

Structural polysaccharides synthesis is associated with the cell and the spore wall and includes mannans, glucans and chitin. The sugar polymerization reactions employ sugar nucleotides as substrates, which are formed via activation by UTP or GTP, depending on the substrate.

A major activity in yeast is the synthesis of storage carbohydrates: glycogen and trehalose. Like in other organisms, glycogen is formed by sequential addition of glucose units from UDP-glucose, employing glycogen synthase for the linear α-1,4 linkage of the backbone chain, and the branching enzyme in the formation of α-1,6 branches. Degradation of gylcogen to glucose-1-phosphate is affected by glycogen phosphorylase (Campbell *et al*, 2006).

An unconventional storage disaccharide found in yeast is trehalose (α,α-1,1 diglucose), present in particularly high concentrations in resting and stressed cell. Trehalose-phosphate is synthesized in yeast from glucose-6-phosphate and UDP-glucose by trehalose-6-phosphate synthase and converted to trehalose by phosphatase. The breakdown of trehalose to glucose is mediated by trehalase. Both synthesis and degradation are regulated via cAMP **(**Reinders *et al.*, 1998).

2.2.2 Fatty acid and Lipid metabolism

Fatty acids, in the yeast, include those derived from microsomal alkaline oxidation or extracellular lipolysis of fats or those exogenously supplied in the growth medium. The fatty acids are catabolized by ß-oxidation in peroxisomes. The series of reactions leading to the synthesis of long-chain fatty acids, starting from acetyl-CoA is achieved by multi-enzyme complex, the fatty acid synthase. The subsequent formation of unsaturated fatty acids, which

are needed for the membrane integrity, involves an oxidative desturation by fatty acid desaturase (Berg *et al*., 2006).

Synthesis of lipid is similar to the reactions known in other organisms, starting from glycerol phosphate and fatty acids. Breakdown of lipids is affected by yeast lipase that generates long–chain fatty acids and glycerol, which latter is catabolized in the glycolytic pathways (Trotter, 2001).

2.2.3 Nitrogen metabolism in yeast

Yeast is capable of using a range of different inorganic and organic sources of nitrogen for incorporation into structural and functional nitrogenous constituents of the cell, such as amino acid (and in turn peptides and proteins), polyamines, nucleic acids and vitamins. Growth media are often supplemented with complex mixtures of amino acids. However, yeast can live on ammonium ions as a sole source of nitrogen, since it has a whole repertoire of genes encoding enzymes to synthesize all amino acids. Ammonium ions, either supplied as nutrient or derived from the catabolism of other nitrogenous compounds, can be directly assimilated into a couple of amino acids, glutamine and glutamic acid, which can then serve as donors of the amino group in other amino acids. The major pathway for assimilation of ammonium is the reaction of the NADPH-dependent glutamate dehydrogenase (GAD) which forms glutamate from α-ketoglutarate and ammonium. Whenever ammonium ion concentration is low, but also as a prerequisite for the synthesis of many nitrogenous compounds, glutamine synthase is activated, which forms glutamine from α-ketoglutarate and ammonium in an ATP-dependent reaction. Glutamine is absolutely required as a precursor in several essential pathways, such as synthesis of asparagine, tryptophane, arginine, CTP, AMP, GMP, glucosamine and NAD^+ (Murray *et al*., 1993).

2.2.4 Minerals metabolism in yeast

Phosphorous requirements of yeast cells are met by the uptake of inorganic phosphate from the nutrient media. The phosphate taken up can be utilized for incorporation of major cell consituents such as phospholipids, nucleic acids and proteins. The intracellular concentration of free phosphate is generally maintained at very low levels. Only when the yeast cells switch from respiratory to fermentative metabolism following a glucose pulse, dynamic changes in cellular phosphate have been observed. The bulk phosphate in yeast is in organic linkage and in form of polyphosphates. These latter are linear polymeres of ortho-

phosphates. As high concentrations of phosphates are accumulated and their hydrolysis gives the same amount of free energy as the hydrolysis of ATP to ADP and P_i, they are important for both phosphorus and energy supply in yeast cell (Ogawa, 2002).

In addition to membrane associated ATPases, yeast cells contain many important enzymes involved in phosphorylation and dephosporylation, kinases and phosphatases are crucial in governing a multitude of cellular processes especially during the cell cycle regulations.

The sulphur requirement of yeast can be met by uptake of sulphates which can be assimilated through reduction into sulphur amino acids. Yeast cells, as all eukaryotes, require transition metals such as iron, copper, zinc and manganese. These metals have to be acquired by cells through specific transport systems that mediate uptake across the plasma membrane. Much of this understanding has resulted from genetic and biochemical studies in yeast and the regulation has been defined at both the transcriptional and posttranscriptional levels (Van Ho *et al*., 2002 and Kosman, 2003).

2.3 Cultivation of *Saccharomyces cerevisiae*

Yeast can be cultivated with different techniques depending on the aim of the investigation, although the most familiar techniques are the batch cultivation and the continuous cultivation (chemostat).

2.3.1 Batch cultivation

Von Meyenburg (1969) has demostrated that *S. cerevisiae* exhibit diauxic behaviour in batch culture when supplied with glucose as the only carbon and energy source. After a brief initial lag phase during which growth occurs, the organism attains its highst specific growth rates, with exponential increase in cell number and production of ethanol via the well known fermentation pathway. Various kinetic studies on *S. cerevisiae* (Woehrer and Roehr, 1981) have clarified that the maximum growth rate available from fermentation is about 0.45 h^{-1}, with a low cell mass 0.15 g per gram glucose consumed. After this growth phase in which all the aviable glucose is depleted, cell growth stops for a finite period. During this lag phase, the cells undergo a switch-over in its enzymatic make-up, synthesizing new enzymes to consume ethanol produced as a byproduct of fermentation. A second exponential growth phase begins, during which the available ethanol is consumed by an oxidative pathway. The maximum growth rate available from ethanol oxidation is about 0.20 h^{-1}, with a high cell mass yield of

0.65 g per gram ethanol consumed. This second growth rate is much slower than that of the earlier fermentation growth phase; however the cell mass yield is much higher during ethanol oxidation. During fermentation much of the available glucose mass was converted to the byproduct ethanol, reducing the cell mass yield on this pathway, while all of the carbon consumed via ethanol oxidation pathway is used for the production of cell mass (Jones and Kompala 1999).

Locher *et al*., (1993) divided the batch cultivations with *S. cerevisiae* into seven different phases on the basis of metabolite production and consumption as shown in Figure (2.3).

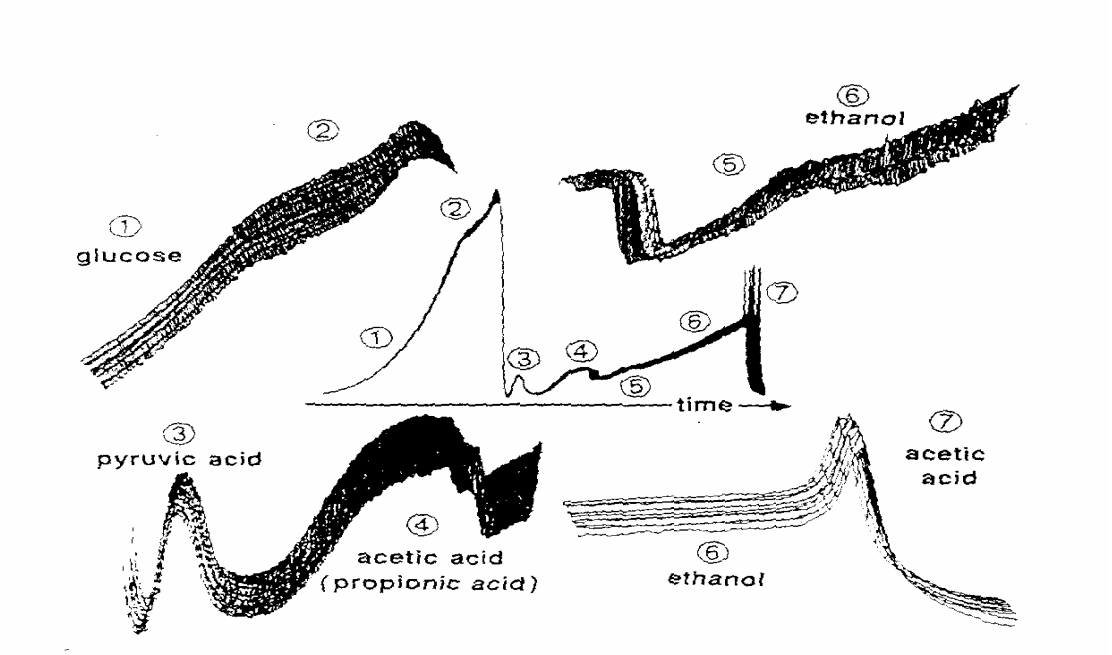


Figure (2.3): Data of CO_2 exhaust gas analysis, where seven regulatory phases can be distinguished in the course of batch cultivation of *S. cerevisiae*. The glucose phase is split into two phases with the second one only determinable under well mixed conditions. Phase 3 and 4 are characterized by the consumption of organic acids (pyruvic, acetic acid, and propionic acid), whereas the metabolic background for the 5th phase is still unkown, but ethanol is dominated and a weak drop in CO_2 signal at the end of this phase. During phase 6, mainly ethanol is metabolised and again acetic acid is accumulated in the medium (as a waste product of ethanol utilization). This is reutilized during phase 7, this is clear by the sharp increase in the CO_2 evolution immediately after the depletion of ethanol from the medium. (taken from Locher *et al*., 1993)

2.3.2 Continuous cultivation

In many instances continuous cultivations are used since they yield higher productivity than batch or fed-batch cultivations. However, continuous cultivation of *Saccharomyces cerevisiae* is known for its ability to exhibit autonomous oscillations over a fairly wide range of dilutions. During such oscillations all microorganism variables oscillate with rather amplitudes as a large fraction of cell population synchronously passes through the cell cycle. The physiological basis and the triggering of oscillations is not understood (Moller and Jorgensen, 1996).

The oscillations seems to be related to the peculiar respiratory-fermentative metabolism of budding yeast growing on fermentable carbon sources such as glucose and are detectable as periodic changes of many reactor bioparameters like dissolved oxygen, CO_2 production, and glucose, ethanol and biomass concentrations (Beuse, 1997).

The chemostat cultivation system allows manipulation of the specific growth rate (which is equal to the dilution rate) while keeping other important growth conditions constant. Similar to industrial fed-batch cultivation, sugar-limited chemostat cultivation allows fully respiratory growth of *S. cerevisiae* on sugars. This is not possible in batch cultures, which by definition require high sugar concentrations, which lead to alcoholic fermentation, even during aerobic growth. Continuous culture data show that there is a sharp maximum in the cellular productivity at a dilution rate near 0.25 h^{-1}. The exact amplitude and location of maximum can change as a result of many factors, such as culture conditions, feed conditions and environmental conditions. A severe problem for process control of a continuous culture of *S. cerevisiae* is its tendency to exhibit oscillatory behaviour. Continuous cultures of Bakers' yeast have long been known to exhibit oscillatory behaviour at dilution rates lower than a critical dilution rate. Sustained oscillations have been observed in continuous cultures when the feed conditions and culture conditions remain constant. Further, these oscillations frequency occur without any obvious triggering such as pH changes or temperature pulses. Simply, oscillations are induced and eliminated by varying the dilution rate, agitation speed and dissolved oxygen level over wide ranges (Scheper and Schügerl, 1986). The oscillations of carbon dioxide production, dissolved oxygen concentration and other variables occur when major parts of the population initiate bud formation at the same time (Martegani *et al*., 1990).

The oscillatory behaviour is induced by a pulsed substrate shift following the initial batch phase. In accordance with Parulekar *et al*., 1986, it was found that batch phase has to be finished by exhaustion of C-sources before the continuous culture is started. It may be useful

to add a starvation phase of one or two hours to ensure that substrate and internal storage carbohydrates are totally consumed. Starting the chemostat in this way will immediately lead to oscillations and after a few periods, the period length becomes constant (Beuse *et al.*, 1998).

The critical amount of the pulsed substrate necessary for the maintenance of forced synchrony is dependent on the pH in the culture. While at pH 4 glucose pulses of 60 mg/L were sufficient to force oscillations, at pH 5 this amount of substrate had no forcing effect at any time in a synchronous culture. Instead 120 mg/L pulses were necessary for forcing oscillations. The pH value in cultures of *S. cerevisiae* was found to determine strongly the spontaneous synchronization characteristics. The lower the pH, the lower the range of dilution rates where spontaneous oscillations occur (Muench *et al*., 1992).

Within cells, a number of biochemical pathways are involved in the occurrence or absence of metabolic oscillations: the ethanol assimilation pathway (Keulers and Kuriyama, 1998), the glutathione redox cycling pathway (Murray *et al*., 1999), the sulphate assimilation pathway (Sohn and Kuriyama, 2001) and the mitochondrial respiratory chain (Sohn *et al*., 2000). None of these can alone explain the behaviour of oscillation in the continuous culture, so there is a strong possibility of crosstalk among the pathways (Murray *et al*., 2001).

Although synchronization seems to be at the core of oscillatory metabolism, neither its control features nor its position in the cause and effect sequence is clear. This made Jones and Kompala in 1999 to raise a fundamental question when they postulated that cell synchrony is not a cause of oscillation, but the result of dynamic competition between three metabolic pathways, glucose fermentation, ethanol oxidation and glucose oxidation. Oscillatory responses were attributed to shifts among these pathways, but this postulation was not proved experimentally which reduced the credibility of this model.

2.4 The yeast cell cycle

The cell cycle is the succession of events whereby a cell grows and divides into two daughter cells that each contains the information and machinery necessary to repeat the process. Between one cell division and the next, all essential components of the cell must be duplicated. The most important component is the genetic material (DNA molecules present in chromosomes), which must be accurately replicated and the two copies carefully segregated to the two daughter cells. The processes of DNA replication and sister chromatid separation occur in temporally distinct phases of the eukaryotic cell cycle. These are known as S-phase

(DNA synthesis) and M-phase (mitosis), In general, S and M phases separated by two gaps, known as G1 and G2 (Campbell *et al*., 2005).

The unicellular budding yeast, *Saccharomyces cerevisiae*, is a model system to study cell cycle regulation. As a yeast cell progresses through the cell cycle, it halts at two major checkpoints:

- **The G1 checkpoint**: If DNA damage is detected, mating pheromone is present, or the cell has not reached the critical size, the cell arrests in G1 and is unable to undergo the start transition which commits the cell to a new round of DNA synthesis and mitosis.
- **The spindle assembly checkpoint**: If DNA damage is detected, DNA is not replicated completely, or chromosomes are not aligned on the metaphase plate, the cell arrests in metaphase and is unable to undergo the finish transition, whereby sister chromatids are separated and the cell divides.

These checkpoints are enforced by **the Cdk/cyclin complexes**, a family of protein kinases. The catalytic subunit of these complexes, the cyclin-dependent kinase (Cdk), is only active when combined with a regulatory cyclin subunit. In budding yeast, there is only one Cdk (called Cdc28); and nine different cyclins (Cln1-3, Clb1-6). Depending on the cyclin partner, Cdc28/cyclin dimers accomplish specific and different tasks. Proper progression through the cell cycle requires the successive activation and inactivation of these Cdc28/cyclin dimers (Oehlen, 1992). There are several different mechanisms for regulating Cdc28 activity in the cell, namely:

- through the synthesis of cyclins by various transcription factors (SBF, MBF and Mcm1).
- through the degradation of cyclins (promoted by Cdc20/APC, Cdh1/APC, and Grr1/SCF).
- through association with stoichiometric CDK inhibitors (Sic1 and Cdc6, and Far1).
- through phosphorylation and dephosphorylation of Cdc28 by Swe1 and Mih1.

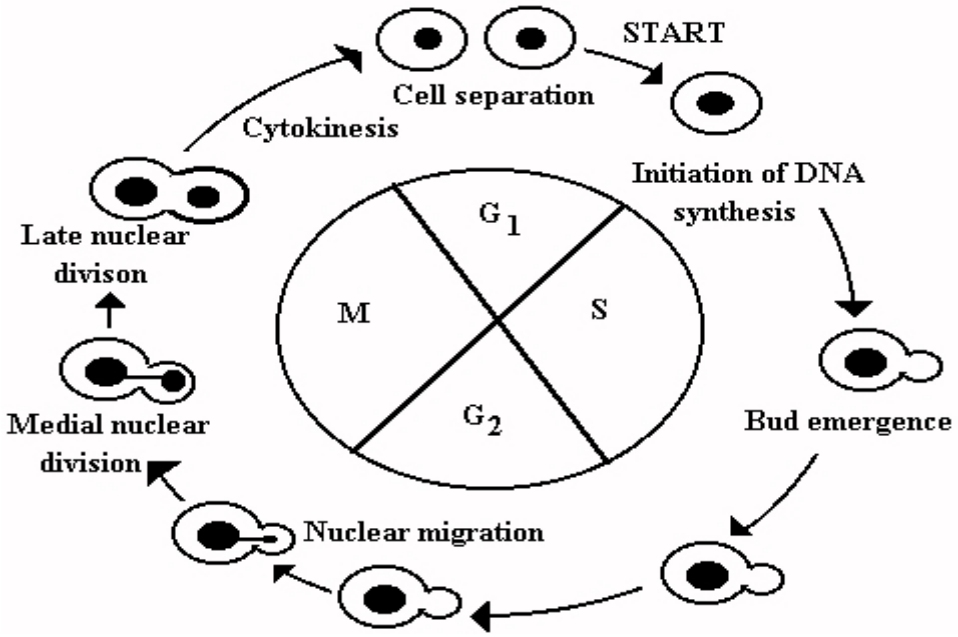


Figure (2.4): Yeast cell cycle different phases

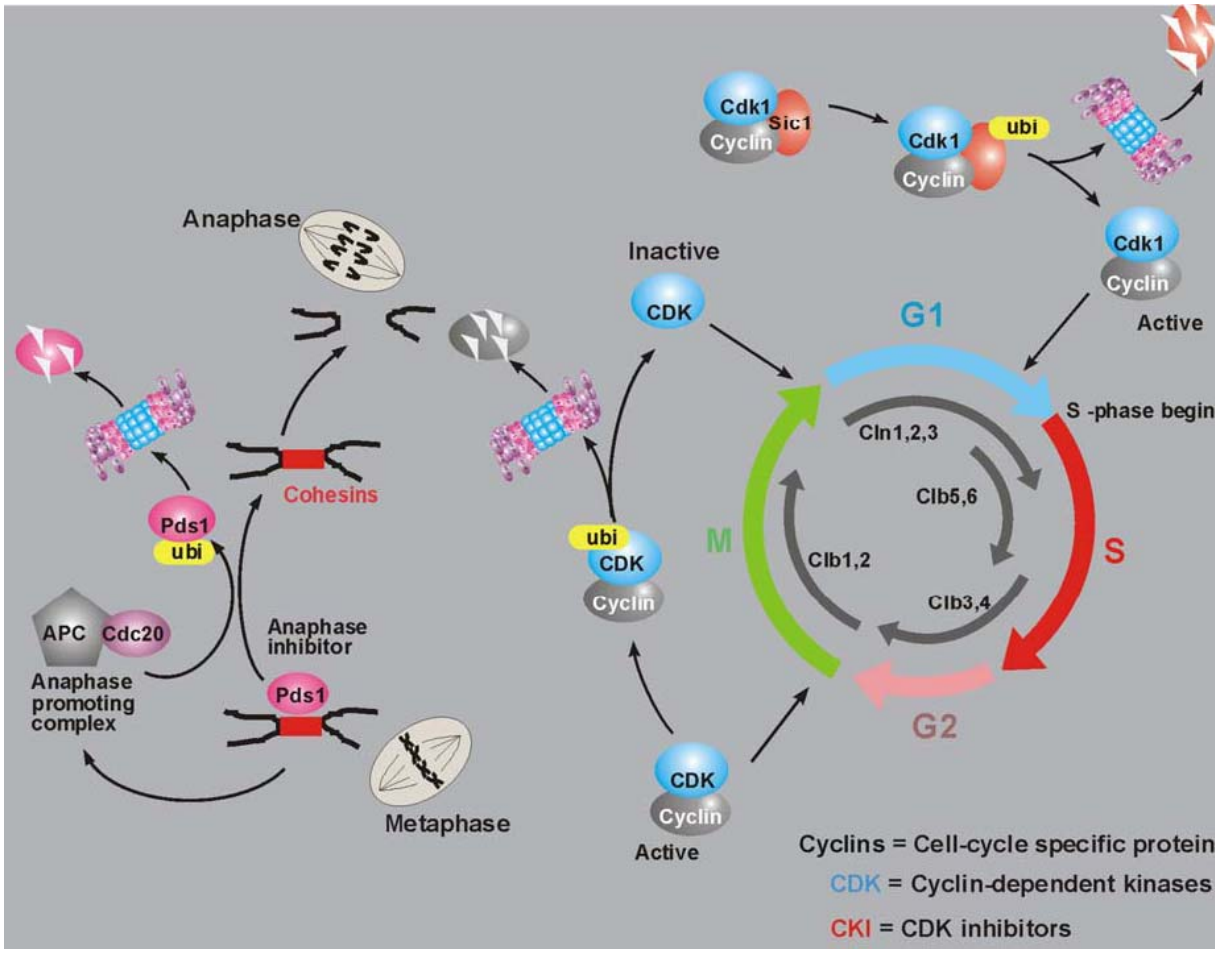


Figure (2.5): Yeast cell cycle regulation (taken from biochemie.web.med.uni-muenchen.de/Yeast_Biol)

As shown in Figure (2.5), Cyclin-dependent protein kinases have a central role in cell cycle regulation. In *Saccharomyces cerevisiae*, Cdc28 kinase and the G1 cyclins Cln1, 2, 3 are required for DNA replication, duplication of the spindle pole body and bud emergence. The amount of Cln3-Cdc28 is constant throughout the cell cycle. However, just prior to start, as cells approach the required minimum size, Cln3-Cdc28 kinase activates two transcription factors, SBF and MBF and induces the transcription of about 200 genes. Among these SBF and MBF targets are two more G1 cyclins Cln1 and Cln2 and two S-phase cyclins Clb5 and Clb6. These cyclins also form protein kinase complexes with Cdc28 which phosporylate various substrates and ultimately push cell into S-phase. One particular well studied event is the phosphorylation of Sci1, a CDK inhibitor, by the Cln1-Cdc28 and Cln2-Cdc28 complex. Once the Sci1 has been phosporylated, it is ubiquitinated and degraded and this allows activation of Clb5-Cdc28 and Clb6-Cdc28 complex and these directly activate the DNA replication (Oehlen, 1992 and Spellman *et al.*, 1998).

The events of late mitosis, from sister chromatid separation to cytokinesis, are governed by the anaphase promoting complex (APC) which functions as an ubiquitin ligase. APC activity raises at metaphase resulting in destruction of key regulatory protein, Pds1, that inhibits sister chromatid separation. This APC-dependent destruction initiates spindle disassembly, cytokinesis and the resetting of the replication origins for the next cell–division cycle. The activity of APC is cell cycle regulated partly through association with activating subunit Cdc20. It has been proposed that DNA damage induced phosporylation of Pds1, renders it resistant to APC degradation. Alternatively, phosporylated Psd1 may function as a negative regulator of APC-Cdc20 complex and Pds1 is stabilized as a consequence of APC inactivation (Wang *et al.*, 2001).

2.5 Yeast Genome

If individual yeast cells were placed side by side it would take approximately 1200 cells to measure 1cm in length. Inside each cell are the following: A liquid solution of protoplasm, protein, fat and mineral matter; one or more dark patches called vacuoles; and a darker spot which is the nucleus. This is where the cell's genetic information is stored as DNA which controls all the operations of the cell.

The genomes of particular non-human organisms such as yeast have been studied for a number of reasons including the need to improve sequencing and analysis techniques. These non-human genomes also provide powerful sets of data against which to compare the human

genome. For example, a gene known to govern the rate of aging in yeast cells has been found to be active in mice, yielding a new insight into why mice and people age and, possibly, ways of enhancing life span.

The *Saccharomyces cerevisiae* (yeast) genome contains 12.1 million base pairs and is estimated to have 6,034 genes. The sequencing of this genome was completed in 1996. The yeast genome is about 200 times smaller than the human genome but less than four times bigger than that of *E.coli*. At the onset of the sequencing project, knowledge about some 1200 genes encoding either RNA or protein products had accumulated. The complete genome sequence now defines about 6000 open reading frames (ORFs) most of which are likely to encode specific proteins. A protein-encoding gene is found every two kb in the yeast genome, with approximately 70% of the total sequence being covered. In addition to the protein-encoding genes, the yeast genome contains some 120 ribosomal RNA genes in a large tandem array on chromosome XII, 40 genes encoding small nuclear RNAs (sRNAs), 274 tRNA genes (belonging to 42 families) which are scattered throughout the genome, and 51 copies of the yeast retrotransposons (Ty elements). Finally, the sequences of non-chromosomal elements, such as the 6 kb of the 2μ plasmid DNA, the killer plasmids present in some strains, and the yeast mitochondrial genome (ca.75 kb) have to be considered (Walker, 1997).

2.6 DNA Microarray

Though most cells in our bodies contain the same genes, not all of the genes are used in each cell. Some genes are turned on, or "expressed" when needed. Many genes are used to specify features unique to each type of cell. Liver cells, for example, express genes for enzymes that detoxify poisons, while pancreas cells express genes for making insulin. To know how cells achieve such specialization, scientists need a way to identify which genes each type of cell expresses (Murray, 1993).

Microarrays are a tool for measuring the expression levels of a large number of genes simultaneously and determine which are expressed in a particular cell type. DNA molecules representing many genes are placed in discrete spots on a microscope slide. This is called a microarray. Thousands of individual genes can be spotted on a single square inch slide. Next, messenger RNA, the working copies of genes within cells (and thus an indicator of which genes are being used in these cells), is purified from cells of a particular type. The RNA molecules are reverse transcribed into cDNA then "labelled" by attaching a fluorescent dye that allows us to see them under a microscope, and added to the DNA dots (spotted oligonucleotides) on the microarray. Due to a phenomenon termed hybridization of base pairs,

cDNA will stick to the gene sequence it came from. After washing away all of the unstuck molecules, we can look at the microarray under a microscope and see which cDNA remains stuck to the DNA spots. Since it is known which gene each spot represents, and the cDNA only sticks to the gene that encoded its RNA, it can be determined also which genes are turned on or off in the cells. During this process the slide is hybridized with two different cDNA samples labelled separately with two distinct fluorescent dyes such as cyanine-3 (cy3), contain nine double bonds and cyanine-5 (cy5), contain ten double bounds (Figure 2.6) (Mueller and Roeder, 2006).

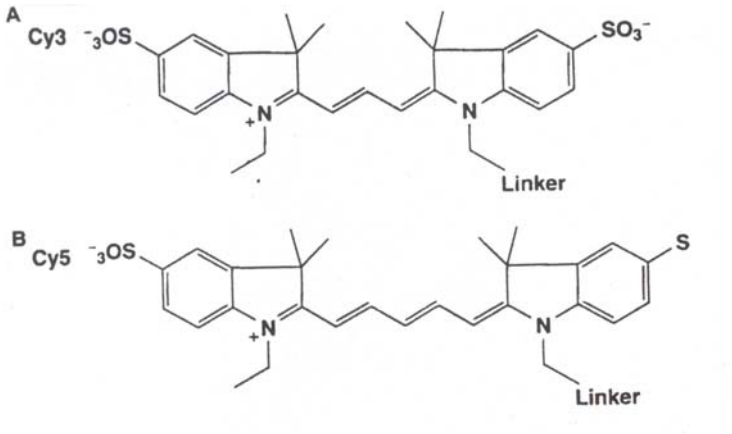


Figure (2.6): Structure images of cyanine-3 (A) and cyanine-5 (B) (taken from Mueller and Roeder, 2006)

The relative intensities of the two flurescent dyes within a spot represent the relative mRNA expression level of the gene. For example, (Figure 2.7), if fluorescent label cy3 (green symbol) and cy5 (red symbol) are used to make each sample's cDNA probe, the expression level of the gene will be displayed as green or red when the gene is differentially expressed, or yellow when the level is the same in the two samples. Some researchers are using this powerful technology to learn which genes are turned on or off in diseased versus healthy human tissues. The genes that are expressed differently in the two tissues may be involved in causing the disease (DeRisi *et al*., 1997).

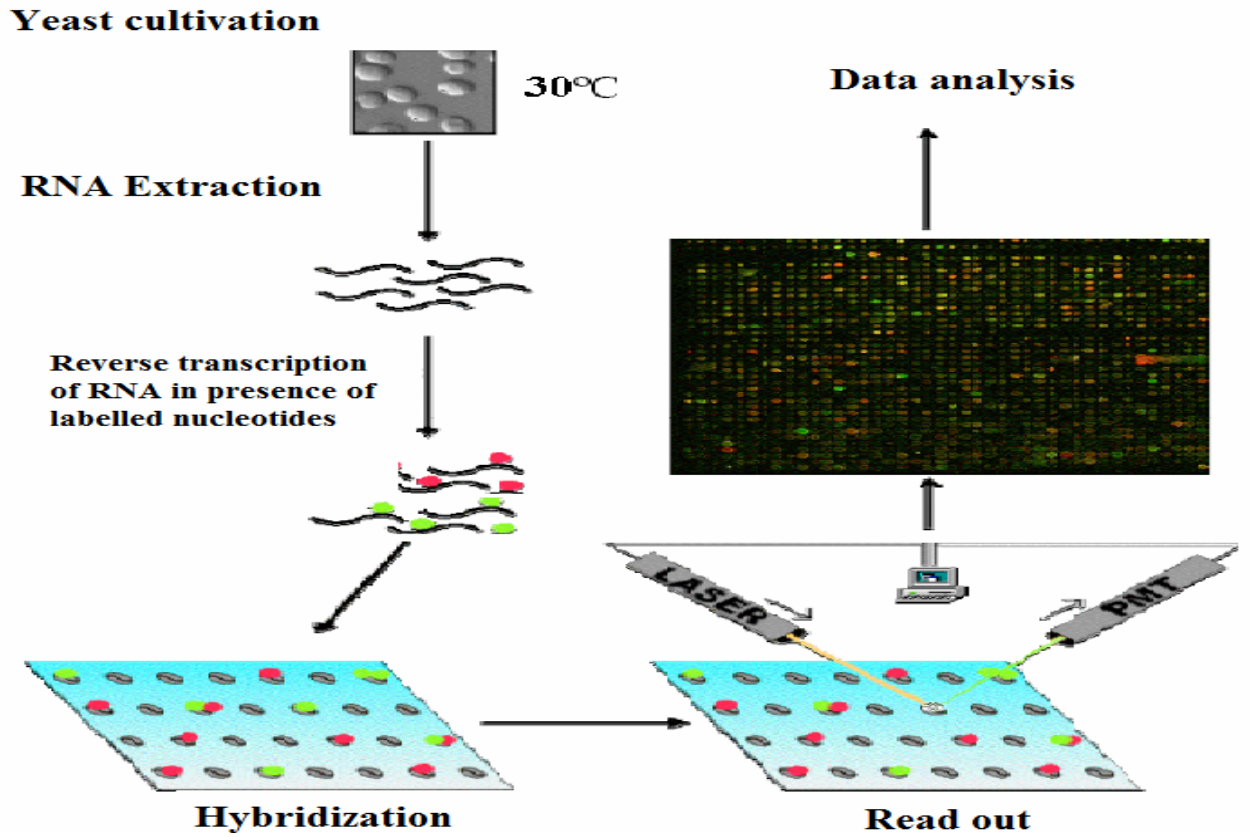


Figure (2.7): DNA microarray technology flow chart, 2 different condition can be tested against each other, like yeast cells expression in G1 against S-phase, taken from (http://web.mit.edu/biology/www/outreach/highschool/pdfs/DNAmicroarray.pdf)

The technological developments in the area of genome science have revolutionized the methods of biological exploration, allowing organisms to be studied on functional genomic scales, characterizing whole genome expression using DNA microarrays provides a snapshot of an organism's genome in action by revealing the relative transcript level of thousands of genes at a time. Exploration of the dynamic nature of genomic expression programs throughout the natural life cycle of cells presents a variety of biological insights, hinting at the underlying cellular processes that mediate or respond to the changes in gene expression.

In a recent study by DeRisi *et al*., 1997, DNA microarrays were used to follow the global changes in gene expression during the natural transition of yeast cells from fermentative to respiration stage, the diauxic shift. Despite decades of study on the metabolic pathways involved, the work demonstrated the potential of genomic expression profiling by revealing many previously unrecognized features of metabolic transition. Nearly 30% of the yeast genome was significantly changed in expression as the available glucose in the medium was depleted, and subsets of genes displayed distinct temporal patterns of transcript variation. DeRisi *et al*., (1997) demonstrated that genes displaying similar expression patterns were functionally related and regulated by upstream sequence motifs common to genes' promoters.

This result established the enormous potential of genomic expression characterization in implicating hypothetical functions for uncharacterized genes, because of their similarity in expression with genes of known functions, while suggesting modes of transcriptional regulation by pointing to factors known to act through conserved promoter elements common to co-regulated genes.

Since then genomic expression studies with microarrays have revealed a wealth of information about yeast biology. Characterization of transcript fluctuation during the yeast cell cycle not only identified 800 cell cycle regulated genes, but also painted a more complete picture of the biological processes that occur during each cell-cycle phase. In addition identification of groups of co-regulated cell-cycle genes led to the subsequent identification of the signalling factors that regulate their expression and the promotor elements through which they execute their control. Investigation of the temporal changes in gene expression during sporulation of diploid cell helped to more accurately define the temporal phases of developmental process, while identifying thousands of genes, many of them completely uncharacterized, that were involved in meiosis. A separate study characterizing genomic expression patterns dependent on ploidy further clarified cellular processes and gene expression patterns that were affected by genomic copy number (Cho *et al*., 1998 and Spellman *et al*., 1998).

The construction of micrroarrays involves four general steps: generation of target DNA or RNA to be spotted, preparation of glass slides, printing of the arrays and processing the final product before use.

2.6.1 cDNA in Microarray

cDNA is a more convenient way to work with the coding sequence than mRNA because RNA is very easily degraded by omnipresent RNases. This is the main reason why cDNA is sequenced rather than mRNA. Likewise, investigators conducting DNA microarrays often convert the total RNA or mRNA into 3´-5´cDNA in order to produce their samples (Berg *et al*., 2006).

By definition, cDNA is double-stranded DNA that was derived from mRNA which can be obtained from prokaryotes or eukaryotes. Once the mRNA is isolated, only a few more reagents are needed, which are dNTPs (dGTP, dCTP, dATP and dTTP), primers, and reverse transcriptase which is a DNA polymerase. The mRNA is mixed with the other reagents and the polymerase is allowed to make a complementary strand of 3´-5 DNA (first strand

synthesis). Next, the mRNA must be removed and the second strand of DNA synthesized. There are many technical details in these steps.

The only issue worth mentioning now is that three different types of primers can be used. If the mRNA has a eukaryotic poly-A 3' tail, then an oligo-dT primer can be used to prime all mRNAs simultaneously. 2) If the target is to produce cDNA from a subset of all mRNA, then a sequence-specific primer could be used that will only bind to one mRNA sequence. 3) If it is wanted to produce pieces of cDNA that were scattered all over the mRNA, then a random primer cocktail is used that would produce cDNA from all mRNAs but the cDNAs would not be full length. The major benefits to random priming are the production of shorter cDNA fragments and increasing the probability that 5' ends of the mRNA would be converted to cDNA. Because reverse transcriptase does not usually reach the 5' end of long mRNAs, random primers can be beneficial (Mueller and Roeder, 2006).

2.6.2 Strategies in the preparation of Microarrays

Microarrays can be produced by spotting DNA or by in situ synthesis of oligonucleotides on a solid substrate. Spotted cDNA arrays are typically produced by depositing PCR amplicons, made from cDNA clones, on modified glass slides. In general, PCR amplicons are several hundred to a few thousands base pairs and one amplicon are used to probe each gene. These arrays can be produced by individual investigators or can be purchased commercially. Production of microarray by in situ synthesis required more sophisticated and costly equipment. One widely used implementation of this technology is the Affymetrix oligonucleotide array (Genechip). Here, photolithography and solid chemistry are used to produce high denistry arrays of 25-mer oligonucleotides.

Various approaches have been used to verify the accuracy of microarray data. Microarray assay technology can be calibrated by spiking known quantities of one or several RNA transcripts into test samples or by using other methods including Northern blotting or quantitative RT-PCR which can verify array measurements. Results from both approach indicated that cDNA arrays and short oligonucleotides arrays quantify gene expression in many cases, but some exceptions have been noted. Some investigators have compared the performance of spotted cDNA arrays and Affymetrix 25-mer arrays across a larger set of genes. Kuo *et al*., (2002) reported that measurements of gene expression by spotted cDNA and Affymetrix 25-mer showed little correlation. Li *et al*., (2002) used both commercial cDNA and Affymetrix GeneChips to analyze gene expression changes induced by tert-

butylhydroxyquinone treatment of human neuroblastoma cells. They conducted that there were very substantial discrepancies between the two array types, and that cDNA arrays often failed to detect the differences in gene expression. Kothapalli *et al.,* (2002) also used both of these arrays types to analyze gene expression in peripheral blood cells from normal and leukemic subject and found substantial discrepancies which have been attributed to several factors including hybridization and misidentification of cDNA probes. There is no clear support for the good agreement between gene expression measurements made with spotted cDNA arrays and in situ–synthesized short oligonucleotide array.

Spotted long oligonucleotides arrays were recently introduced as an alternative to spotted cDNA and in situ synthesized oligonucleotides arrays. Spotted oligonucleotides are produced by deposition of solution containing synthetic oligonucleotides, typically 40-90 bases long, on solid surface. These arrays can be produced and used with methods identical or very similar to those of spotted cDNA arrays. A strong support for the utility of long oligonucleotides probes comes from a study of 60-mer oligonucleotide arrays fabricated by ink-jet oligonucleotide synthesizer (Hughes *et al*., 2001). Results obtained with this type of array correlated with results obtained using yeast cDNA arrays. The overall costs of long oligonucleotides technology will often be lower when labor and other costs associated with obtaining and maintaining cDNA libraries are taken in consideration. Spotted long oligonucleotides arrays can be printed, hybridized and scanned using the same methods and equipment used for spotted cDNA arrays. Barczak *et al.,* (2003) showed a strong correlation between spotted long oligonucleotide array data of human gene expression analysis and in situ-synthesized 25-mer oligonucleotide array data, suggesting that long arrays are a good alternative gene expression analysis platform for many applications.

In general, the hybridization of nucleic acid targets to DNA oligonucleotides arrays depend on a number of parameters. These include: (i) the chemical and physical properties of the support surface; (ii) the nature and length of linkers tethering the oligonucleotides probes to the solid surface; (iii) the attachment density of the probes on the surface; (iv) the length and type of target DNA molecules; (v) the sequence and length of the DNA oligonucleotides; and (vi) the hybridization and washing conditions (Mueller and Roeder, 2006).

2.7 Flow cytometry for cell cycle analysis

Flow cytometry is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus.

A flow cytometry system consists of five main units: a light source (mercury lamp or laser), flow cell, optical filter units for specific wavelength detection over a wide spectral range, photodiodes or photomultiplier tubes for sensitive detection of the signals of interest and a data processing and operating unit (Shapiro, 2003).

A cell suspension is injected into the flow cell where the cells pass one after another across a laser beam. This is achieved by hydrodynamic focusing of the sample stream, wherein the sample stream is injected into the sheath stream inside the flow cell. The velocities of both fluids are in the range of a laminar flow. Since the sheath flow is faster than the sample flow, the sample stream is reduced in its cross-sectional area and therefore the cells are isolated in this focused stream (Rieseberg *et al*., 2001).

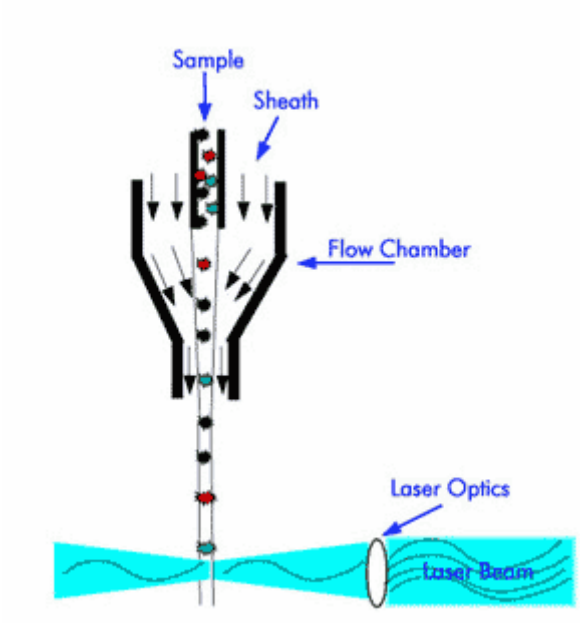


Figure (2.8): Flow cytometers use the principle of hydrodynamic focusing for presenting cells to a laser (taken from biology.berkeley.edu/crl/flow_cytometry_basic.html)

A beam of light (usually laser light) of a single wavelength is directed onto a hydro-dynamically focused stream of fluid. A number of detectors are aimed at the point where the

stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle passing through the beam scatters the light in some way, and fluorescent chemicals, like propidium iodide or sytox green, in the particle may be excited into emitting light at a lower frequency than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to extrapolate various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers on the market have eliminated the need for fluorescence and use only light scatter for the measurement.

Flow cytometers can also be configured as sorting instruments. As cells/particles pass through, they can be selectively charged and on their exit can be deflected into separate paths of flow. It is therefore possible to separate up to 4 defined populations of cells from an original mix with a high degree of accuracy and speed.

In a cell sorter (Figure 2.9), the cells are carried by a thin jet of liquid emanating from a small nozzle orifice. Shortly after the cell leaves the nozzle, it passes through the waist of one or more tightly focused laser beams. The scattered and fluorescence light from these intereactions is collected and analyzed cytometrically.

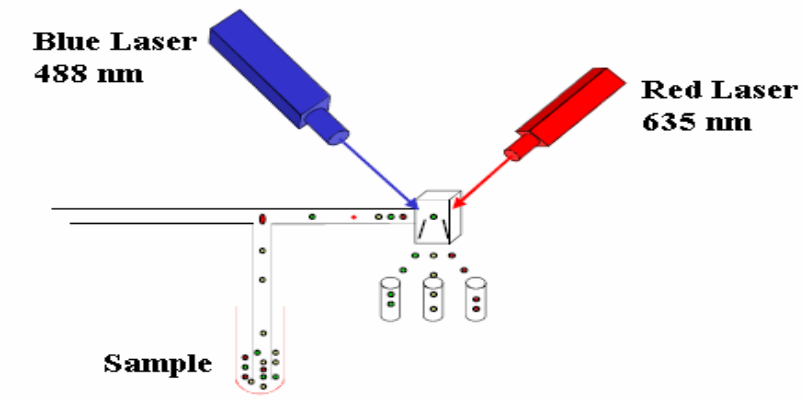


Figure (2.9): Sorting. This figure illustrated the general system for sorting with the flow cytometry (taken from Nunez, 2001).

In order to sort cells, the signals collected for each cell are compared to sorting criteria programmed into the computer. If the criteria are met, an electrical charge is applied to the liquid stream that is being broken up into droplets containing the cells. The charge is applied to the droplet stream at the exact moment that the cell of interest is about to break off from the stream. The charge is removed just when the droplet has broken off from the steam. As the droplets fall, they pass between positively and negatively charged metal plates. Charged droplets get drawn towards the metal plate of the opposite polarity, and can then be deposited in the collection vessel or on a microscope slide. The selected cells can be used for another experiment, or can be grown in cell culture, or can be stained with another fluorescent dye or antibody for reanalysis (Nunez, 2001).

Flow cytometry has proven valuable in the study of the yeast cell cycle. An overview of cell cycle analysis of *S. cervisiae* is given by Dien *et al*., (1994). Cultures of *S. cervisiae* tend to synchronize under special conditions in continuous culture (Scheper *et al*., 1987; Srienc and Dien, 1992) and these systems are ideal for investigating cell cycle and growth behaviour by flow cytometry (Walker, 1999). The changes in the cell cycle can be determined and correlated with other parameters of fermentation to optimize the process strategy. Cell cycle measurements on synchronized cultures have also been performed to study growth kinetics for improved process modelling (Srienc, 1999).

Cell cycle analysis can also be performed on non synchronized cultures. For example a double flow cytometric tag allows the tracking of cell cycle dynamics of newborn *S. cervisiae* cells during balanced exponential growth (Porro *et al*., 1995).

Flow cytometry has also been used to evaluate the total intracellular protein content in yeast populations using fluorescein isothiocyanate (Degelau *et al*., 1992). More interestingly, the the activity of intracellular enzymes was detected using a fluorescently labelled substrate. For example, the activity of ß-galactosidase, an inducible enzyme in yeast, was monitored with fluorescein digalactoside. The fluorescence measured by flow cytometry was proportional to the intracellular enzyme activity (Wittrup and Bailey, 1988).

The brewing industry applied successfully the flow cytometric techniques to monitor the microbiology of the brewing process. Hutter *et al*., 1979 determined the purity of yeast cultures using immunofluorescence and flow cytometry, as well as using antibodies to discriminate the presence of other organisms in fermentations. They also monitored yeast cell cycle in samples to evaluate the state of fermentation (Hutter *et al*., 1996).

3. Materials and Methods

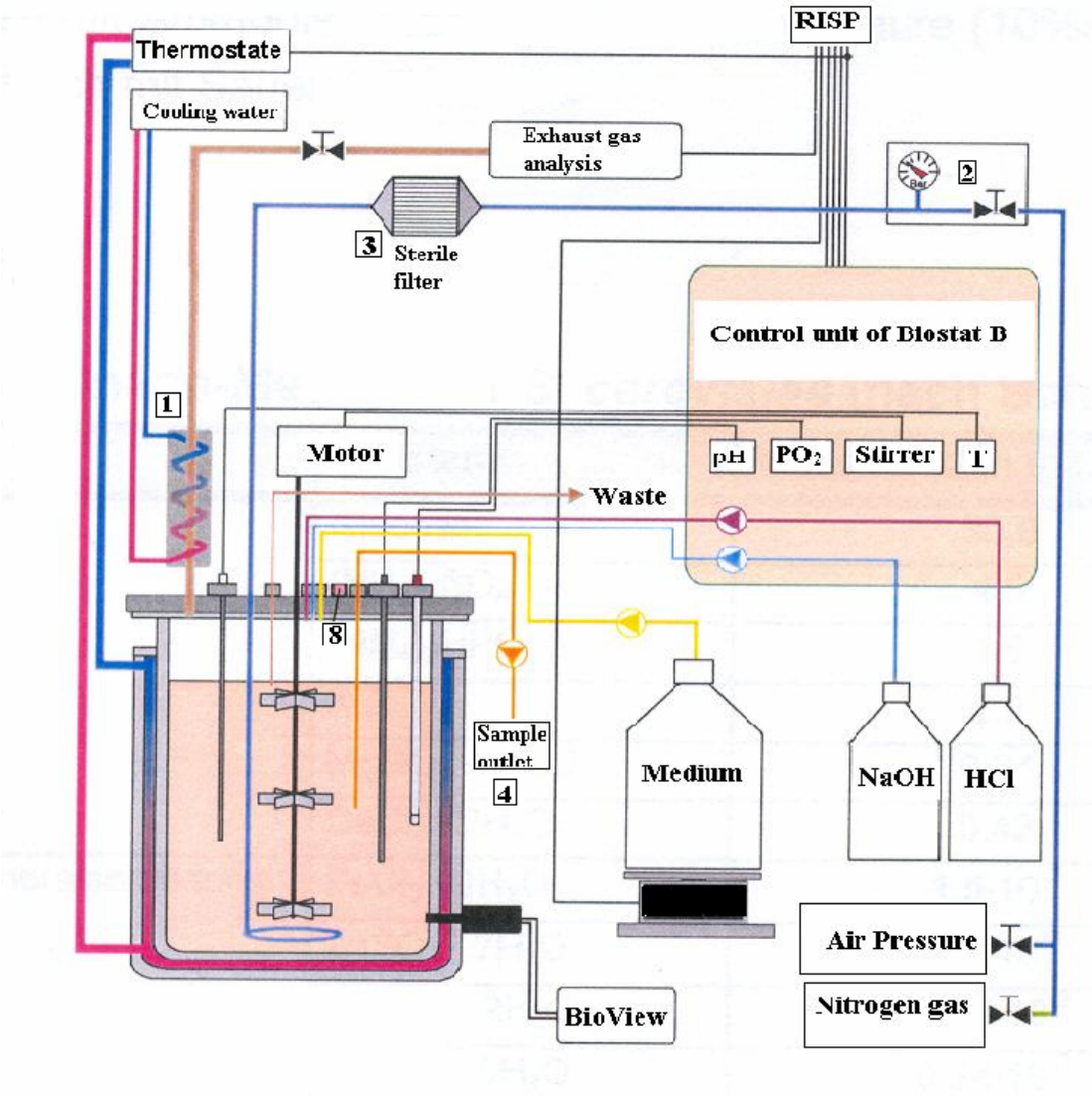
Saccharomyces cerevisiae strain wild type H620, was cultivated in 2 L and 10 L steel tank reactor, Biostat B (Braun Biotech International, Melsungen, Germany), at a temperature of 30 ºC and at pH 5.5 and 4.5 respectively, by using Schatzmann medium supplemented with glucose. During these cultivations, the fluorescence spectra had been collected by BioView spectrometer (Delta Light & Optics, Denmark).

Table 2: Schatzmann-Medium for *S. cerevisiae* continuous cultivation

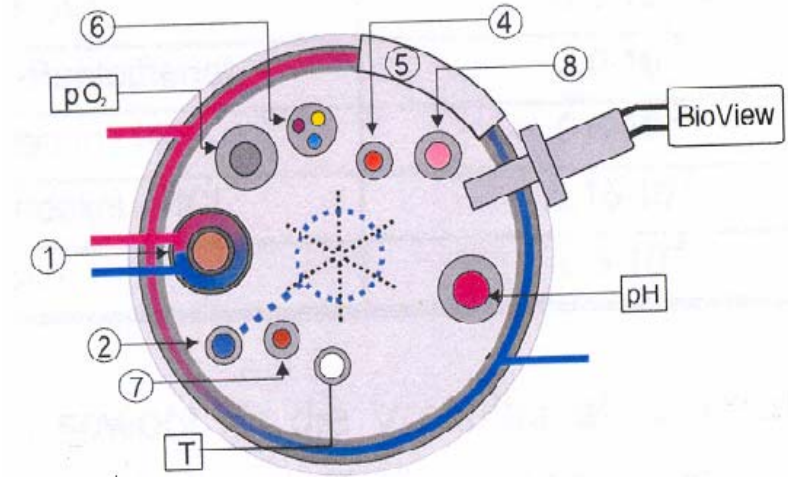
	Substance	Concentration (g/L)
Nutrients	Glucose	30
	$(NH4)_2SO_4$	4.5
	$(NH4)_2HPO_4$	1.9
	KCl	0.9
	$MgSO_4.7H_2O$	0.34
	$CaCl_2.2H_2O$	0.42
Minerals	$FeCl_3.6H_2O$	$1.5x10^{-2}$
	$ZnSO_4.7H_2O$	$0.9x10^{-2}$
	$MnSO_4.2H_2O$	$1.05x10^{-2}$
	$CuSO_4.5H_2O$	$0.24x10^{-2}$
Vitamins	Myo-inositol	$6x10^{-2}$
	Ca-pantothenate	$3\ x10^{-2}$
	Thiamine.HCl	$0.6\ x10^{-2}$
	Pyrodoxine.HCl	$0.15\ x10^{-2}$
	Biotin	$0.3\ x10^{-2}$

3.1.1 Preparation of Inoculum

The microorganism was taken from a sterilized agar slant reserved at 4 ºC. It was cultivated in 50 ml of sterilised Schatzmann medium with addition of sodium citrate in a concentration of 5.8 g/L. This pre-culture was carried out under incubation temperature of 30 ºC on a shaker at 120 rpm for 20 hour before the beginning of the batch cultivation.



A: Side view



B: Overview

Figure (3.1): A Schematic diagram of the bioreactor used for the continuous cultivation of *S. cerevisiae*. 1-Exhaust air cooler , 2- Gassing system, 3- Sterile filter, 4- Sample outlet, 5- Monitoring window 6-Feeding inlet, 7- Waste outlet, and 8- Septum

3.1.2 Cultivation conditions

The 2 L steel tank of the bioreactor was filled with about 1.6 L of Schatzmann medium without addition of glucose or vitamins. The pH electrode was calibrated and installed inside the reactor. The whole system was autoclaved, with separately prepared base (1M NaOH), acid (1%H_2SO_4) and antifoam (1% PEG), for at least 30 min at 121 ºC. The exhaust gas analysis unit, thermostat and the motor were switched on after the sterilization, in the mean time the calibration of the pO_2 electrode was carried out at the same mixing speed. The glucose content of the medium (30 g/L) was prepared and sterilized separately and added shortly before the beginning of the batch cultivation. The minerals and vitamins were prepared as stock solutions and kept at 4 ºC to be added to the medium through the septum with sterilized filters (Minisart high-flow, 0.2 µm, Sartorius, Göttingen). The RISP and Bioview were turned on from the beginning of the batch culture to monitor the cultivation conditions through the obtained data. The Schatzmann medium was prepared for the continuous cultivation during in 20 l autoclaved, sterilized tank. The medium was filled in this tank through two sterile filters (Sartopure PP2, 1.2µm and SartobranP, 0.45 + 0.2 µm Sartorius AG, Göttingen). The medium was begun to be transferred to the bioreactor through a peristaltic pump after 18 hour from the beginning of batch cultivation, that to say, substrate feeds for continuous operation were initiated once the cells had reached the late exponential or stationary phase of batch growth. The experiments were always performed by staring at the lowest dilution and subsequently increasing the dilution rate step by step. The whole process of cultivation was optimized to the level of 10 L using the same conditions.

3.1.3 Calculation of oxygen uptake and carbon-dioxide formation rate

Oxygen uptake rate, carbon dioxide production rates, and respiratory quotient during the cultivation were calculated according to the following equations:

Oxygen uptake rate (OUR),

$$OUR = \frac{V_G * P}{R * T * V_L} * \left[x^e{}_{O_2} - \frac{\left(1 - x^e{}_{O_2} - x^e{}_{CO_2}\right)}{\left(1 - x^a{}_{O_2} - x^a{}_{CO_2}\right)} * x^a{}_{O_2} \right]$$

CO_2 production rate (CPR),

$$CPR = \frac{V_G * P}{R * T * V_L} * \left[x^a{}_{CO_2} - \frac{\left(1 - x^e{}_{O_2} - x^e{}_{CO_2}\right)}{\left(1 - x^a{}_{O_2} - x^a{}_{CO_2}\right)} * x^e{}_{CO_2} \right]$$

Respiratory Quotient (RQ).

$$RQ = \frac{CPR}{OUR}$$

With:

V_G : Air flow L h^{-1}

P : Working pressure (1 atm)

R. : Gas constant

T : Absolute temperature, K

V_L : Working volume

$x^e{}_{O_2}$: Mole fraction of oxygen in inlet air

$x^e{}_{CO_2}$: Mole fraction of carbon dioxide in inlet air

$x^a{}_{O_2}$: Mole fraction of oxygen in off gas

$x^a{}_{CO_2}$: Mole fraction of carbon dioxide in off gas

3.1.4 Samples collection and preparation

During the bioreactor continuous cultivation, aliquots of the culture were removed from the vessel, from 2 stable oscillatory cycles, every 15 min. The sample (usually 15 ml) was withdrawn using a syringe acting through the sample outlet of the bioreactor. Samples were collected in sterilized centrifugation tube of 50 ml. (Falcon, USA), centrifuged at 4ºC with 5000 rpm (1550xg) for 5 min. A small fraction of the supernatant was frozen at -80ºC and used for glucose and ethanol determination. The cell pellets were washed twice using double-distilled water (dd-H_2O), with centrifugation again and frozen immediately at -80 until they were used for RNA isolation. 300 µl of the orginal sample were taken and fixed with 700µl absolute ethanol for the further flow cytometric analysis, another 1ml was taken in a preweighed 1.5 ml reaction tube, centrifuged at 10000 rpm and the pellet dried at 40ºC for determination of cell dry weight.

3.2 Flow cytometric analysis of the yeast cell cycle

The use of flow cytometry to monitor the cell cycle position of individual cells in a population has been a standard tool for cell cycle studies in both mammalian cells and yeast. Fluorescence can be measured for cells by flow cytometry following the fixation and staining with a fluorescent dye that binds DNA quantitatively. The two familiar fluorescent dyes for this purpose are propidium iodide and sytox green (Haase and Reed, 2002).

Propidium iodide (PI) binds to DNA by intercalating between bases with little or no sequence preference and distributes within a range of one dye per 4 – 5 base pairs of DNA. PI also binds with RNA, necessitating treatment with nuclease to distinguish between RNA and DNA staining. Once the dye is bound to nucleic acid, its fluorescence is enhanced 20 to 30 fold and the absorption maximum for PI is 535 but the fluorescence emission maximum is 627 nm. PI can be excited with xenon or mercury-arc lamp or the 488 line of an argon laser. Generally, PI fluorescence is detected in the FL2 channel of flow cytometers (Figure 3.2).

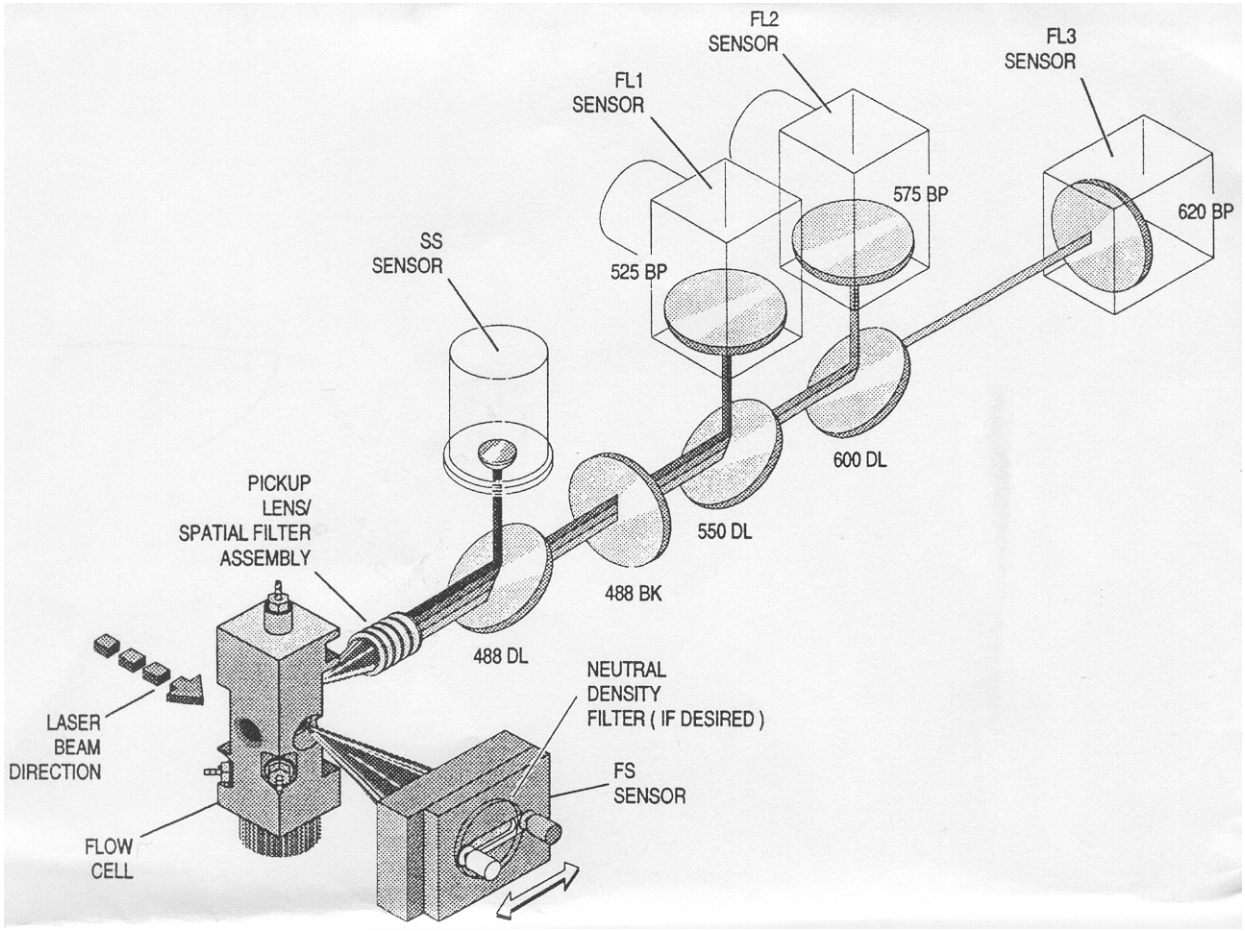


Figure (3.2): A schematic construction of filters and sensors of fluorescence flow cytometry (Coulter, 1998)

Sytox green is also an excellent nucleic acid green fluorescent counter stain and has a high affinity for DNA. It is an effective alternative method for measuring cell viability and antibiotic susceptibility. Sytox green does not cross the membrane of living cells and easily penetrate cells with compromised plasma membrane. This dye is also excited by the 488 nm argon ion laser line commonly used with the flow cytometers. It exhibits an absorption maximum of 504 nm and emission maximum of 523 nm.

3.2.1 Sample preparation for flow cytometric analysis

Samples were taken from fermenter cultures (300 µl; around 1 *10^8 cells) and fixed by adding 700 µl of absolute ethanol and stored at -20 ºC. Before staining for flow cytometric analysis, 50 µl (containing; around 1*10^7cells) of the fixed sample were washed twice in PBS, centrifuged at 10000 rpm, and resuspended in PBS. Samples were then treated with 100 µl RNase (2 mg/ml in PBS) and incubated overnight at 37 ºC in a thermomixer with agitation of 800 min^{-1}. The samples were centrifuged again and washed twice with PBS. The pellets were resuspended in 900 µl PBS and added to it either 100 µl of sytox green solution (5 µM) or in case of using PI, the pellets were dissolved in 1 ml of PI solution (50 µg/ml in 0.9% NaCl). The incubation was carried out for 15 min at 37 ºC in a thermomixer with agitation of 800 min^{-1}. Samples were washed and centrifuged again with PBS at 1000 rpm and finally resuspended in 1 ml PBS to be measured by the flow cytometry to determine percentage of number of cells in each phase of the cell cycle. The particles of each sample were focused hydrodynamically by the sheath flow stream and were excited by an argon ion laser beam (488 nm, 15mW) of the light source in EPICS XL –MCL flow cytometry. Measurement rate was of about 1000 cells per minute.

3.3 Purification of total RNA from yeast

One of the most difficult steps in molecular biology is the preparation of intact RNA. Because degrading RNAses are very common in every tissue and on every surface, much care must be taken to inactivate them and avoid contamination. Disposable plastic ware must be used in every step possible and the wearing of gloves to reduce the likelihood of introducing RNase from our own bodies. Frequent changing of gloves will also reduce the possibilities of contamination.

The samples which were collected from the yeast cultivation and stored at -80ºC were used in this purification. The isolation of RNA from *S. cerevisiae* was carried out using 2 different protocols.

3.3.1 Isolation of total RNA from yeast using hot phenol method

The yeast pellet was thawed by adding 600 µl of sterile ddH_2O and 70µl of 10x high salt solution (3M NaCl, 100mM EDTA and 200 mM Tris, pH 8). The thawed yeast pellet was transferred to a microcentrifuge tube and 30 µl of 10% SDS were added with using a vortex for 15 sec. 600 µl of hot phenol (65ºC) were added with vortexing for 30 sec and incubation time of 4 min at 65ºC and then cooled on ice for 20 min. The samples were centrifuged in a microcentrifuge for 2 min at maximum speed. The aqueous phase (upper phase) was transferred to a fresh microcentrifuge tube. The hot phenol was added again to the aqueous phase and the above steps were repeated again. After that, 600 µl of PCIA (25:24:1 phenol: chloroform: isoamyl alcohol) were added to the aqueous phase with vortexing for 30 sec and centrifugation for 5 min at maximum speed. The aqueous phase was transferred to fresh centrifuge tubes and about 1.5 ml of absolute ethanol were added with optional incubation time of 2 to 12 hours at -20ºC (the RNA yield will be increased with this incubation) and then the centrifugation was carried out for 15 min at 4º C at maximum speed. The supernatant was discarded and the RNA pellet was centrifuged again to bring down any residual ethanol. This pellet was resuspended in about 100 µl of 2 mM EDTA solution and stored at -80 ºC.

3.3.2 Enzymatic lysis protocol for isolation of total RNA from yeast using RNeasy Midi kit

The stored yeast pellets were resuspended in 4 ml of freshly prepared Y1 buffer (1M sorbitol and 0.1M EDTA) containing 100 U/ml lyticase and 0.1% (v/v) ß-mercaptoethanol (ß-ME), with an incubation time of 30 min at 30 ºC with gentle shaking to generate spheroplasts which must be handled gently. The centrifugation was carried out for 5 min at 500 xg to pellet spheroplasts which must be disrupted by adding about 4 ml of buffer RLT and 40 µl ß-ME. The samples were homogenized using a conventional homogenizer for at least 45 sec at maximum speed until the samples were uniformly homogeneous. The yeast lysate was centrifuged for 5 min at 5000 xg. The supernatant was carefully transferred to new 15 ml tube and 4 ml of 70 % ice cold ethanol were added with vigorous shaking. The samples were applied to the RNeasy midi column placed in a 15 ml centrifuge tube and centrifuged for 5 min at 5000 xg, the flow-through was discarded. 4 ml of buffer RW1 were added to the RNeasy column and the centrifugation was carried out for 5 min at 5000 xg to digest any residuals of DNA. 2.5 ml of buffer RPE were added to the RNeasy column and the centrifugation was carried out for 2 min at 5000 xg with discarding the flow-through, this step was repeated twice to dry the RNeasy silica-gel membrane. The RNA was eluted from the membrane with adding 100 µl RNase-free water and centrifugation for 3 min at 5000 xg. The RNA concentration was assessed using the spectrophotometer measurements at 260 nm.

3.3.3 Denaturing agarose gel electrophoresis of RNA

The overall quality of an RNA preparation may be assessed by electrophoresis on a denaturing gel; this will also give some information about RNA yield.

The buffers of agarose gel electrophoresis were:

Reaction denaturating buffer	4µl of 37% Formaldehyde + 10µl of 99.5% Formamide + 2 µl 10x MOPS + 2µl (1mg/ml Ethidium bromide)
Loading buffer (4x)	4 mM EDTA + 20 mM Sodium acetate, pH 7 + 80 mM MOPS + 0.1% Bromophenol blue + 0.1 % Xylene cyanol
MOPS buffer (10x)	0.05M sodium acetate, pH 7 + 0.01M EDTA + 0.2 M MOPS

1.2 g of agarose were added to 65 ml of dd-H_2O and 5 ml of 10x MOPS, and placed in a microwave on high power for 1 min. The molten agarose was cooled and 15 ml of Formaldehyde were added to it. The solution was poured into the gel casting tray and the comb was placed into it. The Agarose solution was allowed to harden at the room temperature for 20 min and covered with 1x MOPS buffer. The assessed RNA samples were prepared by adding 18 µl of reaction denaturing buffer and 1µl of 4x loading buffer, heated to 65 ºC for 5 min and placed immediately on ice. The centrifugation was carried out for the samples at full speed for 5 min before loading it on the wells. The gel was run at 100V for 1.5 hr or until the traking dye migrated about 80% of the distance to the end of the gel. The intact RNA was visualized on the gel under UV illumination as 2 clear bands.

3.3.4 Quality control testing of RNA using Agilent Bioanalyser 2100

The Agilent Bioanalyser is designed for the analysis of total RNA and mRNA samples. Each RNA chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven though it electrophoretically. 12 samples of RNA can be examined together with in one chip for purity with very high accuracy.

3.3.4.1 Preparing the gel and samples

550µl of RNA 600 Nano gel were centrifuged in a spin filter at 1500g for 10 min. 65 µl of the gel were added to 1µl of dye with vortexing and centrifugation at 1300 xg for 10 min at room temperature. The gel-dye mixture was loaded on the chip in certain wells. 5µl of RNA 6000 Nano marker were pipetted in all 12 samples wells and in the well of the ladder. The 12 samples and the ladder were denatured at 70 ºC for 2 min. The ladder and samples were pipetted in their wells on the chip. The whole chip was put horizontally in the adapter with vortexing for 1 min at 2400 xg. The chip was run in the Agilent 2100 bioanalyzer for the RNA analysis and measurement.

3.4 The strategy for the production of DNA Oligonucleotide Microarrays

One aim of this research work is the production of a new microarray chip by self spotting of its oligonucleotides. Therefore a pre-screening experiment using MWG Pan® yeast microarray chips was performed to study the gene expression of the synchronized oscillating yeast. In order to produce this chip, a 5′ amine modified, 70-mer, oligonucleotides were mixed in 96 well PCR plate with Arraylt™ Micro-spotting plus buffer (TeleChem International, Sunnyvale, USA) in a ratio of 1:1. Each nucleotide was spotted three times on nuclease-free silylated glass slides (CEL Associates, Inc. Pearland, Texas, USA) using Affymetrix 417 Arrayer. The mechanism of reaction between oligonucleotides and glass slide is shown in Figure (3.3).

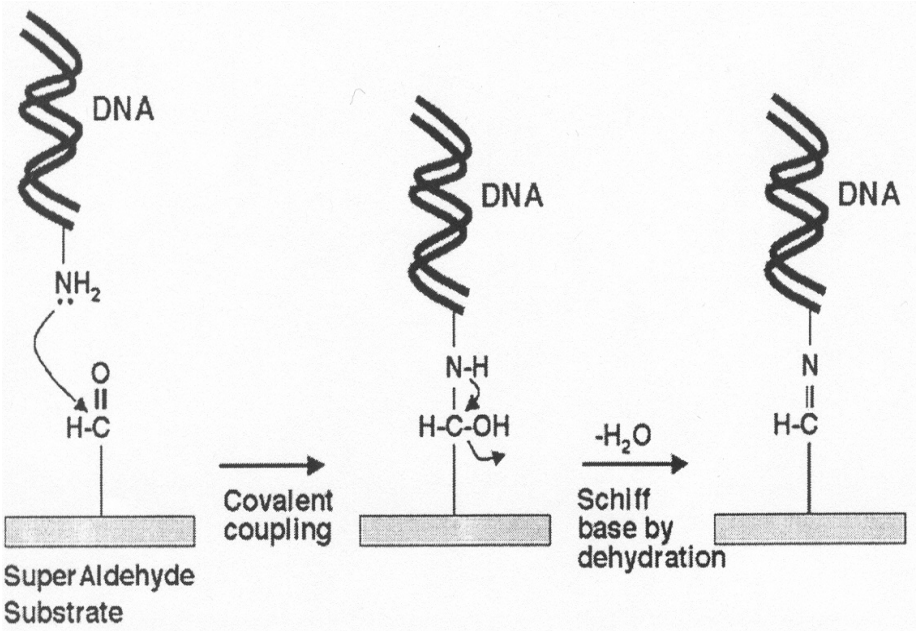


Figure (3.3): Aldehyde coupling chemistry. SuperAldehyde substrates contain aldehyde groups attached covalently to glass surface. Primary amino linkers on the oligonucleotides attach to the aldehyde groups (left panel) forming covalent bonds (center panel). Attachment is stabilized by a dehydration reaction which leads to Schiff base formation (right panel), this provides highly stable attachment of DNA for gene expression and genotyping applications (taken from Microarrays, Röder and Müller, 2006).

The spotted microarray slides were placed under UV-light (254 nm) for 3 min, dried at 80 ºC for 2 h and left in dark on room temperature for about 24 h. On the next day the surface of the slides had been reduced and blocked. The slides were rinsed with 0.2 % SDS for 2 min and then two times in dd-H_2O. The reduction was carried out for 5 min using sodium borohydride reducing solution (1g NaBH4 + 300ml PBS + 100ml absolute ethanol). The

slides were rinsed for 1 min in cold dd-H_2O, 1 min in 0.2 % SDS and finally two times in dd-H_2O for 1 min. The blocking was carried out for 45 min at 42 ºC with (6x SSC + 0.1% SDS + 1% BSA). The slides were washed 5 times, for 1 min each, with dd-H_2O. The chips were dried by centrifugation for 2 min at 1200 rpm. These microarray slides were ready for hybridization and could be stored in dark at room temperature.

3.4.1 cDNA synthesis and direct labelling using LabelStar kit for Microarray experiments

In order to prepare cDNA for a chip experiment, 2 different samples of RNA, one from G1-phase and another from S-phase, were used to observe the different expression of genes within the two phases, each sample must be labelled with a different dye (cyanine 3 or cyanine 5). The concentration of RNA was adjusted to 100 µg with RNase-free H_2O in a volume of 18 µl and 2 µl of Denaturation solution were added with mixing, short centrifugation and then incubation at 65ºC for 5 min and cooling on ice immediately. For each reaction a fresh master mix must be added to complete the reaction volume to 50µl.

The master mix should be freshly prepared as follow:

5 µl 10x Buffer RT,

5 µl dNTPs mix,

1 µl Cyanine 3 or Cyanine 5-labelled dCTP (25 mM),

5 µl oligo-dt primer (100µM) ,

0.5 µl RNase inhibitor (40 U/µl),

11 µl RNase-free water and

2.5 µl LabelStar Reverse Transcriptase (200 U / µl).

After adding this master mix, the reaction mixture was incubated at 37 ºC for 2 h and then stopped by adding 2 µl of stop solution LS. The alkaline hydrolysis of the template RNA was carried out with addition of 10 µl of 1M NaOH and incubation at 65ºC for 10 min. After this step, the reaction mixture was cooled on ice and neutralized with 10 µl of 1M HCl.

3.4.2 Purification of cDNA

The purification step was performed using PCR purification kit of Qiagen. The reaction mixture was mixed with 300 µl of Buffer PB and placed on a spin purification column with a centrifugation for 1min at maximum speed. The flow-through was discarded and 700 µl of 35% guanidine hydrochloride were added to the column, with centrifugation at maximum speed for 1 min. 700 µl of Buffer PE were added to the column with centrifugation

for 1 min. at maximum speed. The flow-through was discarded and the column was centrifuged at maximum speed to be dried from any buffer. The purified cDNA will be eluted from the column with 2 times washing with 25 µl Buffer EB and 1 min centrifugation at full speed after each wash.

3.4.3 Hybridization of cDNA to the chip

The eluted cDNA was lyophilized with speed vacuum centrifugation for 1 h, till the cDNA pellet was formed. This pellet was dissolved in 36 µl hybridizing buffer, and denatured at 95 ºC for 3 min and cooled on ice immediately for 1 min. 4 µl of the top block buffer were added to the sample before loading it on the chip. The chip was covered with a thin slip cover which was fixed on it with fixogum. The chip was placed on moist towel paper in a lidded plastic box and incubated over night at 42 ºC. After the hybridization, the chip was washed with washing buffer 1 (2x SSC, 0.01% SDS) for 5 min, washing buffer 2 (1x SSC) for 5 min and finally with washing buffer 3 (0.5x SSC). The chip was dried with centrifugation for 2 min at 1200 rpm and scanned by the Affymetrix Array scanner 428 ™.

3.5 cDNA preparation, signal amplification and detection using Micromax TSA™ labeling kit

The Micromax cDNA microarray system utilizes tyramide signal amplification (TSA), which requires 20-100 times less RNA than direct cDNA labelling (Karsten et al., 2002). This indirect labelling using TSA employs antibody-antigen binding and enzymatic reactions to label array spots of target-probe hybrids (oligonucleotides – cDNA hybrids). Labeling with TSA labels oligonucleotides-cDNA hybrid and therefore labelling event takes place after hybridization. In addition, TSA generates reactive fluorescent dyes which then couples with the array surface. Two components are critical to this assay: hapten (usually biotin or fluorescein) modified nucleotides and horseradish peroxidase (HRP) conjugated antibody (streptavidin). cDNA are incorporated with hapten modified nucleotides by reverse transcription of total RNA and then hybridized on the array. After hybridization, the array is incubated with HRP conjugated antibodies, which bind to haptens on the target-probe hybrids. The binding of antigen and antibody brings HRP close to the array surface. The array is then incubated with hydrogen peroxide (H_2O_2) which is used by HRP to oxidize tyramide linked fluorescent reagents as Cy5-tyramide. Oxidized tyramide flurorescent reagents are highly reactive and can rapidly attach to the array surface. Therefore, only at the spots where the hybridization between oligonucleotides and cDNA takes place, fluorescent dyes will be

incorporated (Ritcher et al., 2002). As shown in Figure (3.4), hybridized biotin or fluorescein labelled targets are sequentially reacted with HRP and cy3-tyramide or cy5-tyramide, resulting in numerous depositions of these flurophors on the array (Karsten *et al*., 2002).

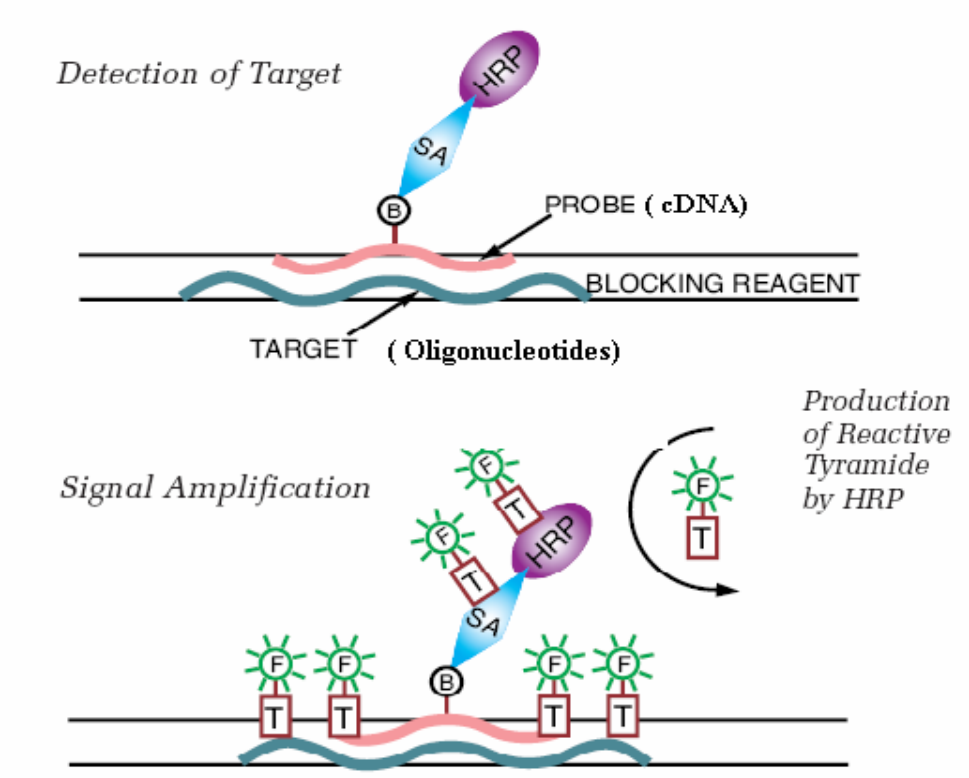


Figure (3.4): Tyramide signal amplification: The mechanism is based on the enzymatic activity of HRP which activates tyramide conjugates.
Key: B = biotin, F= flurophore (cyanine 3 or cyanine 5), T = Tyramide, SA = streptavidin, and HRP = Horseradish peroxidase , taken from (www.perkinelmer.com).

TSA approach amplifies fluorescent signal rather than the target. The advantage of using TSA compared to using reverse transcription method is that it can provide hundred fold signal amplification and therefore minimizing the usage of big amount of RNA samples. One drawback of this protocol is that it is somehow time consuming specially in washing steps compared to other methods (Schena, 2003).

3.5.1 cDNA synthesis

X µl (around 6 µg) of RNA, 1 µl dNTPs, 1 µl Biotin or Fluorescein nucleotide, 1µl of 100 µM oligo-dT, and 1µl of 100 µM random hexamer primer were mixed together with completing the whole volume to 20µl with RNase-free water (DEPC-H_2O). The incubation was carried out for 10 min at 65 ºC , followed by cooling for 5 min at 25 ºC and warming at 42 ºC for 3 min. 2.5 µl of 10x RT buffer and 2.5 RT/RNase inhibitor mix were added to the above mixture with incubating at 42ºC for 2h and then cooling on ice for 5 min. 2.5 µl of 0.5

M EDTA, pH 8 (to stop the synthesis of cDNA) and 2.5 µl of 1 N NaOH (to initiate the hydrolysis of RNA template) were added with incubating at 65 ºC for 30 min and cooling on ice for 5 min. In order to neutralize the reaction, 6.5 µl of 1 M Tris-HCl, pH 7.5 were added. The purification of the cDNA was carried out exactly as stated in the previous technique using QIAquick PCR purification kit. The hybridization of cDNA on the chip was also performed as in the previous technique with incubation at 42 ºC overnight.

3.5.2 Washing and signal detection of the chip

The washing and TSA detection steps were all carried out at the room temperature. The chip was washed with washing buffer 1 (0.5 X SSC, 0.01 % SDS) for 5 min, washing buffer 2 (0.06 X SSC, 0.01% SDS)) for 5 min and washing buffer 3 (0.06 X SSC) for 2 min. The edges of the chip were determined by ImmEdge^{TM} Pen to prevent the leakage of buffers from the glass surface. TNB buffer (0.1 M Tris-HCl, 0.15 M NaCl and 0.5 g blocking reagent per 100ml dd-H_2O) was prepared by heating gradually to 60 ºC with continuous stirring to completely dissolve the blocking reagent, cooled at room temperature and stored at -20 ºC for long term storage. The chip was blocked with about 300 µl TNB containing 10% Goat serum buffer (4.5 ml TNB + 0.5 ml goat serum) for 10 min and rinsed with TNT buffer (0.1M Tris-HCl, pH 7, 0.15 M NaCl and 0.05%Tween-20) for 1 min. The chip was incubated with 200 µl of Anti-Fl-HRP conjugate solution (2 µl Anti- Fl-HRP conjugate + 200 µl TNB-10% goat serum buffer) for 10 min, followed by 3 times TNT washing. The first labelling step was with 250µl cyanine-3-tyramide solution (0.5µl of cyanine-3-tyramide diluted in 20µl DMSO + 250 µl amplification diluent) for 10 min and followed by washing 3 times with TNT, 5 min each. The inactivation of chip was with 300 µl HRP inactivation solution (10µl 3M Na acetate +100µl H_2O_2 + 190µl dd-H_2O) for 10 min and followed by 3 times rinsing with TNT, for 1 min each. The chip was incubated with 200µl of Streptavidin-HRP solution (2 µl Streptavidin-HRP + 200µl TNB-10% Goat serum buffer) and washed 3 times with TNT, 1 min each. The second labelling step was with 250 µl of cyanine-5-tyramide (0.5µl of cyanine-5-tyramide diluted in 20µl DMSO + 250 µl amplification diluent) for 10 min and followed by washing 3 times, for 5 min each, with TNT. The chip was fnally rinsed for 1 min with 0.06x SSC washing buffer, dried by centrifugation for 2 min at 1200 rpm and scanned with the array scanner at 2 different wave lengths to measure cy3 spots by green laser at 532 nm and cy5 spots by red laser at 635 nm excitation. .

4. Results and Discussion

The budding yeast *Saccharomyces cerevisiae* exhibit autonomous oscillation when grown aerobically in continuous culture with glucose as the primary carbon source under certain environmental conditions. The oscillations are autonomous in the sense that external force is not required to maintain cycle's dynamics (Henson, 2004). Three distinct types of yeast oscillations have been reported: glycolytic oscillations (Aon *et al.*, 1992 and Ghosh *et al*., 1971), respiratory oscillations (kreulers *et al*., 1996) and cell cycle related oscillations (von Meyenburg, 1973 and Parulekar *et al*., 1986). Improved understanding of the cellular mechanisms involved could clarify the picture of dynamic regulation of metabolism in yeast cell cycle and in eukaryotic cells present in higher organisms.

Single cell oscillation must be synchronized to be observable at the population level. A perfectly synchronized population is an idealized concept in which every cell oscillates with exactly the same phase and amplitude. Actual experiments yield partially synchronized populations comprised of oscillating cells.

When *S. cerevisiae* is grown aerobically in a chemostat under glucose limitation, a strictly respiratory metabolism is obtained at very low dilution rates. However, as a dilution rates increased above certain critical value, fermentation in addition to respiration becomes apparent, a behaviour which has been attributed to the limited respiratory capacity of *S. cerevisiae*.

4.1 Continuous cultivation of *Saccharomyces cerevisiae* in 2 liter chemostat bioreactor

The yeast bioreactor was operated, as shown in Figure (4.1), at a constant aeration rate of 2.85 L/min and agitation rate of 1200 rpm. The pH was maintained at a value of 5.5 by addition of 2M NaOH and the temperature was controlled at 30 ºC. The cells were grown on Schatzmann-medium, which was added to the reactor with a dilution rate of 0.14 h^{-1}. The working volume was around 1.8 L during the whole cultivation. Glucose medium was supplemented with 30 g/L glucose monohydrate and 1 ml/L Antifoam. Dissolved oxygen levels and carbon dioxide production rate were measured through RISP every 10 sec. The Bioview® was connected to the bioreactor to detect every 3 min the intracellular fluorescence of NADH, flavines and proteins.

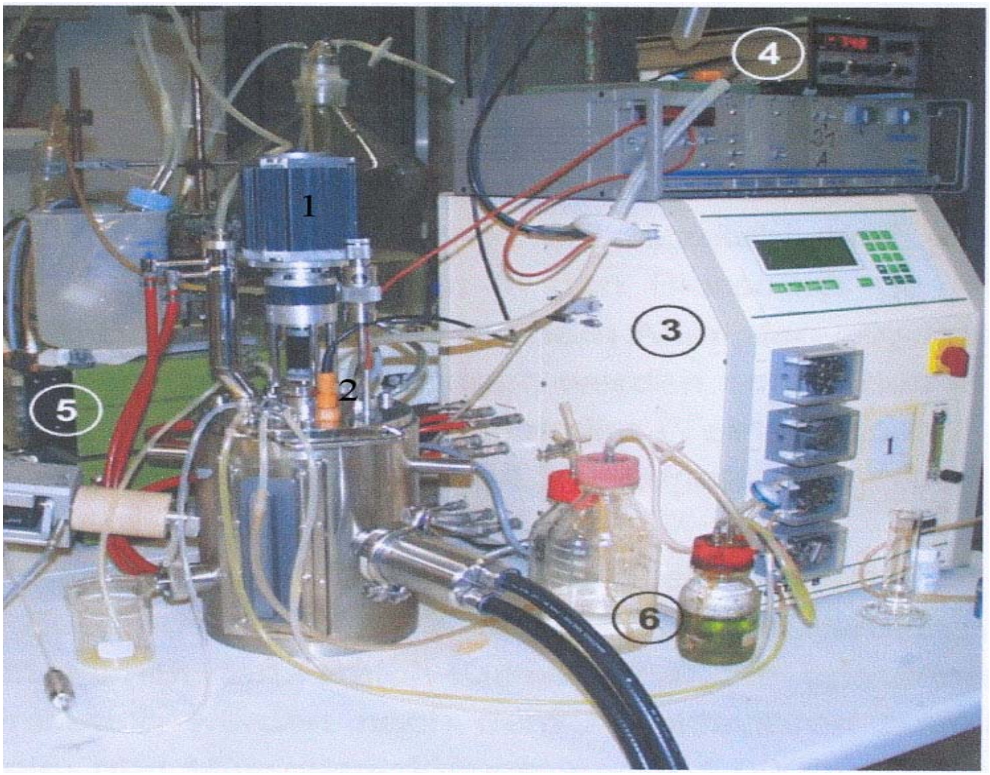


Figure (4.1): A photo, showing the construction of the 2 L bioreactor used for the continuous cultivation of *Saccharomyces cerevisiae* showing; 1: Motor, 2: pH-electrode, 3: Biostat B control unit, 4: Gassing system, 5: Peristaltic pump for the feeding medium, & 6: Acid and base

4.1.1 The relation between carbon dioxide production rate, dissolved oxygen and biomass during oscillations in 2 L continuous culture of *S. cerevisiae*

The samples were collected every 15 min., for 5 h, from 2 consecutive cycles. 1ml of each sample was taken and centrifuged at 10000 rpm for 1 min and the pellet was dried at 40 ºC over night for determination of biomass (cell dry weight). As shown in Figure (4.2), the biomass concentration varies between the value of 12.3 and 13.5 g/L. The base line for exhaust CO_2 % was approximately constant at 0.8 % for about 1.3 h and then it increased quickly through few minutes to a value of about 2.2 % , flowed by a decrease to a value of 1,5% which was in plateau form for about 1 hour and then decreased again to its base line value. The dissolved oxygen was in range between 60% and 80%. Studies with partly synchronized continuous cultures have shown significant changes in CO_2 production and % of dissolved oxygen inside the reactor during the cell cycle (Oehlen, 1992). There was a clear inverse relationship between dissolved oxygen percentage and carbon dioxide production rate as it was shown in Figure (4.2), where the dissolved oxygen % was at its maximum values in the mean time of the exhaust CO_2 % minimum values. As carbon dioxide production decreased to its minimum values at 190 h and 193 h in both cycles respectively, It was an

indicator for the low metabolic activity at these points where the cells might be in G2/M phase in which the division process between parent cell and daughter cell took place which in turn lead to cells with lower biomass of about 12.3 g/L and 12.7 respectively at the same time.

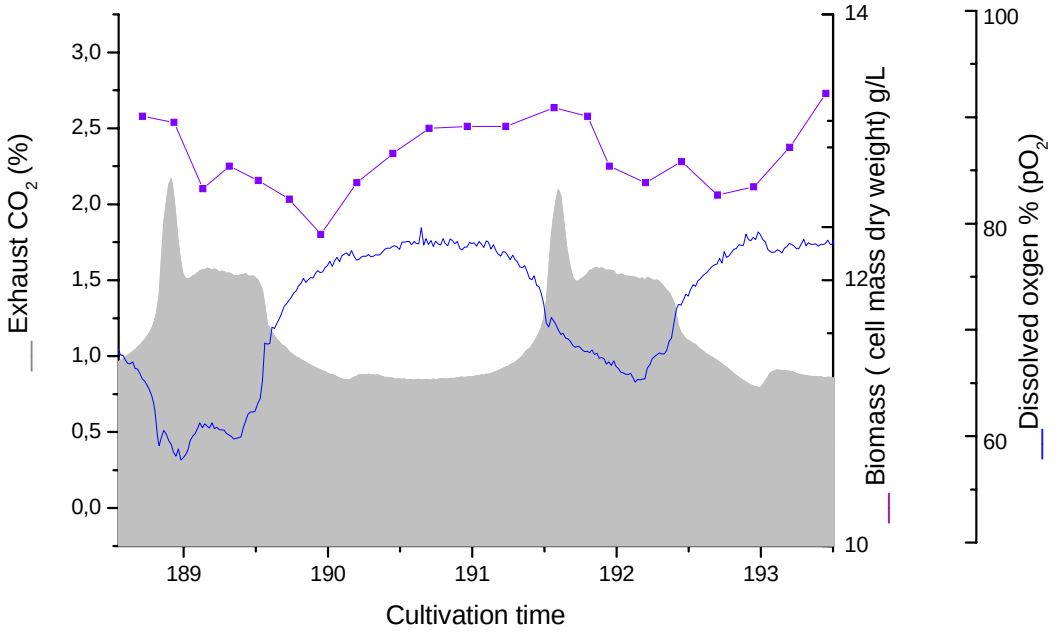


Figure (4.2): Oscillation of carbon dioxide production rate, dissolved oxygen and biomass in 2 L culture of synchronised yeast inside a chemostat at D= 0.14 h^{-1}, pH = 5.5 and T = 30

This was ascertained with the flow cytometric analysis which indicated that the highest percent of G2/M cells at these points. After the mitotic division, the cells began to grow and in turn the metabolic activity increased, and by the way, the carbon dioxide production rose up to its highest value in the mean time of the maximum value of biomass which occurred during G1-phase, since the cells should grow well before the replication process of DNA in S-phase. It can be concluded that the rising of biomass concentration can be a good indicator for the synchronization of cells in G1-phase during the same time.

4.1.2 Online monitoring of NADH, protein and flavines oscillations using BioView®-Sensor (robotic fluorescence spectrometry) in 2 L continuous culture of *S. cerevisiae*

Fluorescence signals consistent with nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), tryptophan and tyrosine were measured to monitor yeast cellular activity and protein content. The measurement of NADH-dependent culture fluorescence was an important tool for process monitoring and modelling of biological parameters (Scheper and Schügerl, 1986). NADH and FAD are key fluorophores that

correlate specifically with cellular activity. Although NADH and NADPH are distributed thoughout the cytosol, studies have shown that the predominant fluorescence signal originates from mitochondria consistent with NADH fluorescence. In this research work, it was referred to NAD(P)H fluorescence as NADH fluorescence, attributed mainly to mitochondrial NADH concentrations.

NADH and FAD are metabolic factors that act as electron donors and acceptors in electron transport chain of mitochondria. When oxidized NADH and $FADH_2$ (reduced FAD) release electrons, and then NAD^+ and FAD return to their reduced forms by accepting electrons by the way of citric acid cycle. Of particular interest is the reduced NADH, which exhibits strong fluorescence with maximum at 350 nm excitation and 430 nm emission, whereas the oxidized form, NAD+, does not elicit fluorescence. Conversely, the oxidized FAD fluoresces at maximum 450 nm excitation and 535 nm emission, whereas $FADH_2$ fluorescence is minimal. It has been shown that flavine fluorescence was predominantly attributed to electron transfer flavoprotein and lipoamide dehydrogenase, a molecule that associated with FAD (Berg *et al*., 2007).

Among aromatic amino acids, tryptophan, in addition to tyrosine, contributed to the most observed intrinsic fluorescence signal. It can be hypothesized that cellular protein production can be estimated with tryptophan-related fluorescence signals (Kirkpatrick *et al*., 2005).

In this study, fluorescence spectra resulting from the combination of tryptophan, NADH and FAD were analyzed as shown in Figure (4.3). The NADH dependent fluorescence was oscillated continuously during the cultivation. The NADH dependent fluorescence began to increase during G1 phase and reached its maximum during the S-phase which is attributed to the increase of the cellular metabolic activity. The oscillation peaks of NADH dependent fluorescence was more indicative in its oscillation behaviour than those of protein or flavine during this cultivation, although the protein content oscillation was also clear as observed in Figure (4.3).

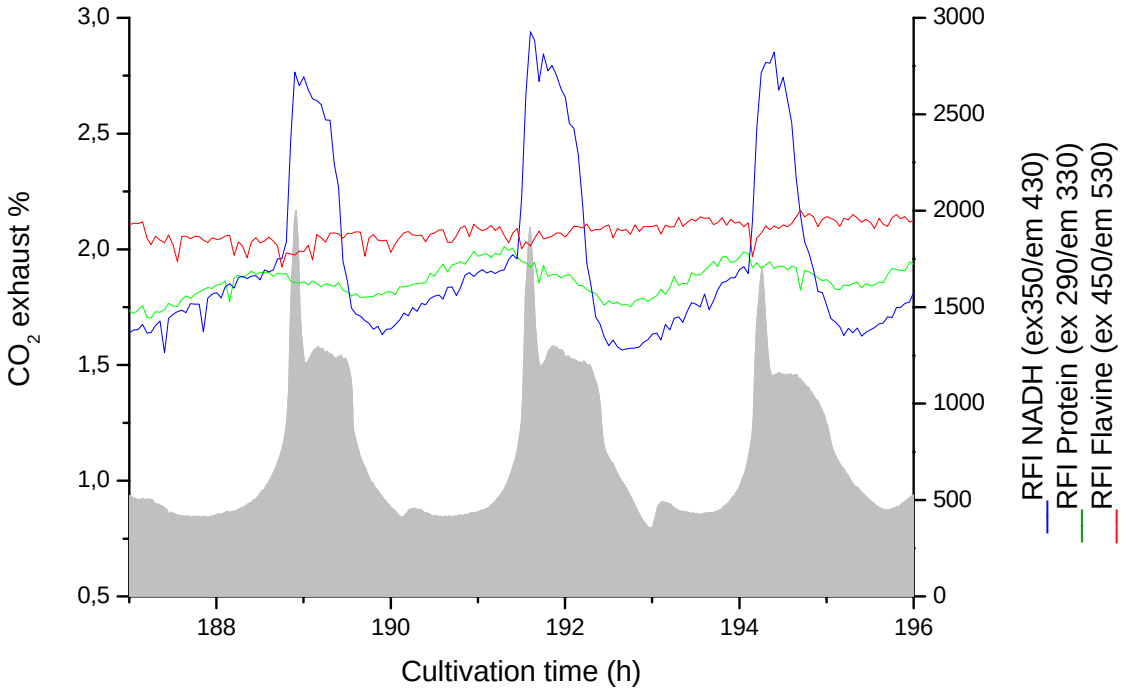


Figure (4.3): Online Bioview-Sensor data for monitoring the relative fluorescence intensity of NADH (ex350/em430), protein (ex 290/em330) and flavin (ex 450/em 530)

4.1.3 Respiratory variables during oscillating chemostat culture of *S.cerevisiae* in 2 L bioreactor

A close inspection of profiles of carbon dioxide production rate CPR, oxygen uptake rate OUR and respiratory quotient RQ revealed clearly the continuous oscillatory behaviour of the yeast during the cultivation. The maxima in CPR, RQ, and % of carbon dioxide in exhaust gas were reached at the same time and in the same phase, whereas the oscillations in dissolved oxygen and OUR were out of this phase as it was clearly shown in figure (4.4). The RQ values were between 0.8 and 1.6. Satroudinov *et al.*, (1992) stated that glucose is oxidatively metabolised during G1-phase and RQ values were less than 1 during this phase. At the beginning of budding process during S-phase, RQ values increased to greater than one and then decreased again to less than one at the end of this phase (Bellgardt, 1994 and Beuse *et al*., 1998). This indicated that the cells directed its metabolism for a short time to the oxido-reductive pathway during the budding process.

The oxygen uptake rate (OUR) should always linearly correlate with the specific glucose uptake rate under oxidative metabolism only. Under oxido-reductive condition of glucose where ethanol is produced by the cells, the oxygen uptake rate must remain constant

at its maximum values (Sonnleitner and Käppeli, 1985). The results of OUR curve during this cultivation were in agreement with these observations as illustrated in Figure (4.4).

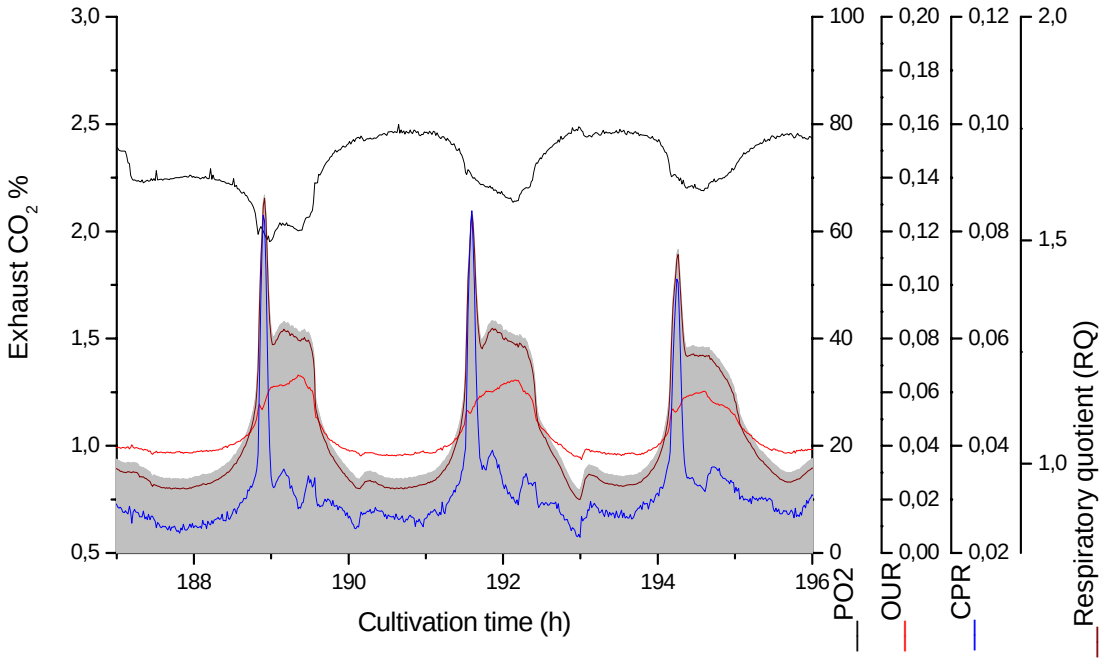


Figure (4.4): Profile of respiratory variables during oscillating chemostat culture of *S.cerevisiae* in Schatzmann medium with D = 0.14, pH = 5.5 and T = 30

4.1.4 The flow cytometric analysis of different populations of synchronized yeast cell cycle in the 2 L chemostat continuous culture

The flow cytometric cell cycle analysis was carried out for samples collected every 15 min during the stable continuous cultivation process from 188h till 193 h. The samples were taken directly from the bioreactor and fixed with ethanol and then treated with RNAse. Sytox green was used for samples dying before measuring it with flow cytometry to determine the percent of different cell population, G1, S, and G2/M in each sample. The results of cell cycle analysis in combination with carbon dioxide production rate in the exhaust gas were shown in figure (4.5), each cycle was about 3 hours in length.

In G1-phase, which represented the growth phase in which the cells must reach a minimum size before progressing through the rest of the cell cycle, the % of exhaust CO_2 began to increase from its minimum levels at 0.8%, at time 188.5 h to reach its maximum level of 2.2% at 189 h, by the end of this phase and beginning of S-phase.

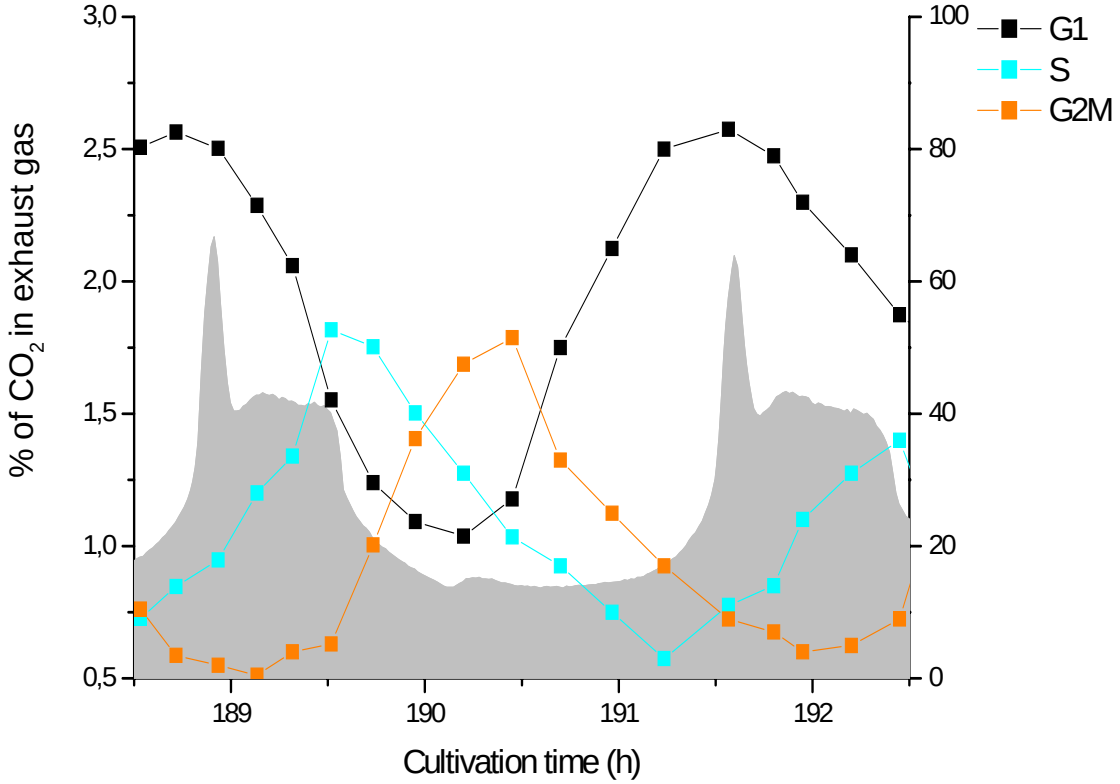


Figure (4.5): Exhaust CO_2 % and distribution of different cell population (G1, S, and G2/M phases) during two periods of oscillation in a chemostat at dilution rate of 0.14, pH = 4.5 and T = 30

During S-phase, where the energy was needed for the synthesis of DNA, CO_2 production rate remained in a high plateau form at about 1.5 % as it was clear in the above Figure (4.5). With the beginning of G2/M-phase, in which the cells division and mitosis process took place, the CO_2 production rate returned again to its minimum level. The results clarified the periodic change of the cell cycle phases in the continuous culture. The synchronization of cells in G1-phase reached its maximum value of 82%, whereas the maximum synchronization percent of cells in S-phase was 55 % and that of G2/M was 45 %.

4.2 The optimization of continuous cultivation of *Saccharomyces cerevisiae* in 10 L chemostat Bioreactor

The optimization process was performed in a 10 L bioreactor as illustrated in Figure (4.6). The reactor was equipped with standard measurements of pH, dissolved oxygen and temperature. The air flow rate was 2.85 L / min. Temperature and pH were controlled at 30 ± 0.1 and 4.5 ± 0.1, respectively. The medium, in both batch and continuous cultures, was simple as well as the one used by Schatzmann (1975). The glucose concentration was 30 g/ L in the continuous feed supply. The agitation rate was fixed to 800 rpm. The working volume was around 9.5 L during the whole cultivation. Dissolved oxygen level and carbon dioxide production rate were monitored through the RISP every 10 sec. The Bioview® was connected to the reactor to detect the intracellular fluorescence of NADH, flavines and protein.

Figure (4.6): A photo, for the construction of the 10L bioreactor used for the optimization of *Saccharomyces cerevisiae* continuous cultivation a dilution rate of 0.18 h^{-1}, pH 4.5 and temperature of 30ºC

4.2.1 The transition from batch to continuous yeast cultivation in the 10 L bioreactor

Sacchromyces cervisiae exhibited diauxic behaviour in batch culture when supplied with glucose as a sole carbon and energy source. The batch step was characterized with 2 growth phases; each of these phases was distinguished with its peak of carbon dioxide production, in the beginning of the cultivation process as shown in Figure (4.7). During the first phase, yeast consumed only the glucose and attained its highest growth rate. After this growth phase, in which all the available glucose depleted, a second exponential growth phase began as clear in Figure (4.7) with its characteristic peak which was lower than the first peak. Ethanol was consumed as an energy source for the growth during this phase (Locher *et al*., 1993).

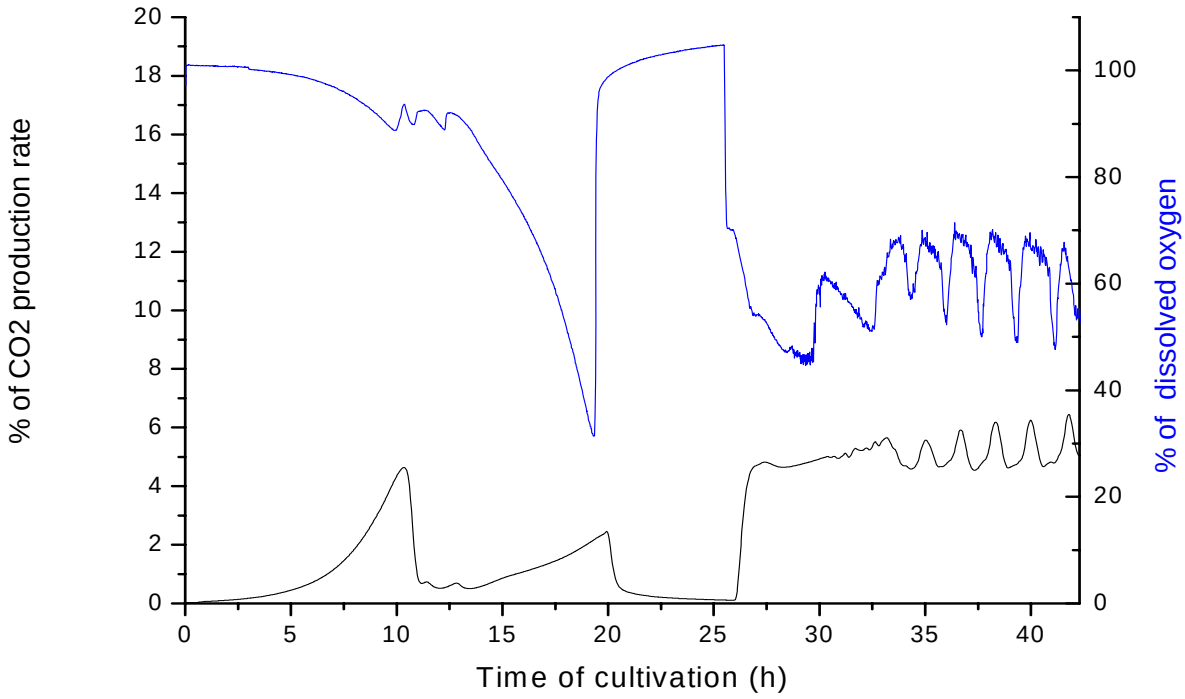


Figure (4.7): Induction of oscillations with transition from batch to continuous cultivation. The continuous process is started with D = 0.04 h^{-1} after an initial batch phase.

Lievense (1984) conducted several experiments in which oscillation were either induced or dampened by a step change in the dilution. These experiments showed that oscillatory behaviour is possible only in the oxidative region. This constraint has also been observed by von Meyenburg (1969). Figure (4.7) shows the transition from batch mode to continuous mode was made at 25 hour just after a sharp drop in CO_2 production, which indicated that exponential growth phase on ethanol had ended. It was necessary to have a

sufficiently low glucose concentration to initiate oscillations in continuous culture of *S. cerevisiae*. A starvation time of 1.5 hour was necessary to ensure that substrates and internal storage carbohydrates were totally consumed.

The feed was initiated with dilution rate of 0.04 h^{-1}. The oscillations were induced in this continuous culture using 2 step changes in dilution rate. The dilution rate was about 0.04 h^{-1} for 6 hours following the starvation phase and with raising it to 0.09 h^{-1} at 31 h from beginning of the cultivation, stable oscillations with in a period of two hours were observed. After 195 h from the beginning of the cultivation, the dilution rate was increased to 0.18 h^{-1}, where the oscillation became very stable and remained till the end of the cultivation process, Figure (4.8).

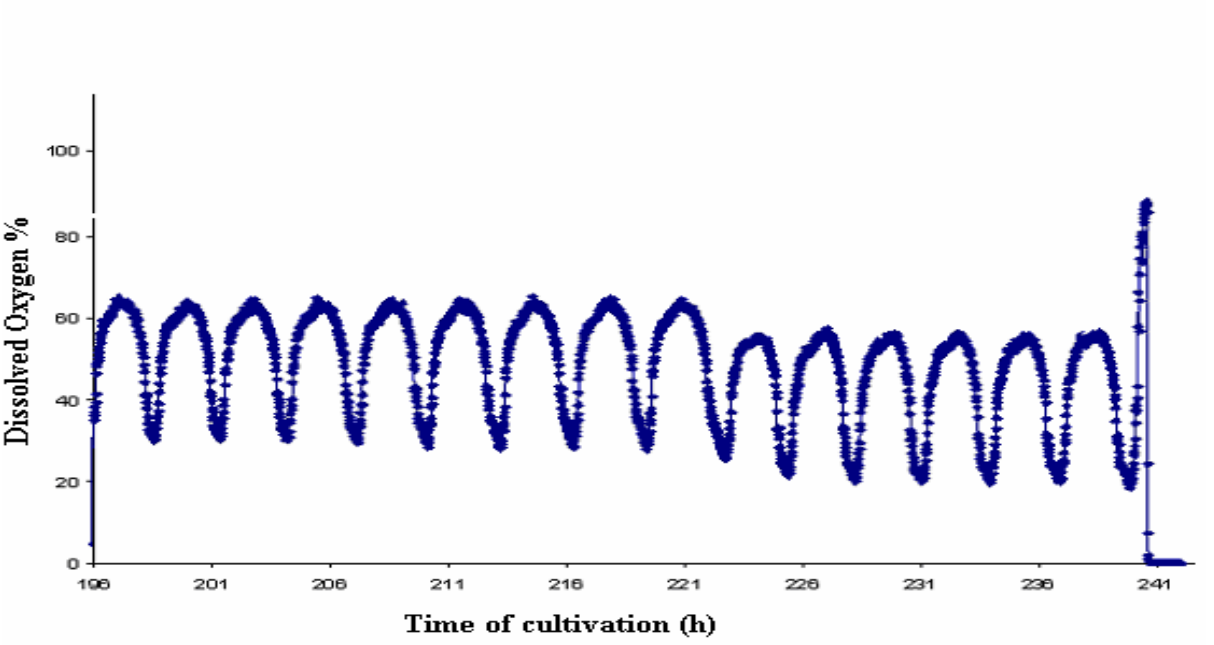


Figure (4.8): Profile of the dissolved oxygen for a continuous culture of *S. cerevisise* in 10 L bioreactor at a dilution rate of 0.18 h^{-1}, pH 4.5 and temperature of 30 ºC

The dissolved oxygen saturation percent in the culture exhibited oscillatory behaviour. It is obvious in the above figure that the DO values were between 20 and 70 % at dilution rate of 0.18 h^{-1}. The oscillations were seen to be induced at dissolved oxygen levels that were neither high nor low. This result can be explained by earlier findings of Parulekar *et al*., 1986 who stated that the oscillations were seen to be connected to the growth kinetics of the micro-organism and were induced at very low glucose concentrations and at DO values between 20 and 78 % air saturation at a dilution rate of 0.2 h^{-1}. The oscillations can be eliminated by raising the DO levels above a critical value or by lowering the DO level below a critical value.

4.2.2 The relation between dissolved oxygen and biomass during oscillations in 10 L continuous culture of *S. cerevisiae*

The cell mass dry weight values were monitored during 3 sequential cycles where they varied between 11.3 g/L as a minimum value and 13.5 as a maximum as shown in Figure (4.9). The yield of biomass depends upon the metabolic pathways employed for the utilization of the carbon source. The maximum values were observed during the G1-phases, where the oxidative metabolism of the glucose was predominated, the cell mass yield via this pathway was much higher than that of the fermentation because glucose was consumed primarily for the production of cell mass (Parulekar *et al*., 1986).

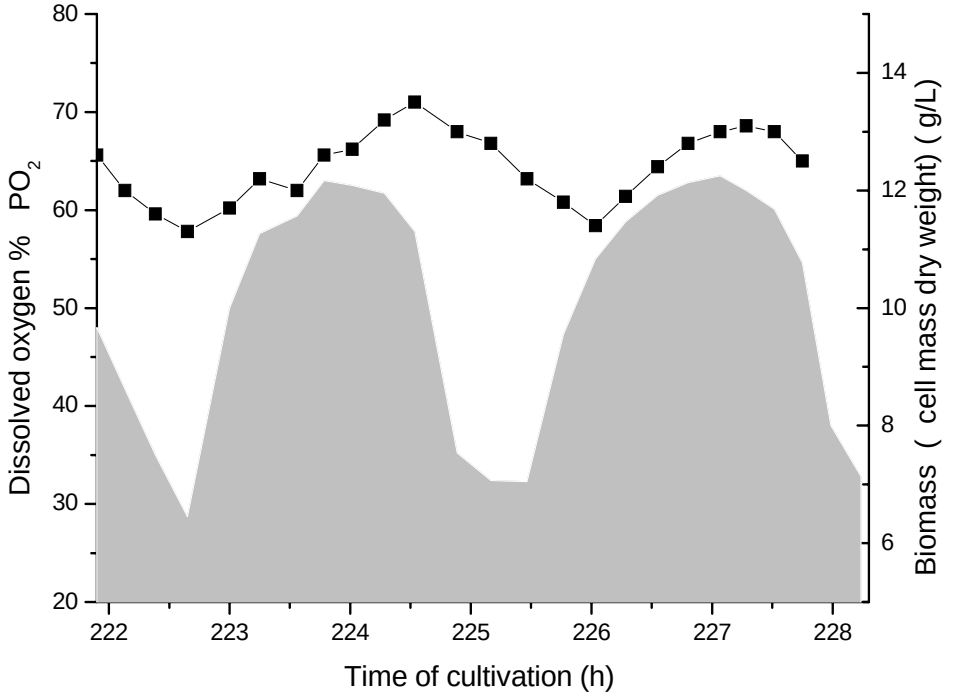


Figure (4.9) : Offline-data of biomass in combination with dissolved oxygen during two periods of oscillation in a 10 L chemostat at dilution rate of 0.18 h^{-1} , pH = 4.5 and T = 30 .

The maximum values reached 13.5 g/L and 13.1 g/L during the 2 cycles and both of these values were in the mean time of the maximum values of cell in G1-phase, the biomass values began to decrease continuously during S-phase, since the carbohydrate and protein synthesis was at its minimum levels (Duboc *et al.*, 1996). According to the biomass values, the growth process may divided into two phenomena: the formation of new cells during mitosis with low biomass yield, followed by size increase of new born cells in G1-phase with high biomass yield (Duboc *et al*., 1996).

4.2.2 Respiratory variables during oscillating chemostat culture of *S. cerevisiae* in 10 Lreactor

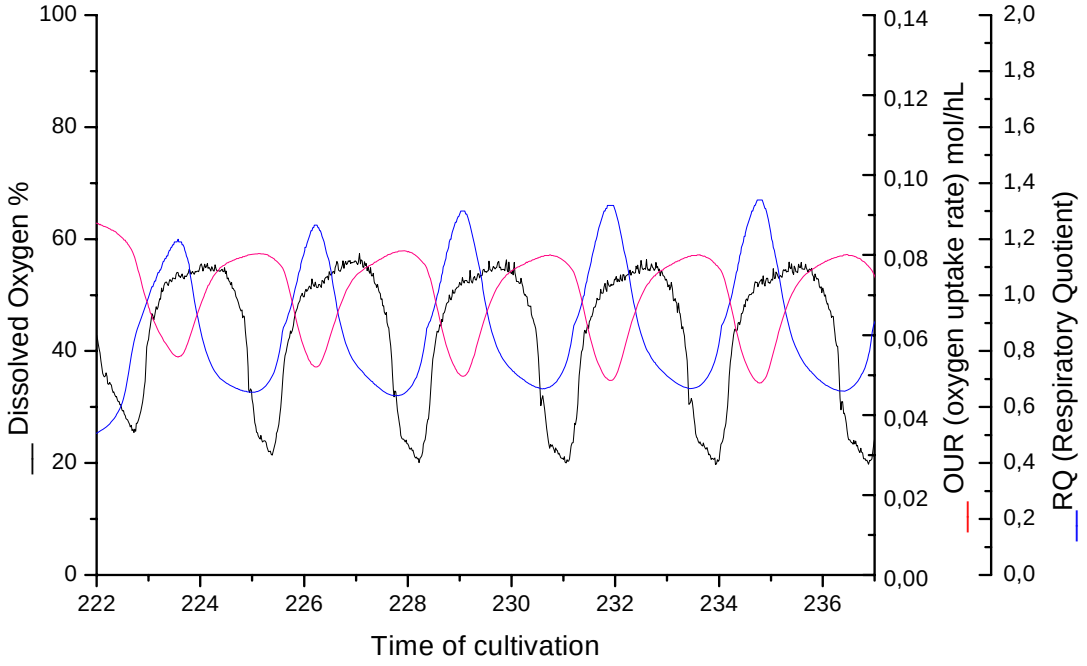


Figure (4.10): Profile of respiratory variables during oscillating chemostat culture of *S.cerevisiae* in Schatzmann medium with D = 0.18, pH = 4.5 and T = 30

As shown in the above Figure (4.10), RQ has been found to be a good indicator of the type of metabolism in aerobic cultures of *S. cerevisiae*. For RQ values between 0.5 and 1, Woehrer and Roehr (1981) observed that both glucose and ethanol will be oxidized with glucose being preferentially. At the beginning of budding phase, RQ value was observed greater than one and at the end less than one (Beuse *et al*., 1998). This indicated a change from respiro-fermentative to respiratory growth. During the respiratory phase, cells accumulated glycogen and assimilated extracellular ethanol, whereas the yeast cells assimilated glycogen and produced ethanol during the respire-fermentative phase (Satroutdinov *et al*., 1992).

As shown in Figure (4.10), the respiratory quotients of the continuous culture in the present experiment were between 0.6 and 1.3. It is therefore believed that there was a continuous shifting between growth on glucose mainly and ethanol shortly (as evidenced by sustained oscillations in RQ) which leads to self sustained oscillations. There is evidence that even the yeast cells, grown in the presence of high glucose concentration, retain a low level of activity for ethanol oxidation during the whole process.

4.2.3 Offline monitoring of ethanol production during the continuous cultivation of *S. cerevisiae* in 10 L bioreactor

The ethanol concentration was determined in all of the collected samples using gas chromatograph GC-14B (Fa. Shimadzu, Japan). Glucose was measured also in the mean time using YSI analyzer 2700 (Yellow Springs Instruments, USA). The results are shown in Figure (4.11).

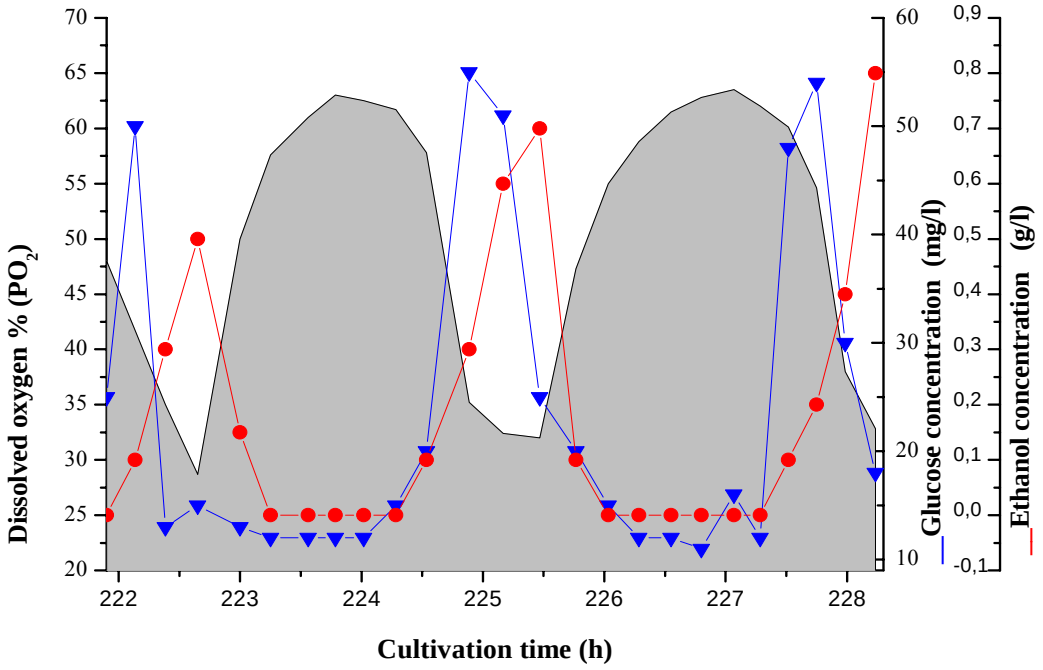


Figure (4.11): Dissolved Oxygen and concentrations of glucose and ethanol during a period of stable oscilations in a chemostat at D= 0.18, pH = 4.5 and T = 30

Ethanol is not detectable at all during certain periods of the cultivation. Simultaneously with decreasing DO, the concentration of ethanol increases dramatically, the maximum concentration of ethanol reaches 0.8 g/L. It is evident that a fast ethanol production was just in coincidence with the bud emergence, an event that in *S. cerevisiae* occurs closely associated to the beginning of the S-phase (Porro *et al.*, 1987). Many authors have tried to give a metabolic explanation of the oscillatory behaviour, and it is clear that oscillation are related to a condition of growth in limiting oxygen supply that does not allow fully respiratory metabolism of glucose during specific phases of the cell cycle such as bud emergence and the beginning of the S-phase. Porro *et al.*, 1987 proposed a mechanism for the origin of the self sustained oscillations. It was based on a limit–cycle driven by an ethanol production before budding, which cause a change in environments and a shift to growth on ethanol with an

alternation of cell cycle parameters. Fermentation of glucose to ethanol is an anaerobic process, However ethanol has been found here in aerobic processes near the high concentration of glucose. This was the crabtree effect and it can be considered as catabolite repression, i.e. the excess glucose repressed its use in the pathways that lead to ethanol. The yeast can not pass the excess glucose through the main pathways and simply shunt it through an alternate route to ethanol. Porro *et al*., (1988) showed that ethanol is a trigger which has a profound impact on the occurrence of oscillations.

It was evident that cells grown in presence of glucose as a carbon source retain an activity for oxidation of ethanol. The role of ethanol is sustained by the observation that in one of the experiments, the addition of a large amount of ethanol to a culture exhibiting oscillations did not result in their elimination. This leads to a conclusion that oscillations should be caused by a repeating shifting between the respiratory growth on glucose and the fermentative growth on glucose plus respiratory growth and ethanol (Parulekar et al., 1986).

4.2.4 Online monitoring of NADH, protein and flavines oscillations in 10 L bioreactor using BioView®-Sensor

NADH fluorescence was monitored continuously during the cultivation. The output was shown in Figure (4.12), and compared to the dissolved oxygen percentage. The NADH fluorescence was observed inversely correlated with the dissolved oxygen. In other words, NADH fluorescence oscillates out of phase with dissolved oxygen. It was obvious that the increase in NADH-fluorescence occurred in the beginning of S-phase at which the metabolic activity was so high and there was also a quick short change in cell state metabolism from oxidative to oxidative–reductive catabolism.

NADH is used in the last step of ethanol synthesis, in order to produce NADH for this synthesis, citric acid cycle is probably broken shortly which resulted in the increase of NADH amount till it was used again in the conversion of acetaldehyde to ethanol and NAD+ (Hantelmann, 2005). Murry *et al*., 1998 suggested that the intracellular redox state plays an important role in the regulation of oscillation. Protein oscillation was also clear and indicative in Figure (4.12) than that of flavines.

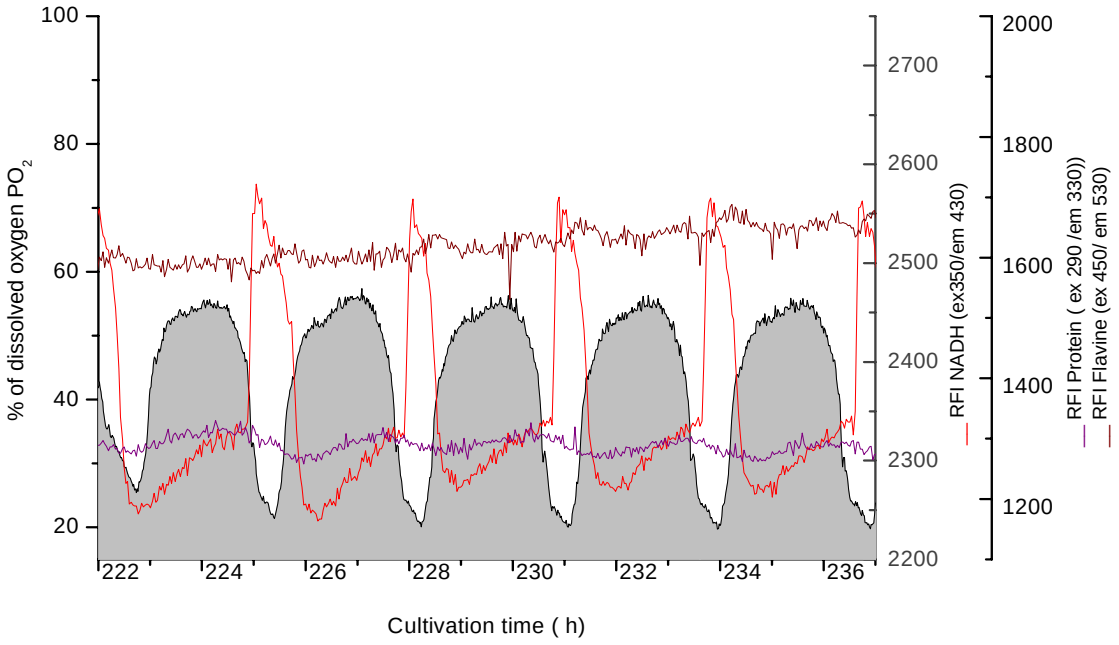


Figure (4.12): The relative fluorescence intensity of NADH, protein and flavines during continuous aerobic yeast culture at D = 0.18, pH = 4.5 and T = 30

4.2.5 The flow cytometric analysis of cell cycle in the 10 Lchemostat

The stable oscillation of the yeast cell cycle was studied during the cultivation time of 221.9 h till 228 h, in which the oscillation was very stable. During this period of time, 3 cell cycles were monitored, each of about 3 hours length. In Figure (4.13), the results of the flow cytometric cell cycle analysis was plotted in combination with dissolved oxygen percent in the culture.

In G_1 phase, in which cells were in growth phase, the dissolved oxygen was found in its maximum phase. On contrary, in S-phase, the dissolved oxygen values were in its minimum during this budding process, in which the cells were in strong need for using oxygen and energy in order to complete the DNA replication process, so the cells tended shortly to the oxidative-reductive pathway to compensate the low level of the dissolved oxygen and in turn ethanol was already produced and consumed during this phase as it was shown in Figure (4.12). During G2/M, the dissolved oxygen PO_2 in the culture began to increase again after its sharp drop during the S-phase and the cells were in division phase specially the nuclear material division between the mother and daughter cell (Beuse, 1997).

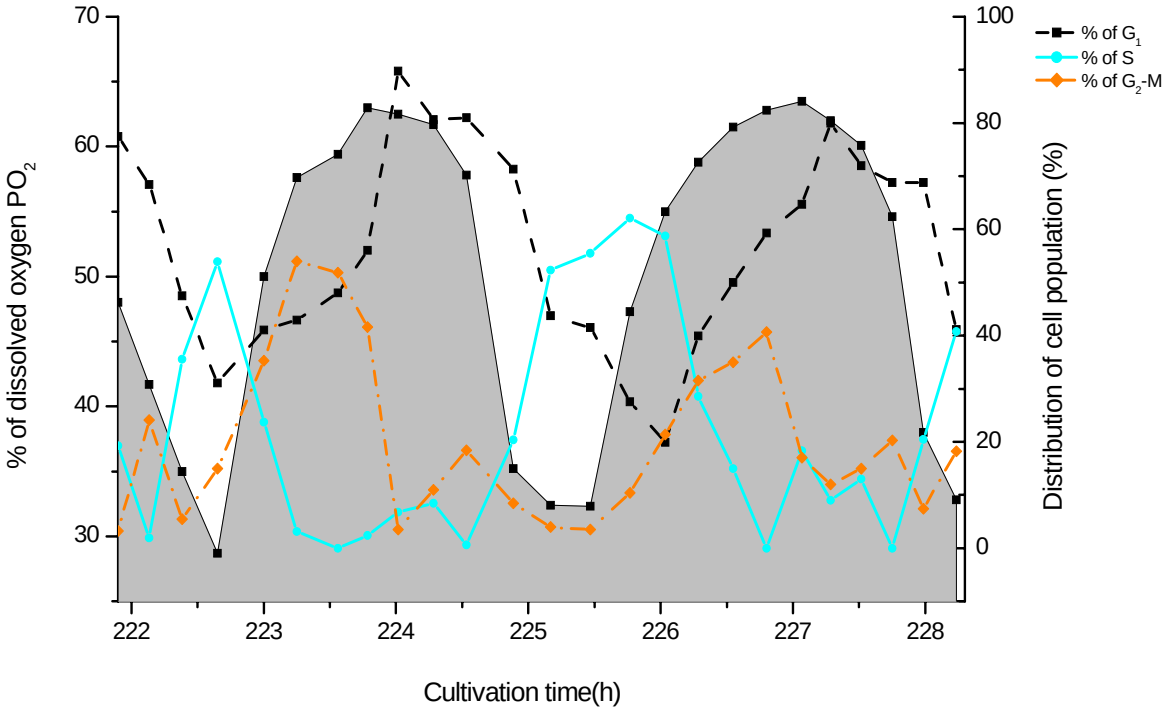


Figure (4.13): Dissolved oxygen and distribution of different cell population (G1, S, and G2/M phases) during two periods of oscillation in a chemstat at dilution rate of 0.18 , pH = 4.5 and T = 30

The results showed that the different phases of the cell cycle were also in oscillation, the synchronization of cells in G1 reached its maximum value of 89.72% and 80 % during the studied cell cycles respectively. The maximum synchronization percent of cells in S-phase were 53.9 % and 62.1 and that of G2 /M were 51.8 % and 40.7% respectively.

4.3 Yeast total RNA purification and characterization

The quality of purified RNA is the single most important factor in determining the outcome of microarray analysis. RNA is generally not stable and RNAases exist in abundance in cells and in laboratory environments, which may cause the degradation of RNA samples. Therefore, RNA samples should be handled with great caution. The quality of RNA will determine the quality of ultimate gene expression data from DNA hybridization. The total RNA should be intact (not degraded) and completely free of any protein or DNA contamination. The yeast RNA samples purity must be checked by a UV spectrometer with measuring the OD 260/OD 280 ratio (260 nm and 280 nm are the wave lengths at which the RNA absorbance is recorded). RNA quality was accessed by electrophoresis on an agarose gel and recently by Agilent Bioanalyzer. Agilent has developed software that assigns the quality of purified RNA sample based on its electrophoretic profile. Only high quality RNA samples with very good electrophoretic pattern and OD 260/OD 280 greater than 1.8 was considered for further microarray analysis. The concentration of RNA was quantified as shown in Figure (4.14) using Nanodrop spectrophotometry.

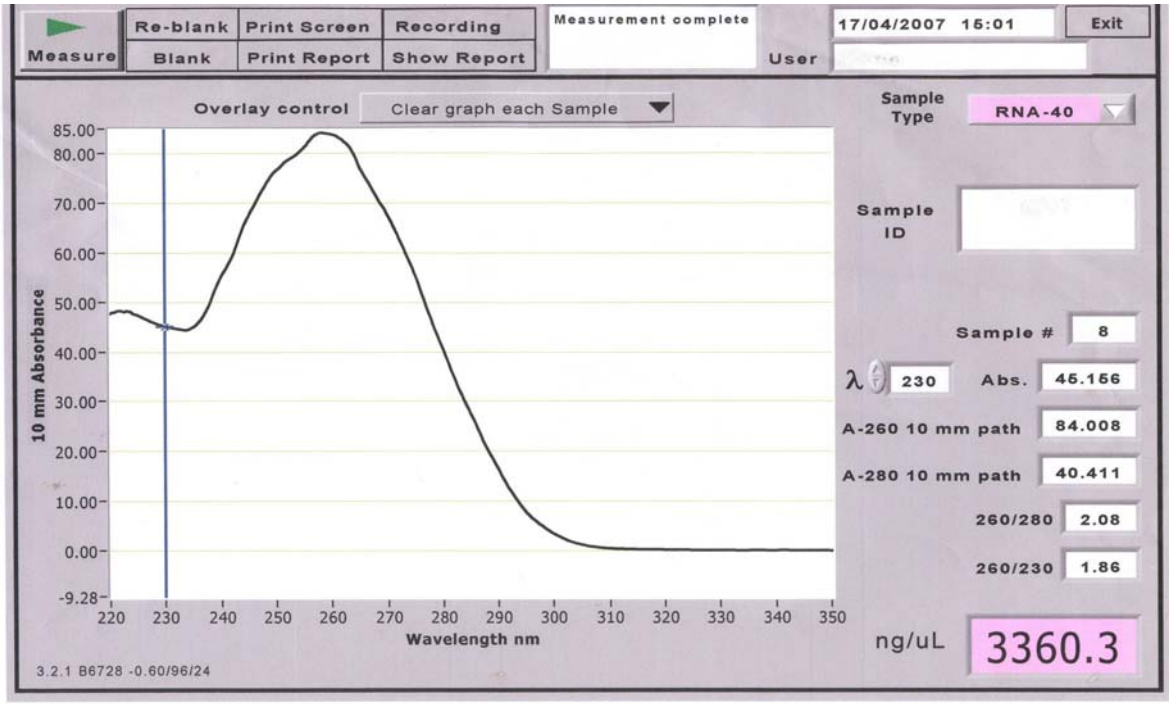


Figure (4.14): A chart taken from Spectrophotometer Nano-Drop-1000 for yeast RNA sample purified from 10 liter bioreactor, showing a concentration of 3.3 µg/µl and the purity testing using the ratio between optical density at 260 and 280 nm.

4.3.1 The quality control testing of RNA samples

The assessment of RNA integrity is a critical step in obtaining meaningful gene expression data. Using intact RNA is a key element for successful microarray or RT-PCR analysis. The quality control of the samples, prepared for microarray analysis, were tested with Agilent RNA 6000 Nano assay which has become the standard in RNA quality assessment and quantitation (Mueller *et al*., 2000 & 2004). Each sample was diluted 10 times by adding RNase free water. Using electrophoretic separation on microfabricated chips, 12 RNA samples were separated and subsequently detected via laser induced fluorescence detection. The bioanalyzer software generated an electropherogram as shown in Figure (4.16) and gel like image as clear in Figure (4.15) and displayed also a detailed report for each sample containing the concentration ,the so called RNA integrity Number (RIN) as shown in one of purified samples Figure (4.17) and rRNA ratio (the ratio between 28s and 18s). The purification process was successful since only two ribosmal peaks resulted in all sample with no other observable peaks for any impurities or degradable RNA as clear in Figure (4.16).

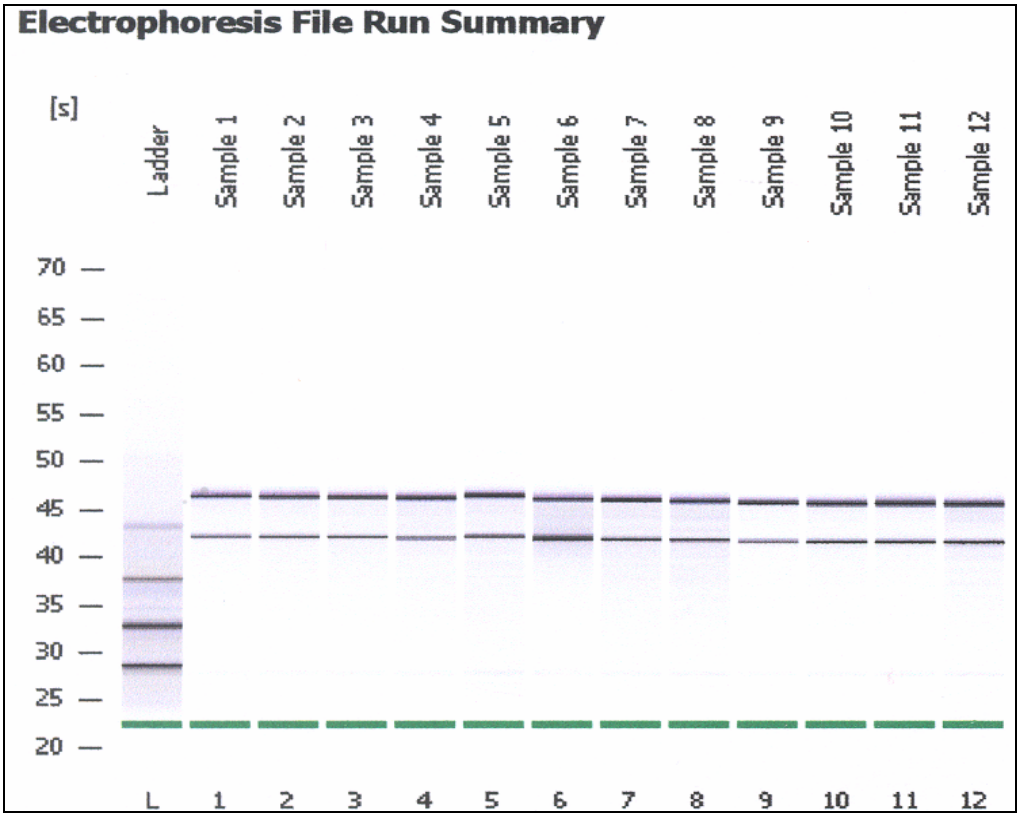


Figure (4.15): A chart taken from Agilent 2100 bioanalyzer for 12 yeast RNA samples purified from 2 liter bioreactor using enzymatic lysis protocol of Qiagen RNeasy Midi kit showing the two ribosomal peaks.

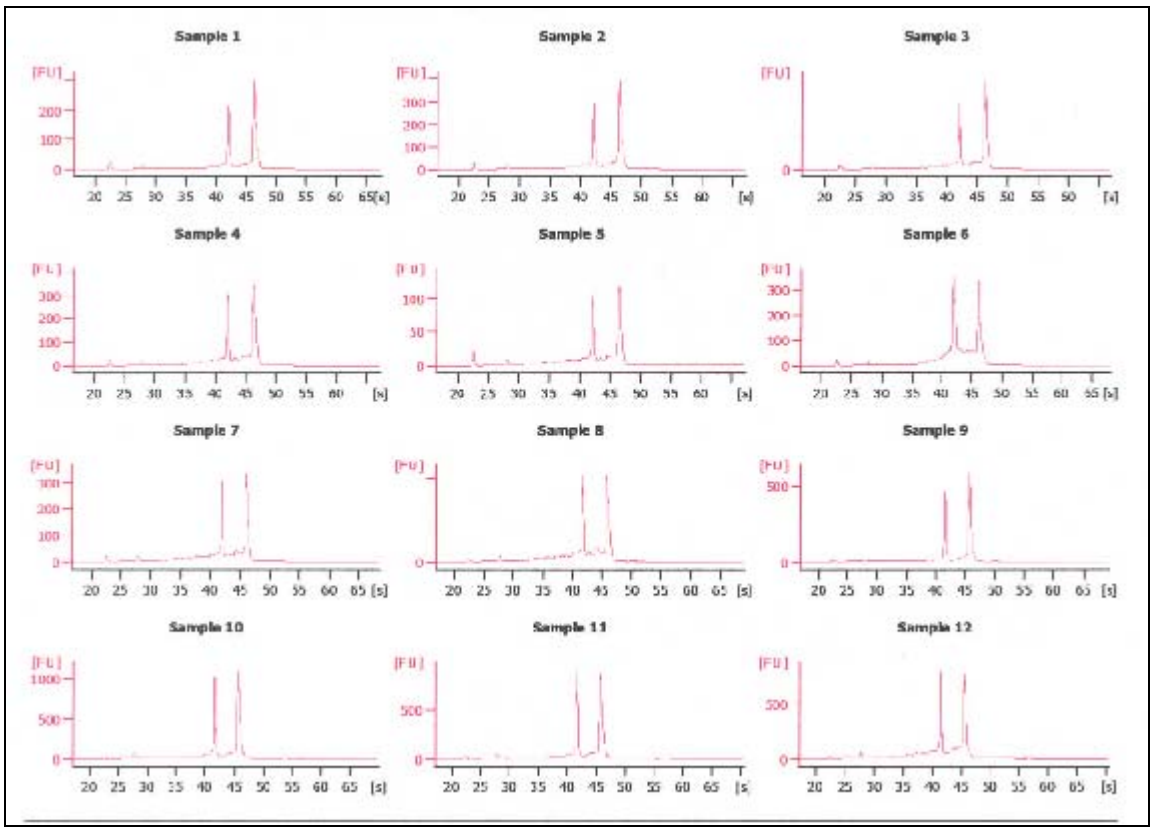


Figure (4.16): Electropherogram chart of 12 yeast RNA samples purified from 2 liter bioreactor showing 18S fragment peak and 28S fragment peak for each sample

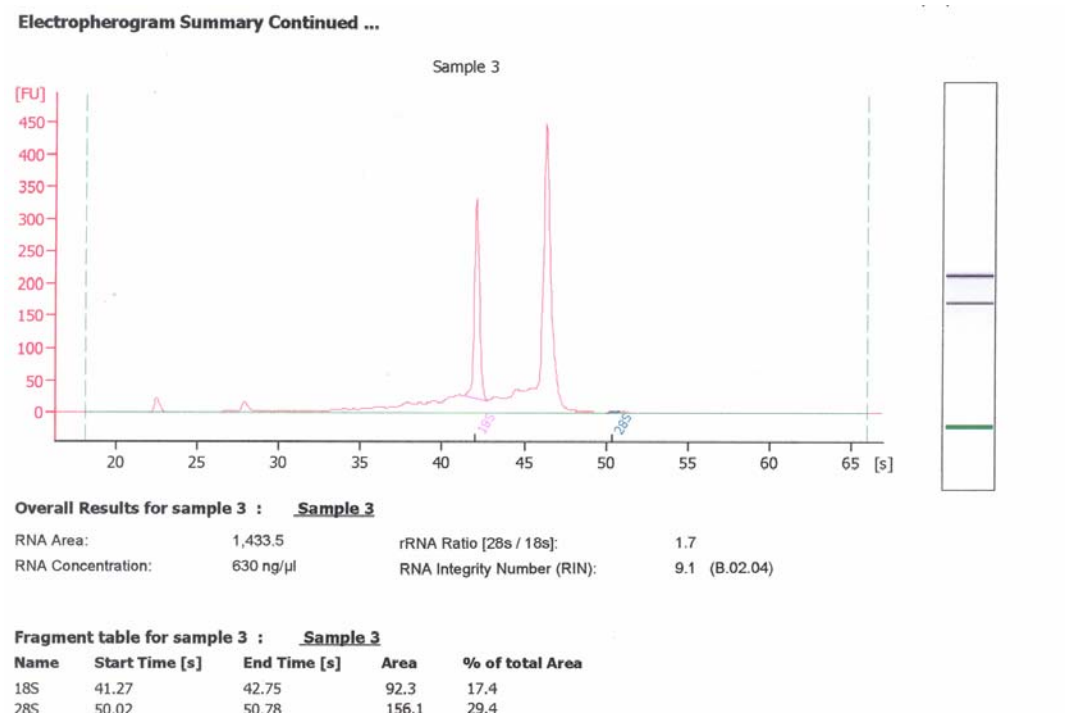


Figure (4.17): Overall results for one of the yeast purified RNA samples from the 2 L bioreactor showing the concentration, RIN and the ratio of 28S to 18S ribosomal subunits

4.3.2 Purification methods of yeast RNA

Most methods of isolation of RNA from yeast require tedious vortexing with glass beads and give low yields (Schmitt *et al*., 1990). In this research work two fast and reproducible methods, without any beads, were used and compared together with respect to the yield and purity. Hot phenol technique and enzymatic (lyticase) lysis protocol of RNeasy Midi kit were the methods of choice to prepare the pure RNA for further microarrays analysis. In Midi kit, the yeast cells were incubated at 30ºC with gentle shaking in a buffer solution containing lyticase for 30 minutes to generate a spheroplast. All the following steps of this protocol were carried out quickly in another 30 min at room temperature. DNA digestion was not required since RNeasy silica membrane removes DNA without DNase treatment. The isolation of RNA using hot phenol and SDS technique was also performed in about 60 min. The isolated RNA was extracted in the upper aqueous phase. Five samples were taken from the 2L continuous culture of the yeast, and purified with both techniques in the same day. Both of the procedures gave a sufficient amount of RNA as clear in Figure (4.18), although the yield of Midi-Kit was somehow higher than that in case of hot phenol. The highest yeast concentration obtained by Midi-kit reached was about 9.4 µg/µl and that of phenol was 7.2 µg/µl.

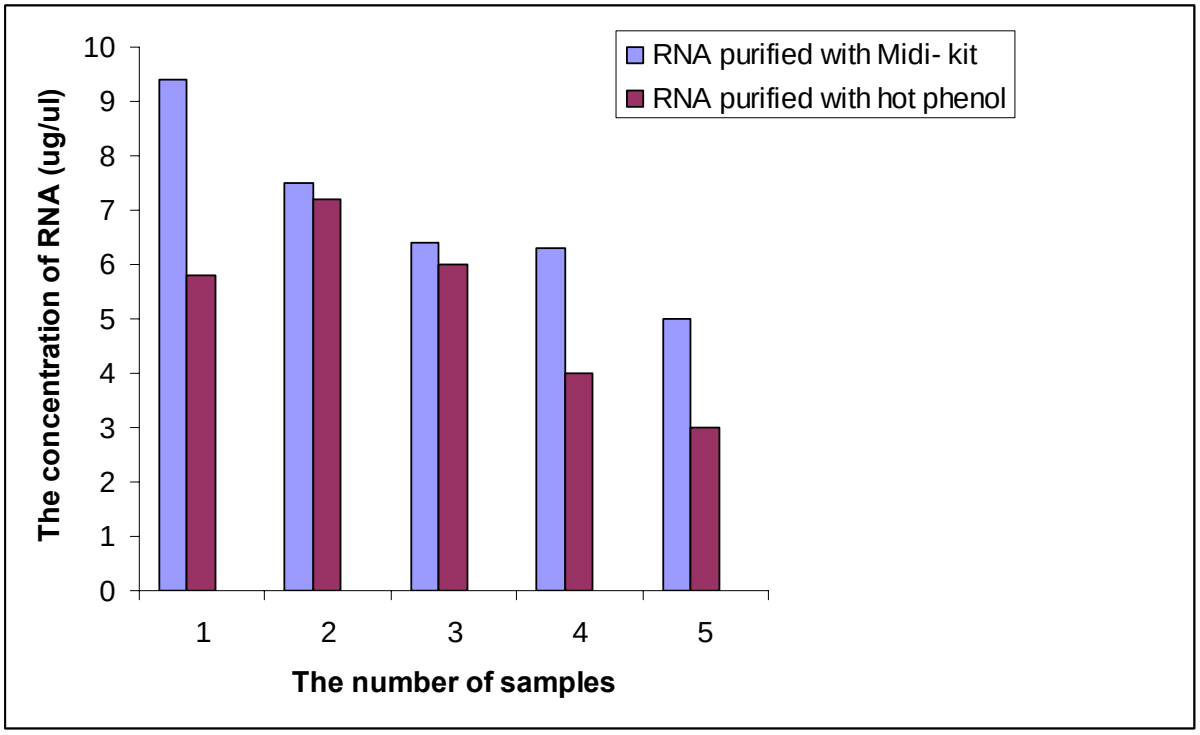


Figure (4.18): The yield of five RNA samples, from the yeast 2 L continuous culture, purified with enzymatic lysis protocol of Midi-kit and hot phenol technique respectively.

Purity (OD 260/280) of the purified RNA samples using the Midi-kit was also higher than those which isolated by hot phenol. All of samples purified with the kit showed a ratio between 1.9 and 2.1, on the other side the RNA samples purified with hot phenol were in range between 1.7 and 1.82 as clear in Figure (4.19)

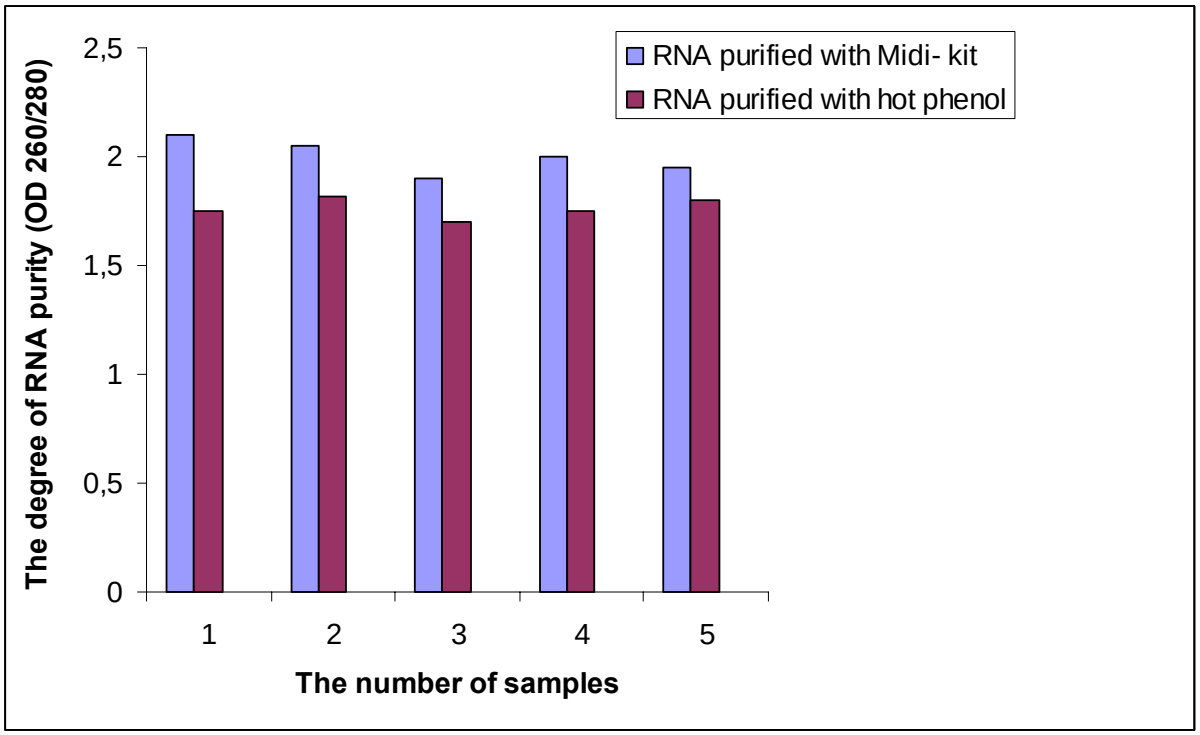


Figure (4.19): A comparison between OD 260/280 ratio of yeast RNA samples purified using enzymatic lysis protocol of Midi-Kit and hot phenol method respectively.

1.2 % Formaldehyde-agraose electrophoresis was used to examine the purified RNA as shown in Figures (4.20 and 4.21). Both techniques of purification showed 2 intact bands of total RNA at 18s and 28s without any obvious degradation for the samples. Although the samples purified with hot phenol had some faint bands in addition to the original bands which may be the reason for their low OD 260/ 280 ratio with respect to the Midi-kit. Based on the comparison between these 2 methods, it was concluded that both of them were good choice to purify the yeast RNA, but depending on the obtained result, the Midi-kit protocol was used to prepare the required RNA for further microarray hybridization, since it can be used directly without any further steps of DNA degradation, in addition if any phenol was still in samples after the isolation process, it should interfere the labeling efficiency of cyanine fluorescent dyes (Zhang *et al.,* 2004). Hot phenol method is an economic method in comparison with Midi-kit, and can be used safely in microarray analysis with addition of DNA degradation step and avoiding the interference of any part of the organic phenol layer during the purification steps.

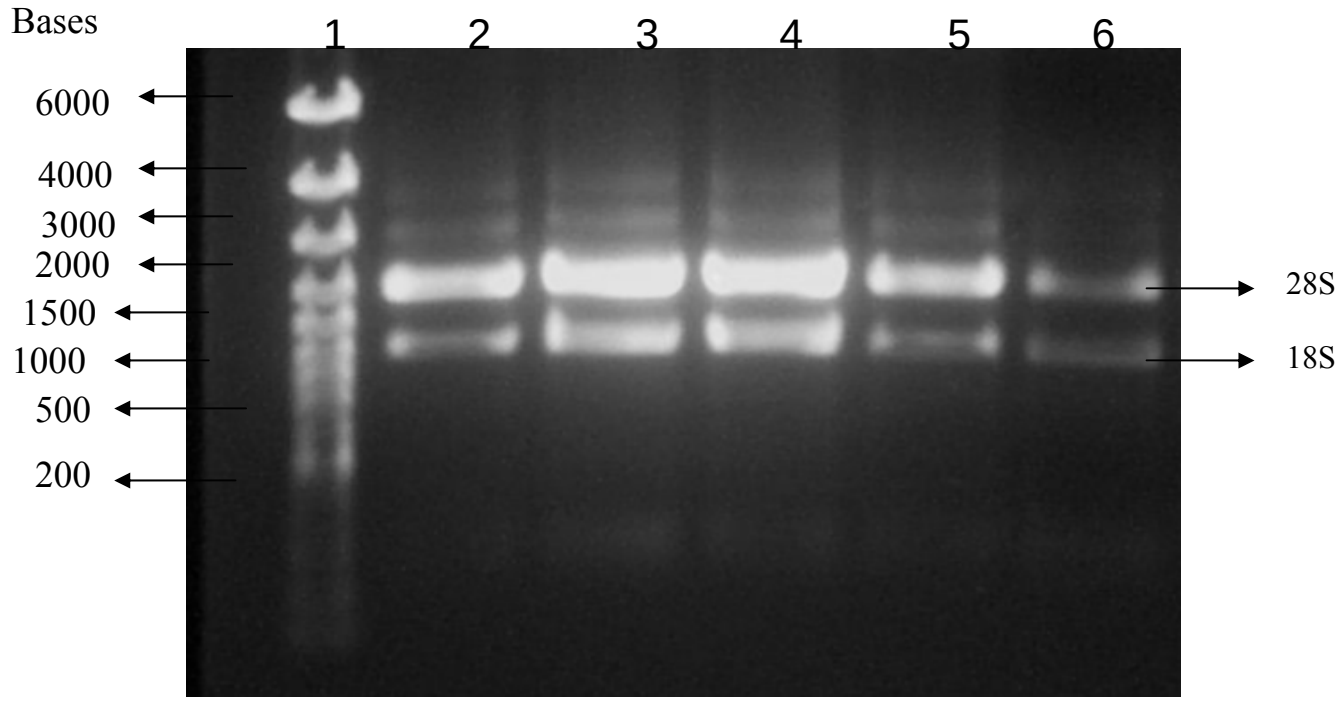


Figure (4.20): Denaturing agarose gel electrophoresis (1.2 %) for *S. cerevisiae* RNA samples purified with hot phenol. Lane 1 represents the molecular weight marker (Fermentas high range). Lane 2 and 3 represent RNA samples of G1-phase, lane 4 and 5 represent RNA samples of S-phase and lane6 represents RNA sample of G2/M sample.

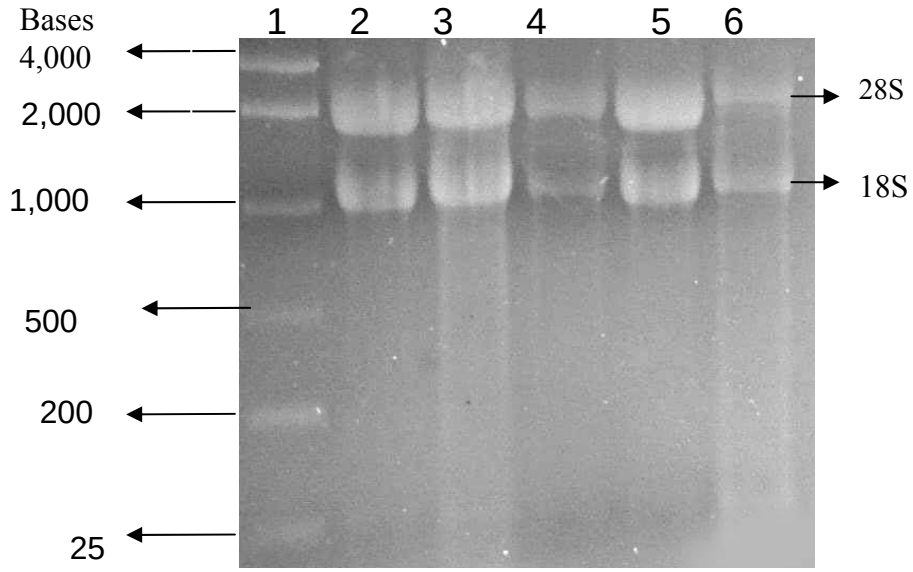


Figure (4.21): Denaturing agarose gel electrophoresis (1.2 %) for *S. cerevisiae* RNA samples purified with the enzymatic protocol of Midi-Kit. Lane 1 represents the molecular weight marker, RNA6000 Ladder (Aligent 2100). Lane 2 and 3 represent RNA samples of G1-phase, lane 4 and 5 represent RNA samples of S-phase, and lane 6 represents RNA sample of G2/M sample.

4.3.3 Monitoring RNA level during different stages of *S. cerevisiae* oscillating cell cycle

The total RNA extracted from synchronously growing yeast cells, from all samples which are collected from 2L and 10L bioreactors respectively, by a Midi-kit method which gave a higher purity and more consistent yield than any other method. RNA concentration was determined by Nano-Drop 1000 spectrophotometer and the samples were stored at -80ºC till they were used for further microarray analysis. The concentration of total RNA of samples collected from the 2 L bioreactor ranged between 3.4 µg/µl and 9.4 µg/µl as shown in Figure (4.22). The highest concentration of RNA was in the mean time of S-phase, whereas the lowest was observed during the G2/M. The cells in G1-phase reached a value of 6.4 and 6.3 µg/µl during its 2 peaks respectively.

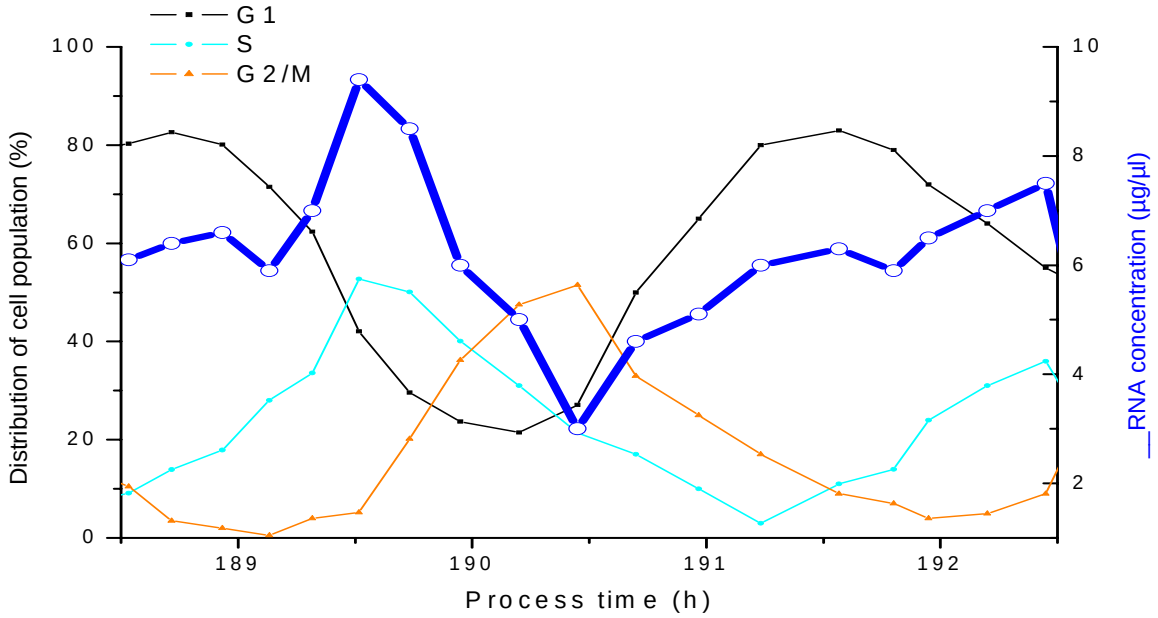


Figure (4.22): RNA concentration (µg/µl) of different cell populations (G1, S, and G2/M) of samples collected from 2 liter bioreactor

In the 10 L bioreactor, the level of RNA in the cells ranged between 3.9 µg/µl and 10.5 µg/µl during the time course of sample collection. As shown in Figure (4.23), the RNA concentration was at its maximum level in the mean time of S-phase peaks, where it reached 7.5 µg/µl and 10.5 µg/µl respectively. At G2/M peaks, it ranged between 6.3 µg/µl and 3.9 µg/µl respectively, whereas the RNA concentrations were 5.1 µg/µl and 5.5 µg/µl at the two G1 peaks repectively.

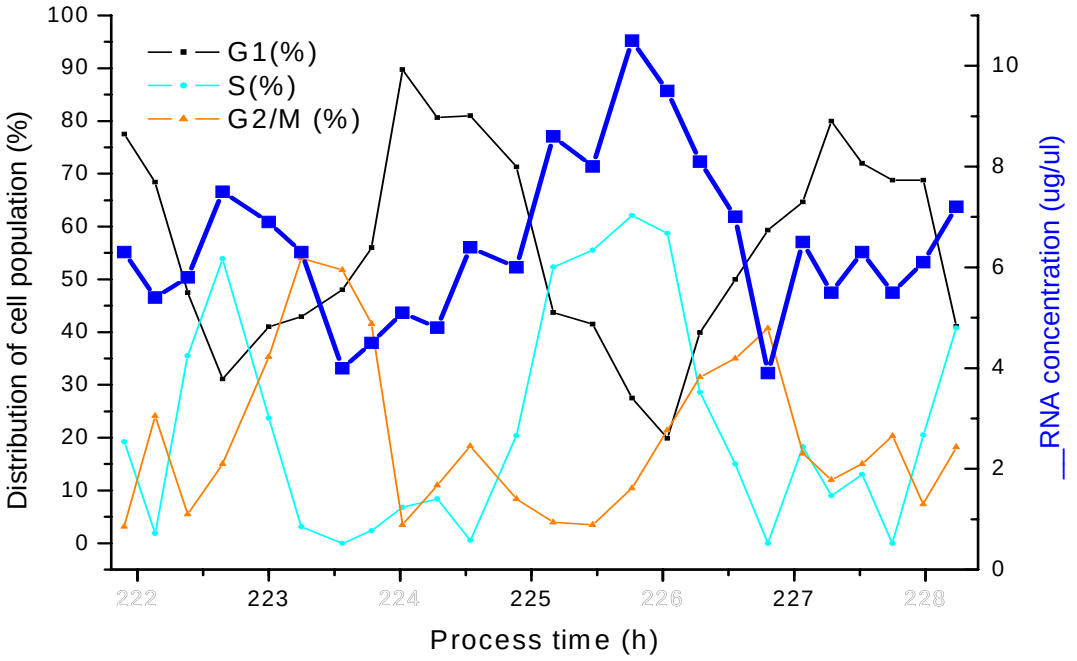


Figure (4.23): RNA concentration (µg/µl) of different cell populations (G1, S, and G2/M) of samples collected from 10 liter bioreactor.

RNA metabolism must have a key role in the regulation of yeast *S. cerevisiae* cell cycle divison (Johnston and Singer, 1978). Several types of RNA can down-regulate gene expression by being the complementary part of a gene (Matzke, 2004). The ratio of RNA to DNA in a rapidly growing cell of *S. cerevisiae* is 50:1 (indeed, the original name for RNA was yeast nucleic acid). The approximate distribution of total RNA is 80% rRNA, 15% tRNA, and 5% mRNA (Warner, 1999). The rRNA genes of *S.cerevisiae* make up 10% of the entire genome, the transcription of rRNA by RNA polymerase I appears to represents nearly 60% of total transcription in the yeast cell (Woolford and Warner 1991). Fraser and Carter (1976) reported a doubling in the rate of synthesis of rRNA and poly-A containing RNA in *S. cerevisiae* during S-phase. This can explain the above results in Figures (4.22 & 4.23), since the S-phase cells showed the highest level of total RNA in comparison with G1 and G2/M cells during the continuous cultivations on the both levels. The doubling in the concentration of total RNA had been seen once in 10 L bioreactor from 5.5 µg/µl during G1 phase to 10.5 µg/µl during the following S-phase in the same cycle (Figure 4.23). It came as a surprise that DNA synthesis also required the cell to be synthesizing RNA. A specific enzyme, primase is involved. All DNA synthesis is initiated by the synthesis of a short chain RNA, in the direction 5´-3´, using nucleoside triphosphates (NTPs) as substrate (Campbell *et al*., 2005).

Core histones in *S.cerevisiae* are encoded by 4 different gene pairs. The expression of these genes is under tight cell cycle control such that their mRNA strongly accumulated only during S-phase and rapidly degraded as cells enter the G2-phase (Canavan and Bond, 2007) which can explain the low values of total RNA during G2/M in the above results. This ensures also that histones are present only when they are needed, as the new DNA is being made and readied to be pack into chromatin. This histone mRNA increases in turn the total RNA amount during S-phase (Sutton *et al.*, 2001, Osley *et al*., 1986 and Hereford *et al.,* 1981). Despite the intense noise in the level of mRNA, the yeast cell cycle is stable enough to bring the largely perturbed cells back to physiological cyclic oscillations (Okabe and Sasai, 2007).

4.4 Gene expression of *Saccharomyces cerevisiae* during the cell cycle

Microarray gene expression patterns are biologically infomative and provide direct clues to cellular functions. Correlating changes in gene expression with specific changes in physiology can provide mechanistic insights into the dynamics of various processes in an organism like yeast. Microarray technology can be used for simultaneously detecting expression of many genes in different developmental cell cycle stages. Microarrays for gene expression analysis were from the first biological application of DNA chip technology (Lemieux *et al.* 1998).

Following the complete sequence determination of the *S. cerevisiae* genome, Affymetrix DNA microarrays have emerged as a powerful tool for examining the simultaneous expression pattern of more than 6000 yeast genes (DeRisi *et al.,* 1997 and Wodicka *et al*., 1997). Existing information about yeast biology, including functional annotations for each gene, were captured and efficiently presented in databases such as the *Saccharomyces* Genome Database (SGD) (Ball *et al*., 2000 and Weng *et al*., 2003) and Munich Information Center Yeast Genome Database (MIPS) (Mewes *et al.*, 2000). Consulting these databases to retrieve known details about gene function and regulation vastly facilitated interpretation of the yeast genomic expression data, allowing biological hypotheses to be formulated and tested. The aim of this study was to analyse how the gene expression changes during different cell cycle phases especially during G1 and S-phases. The basic procedure of the yeast whole genome expression analysis using microarrays can be summarized during this study as follows: total RNA was extracted from the synchronous continuously cultivated yeast cells especially for cells in G1 and S-phases. The RNA was then reverse-transcribed to cDNA. cDNA was then labelled and hybridized to the whole yeast genome array (MWG PAN yeast II array). The relative level of expression for each sample was scanned and digitally acquired for signal intenisty using the fluorescent labelling pattern.

4.4.1 The whole yeast genome expression in G1 and S-phases during the 2 L-continuous culture

The isolated RNA from 2 samples from the same cell cyle, shown in Figure (4.22), was used for this experiment, the first sample (containing 82 % cells in G1-phase) represented G1-phase and the second one (containing 53% of cells in S-phase) represented S-phase. Each of these samples was used to hybridize the yeast whole genome chip (MWG PAN Yeast array II, MWG Ebersberg, Germany). cDNA was synthesized from 100 µg of total RNA using

LabelStar reverse transcriptase and oligo (dT) primers. cDNA was labeled using cyanine 3- or cyanine 5-conjugated dCTP. The cDNA was purified using Qiagen purification kit. The cDNA was hybridized to the whole genome chip over night at 42°C, and scanned by the laser Affymetrix Array scanner 428 ^{TM}. The results of chip scanning for cells at G1 and S-phases are shown in Figures 4.24 & 4.25 respectively.

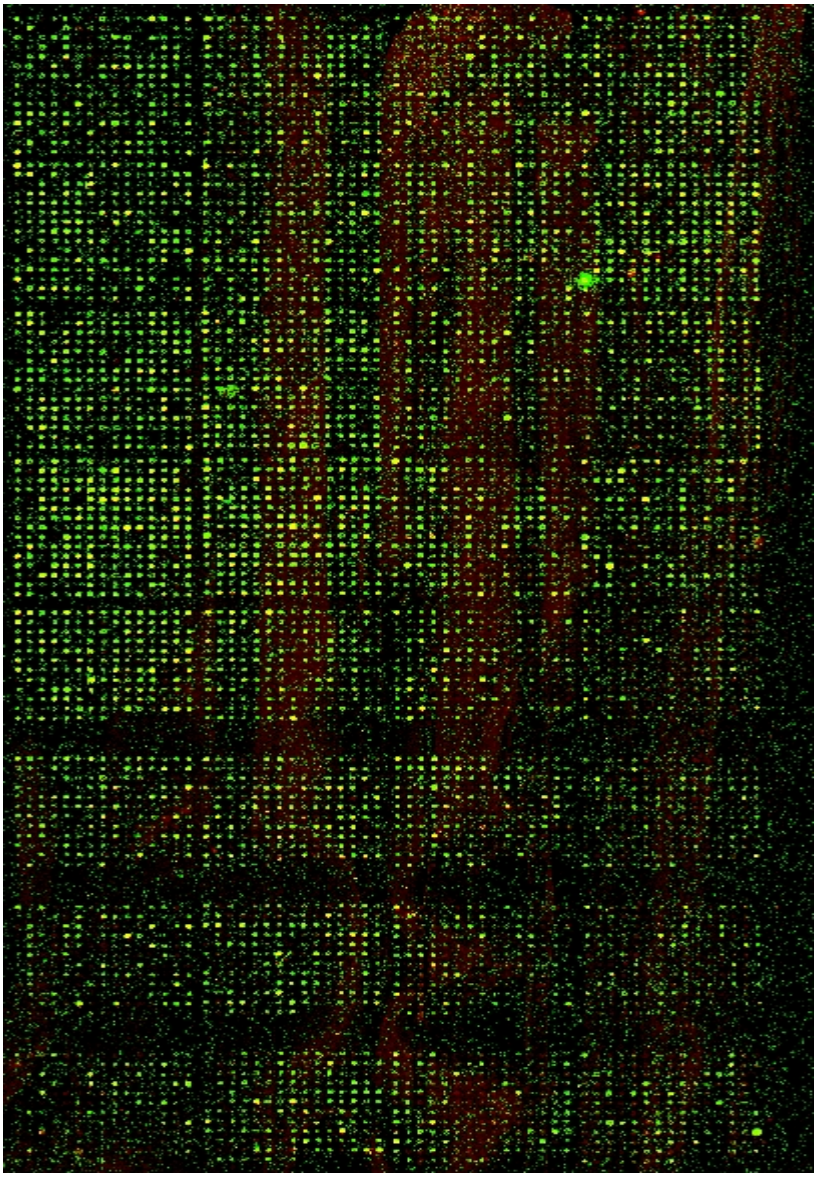


Figure (4.24): Yeast (*S. Cerevisiae*, wild type, H620) whole genome expression for cells in G1 which were labelled with cy3 and cy5, the cells was taken from 2 L- bioreactor continiuous culture.

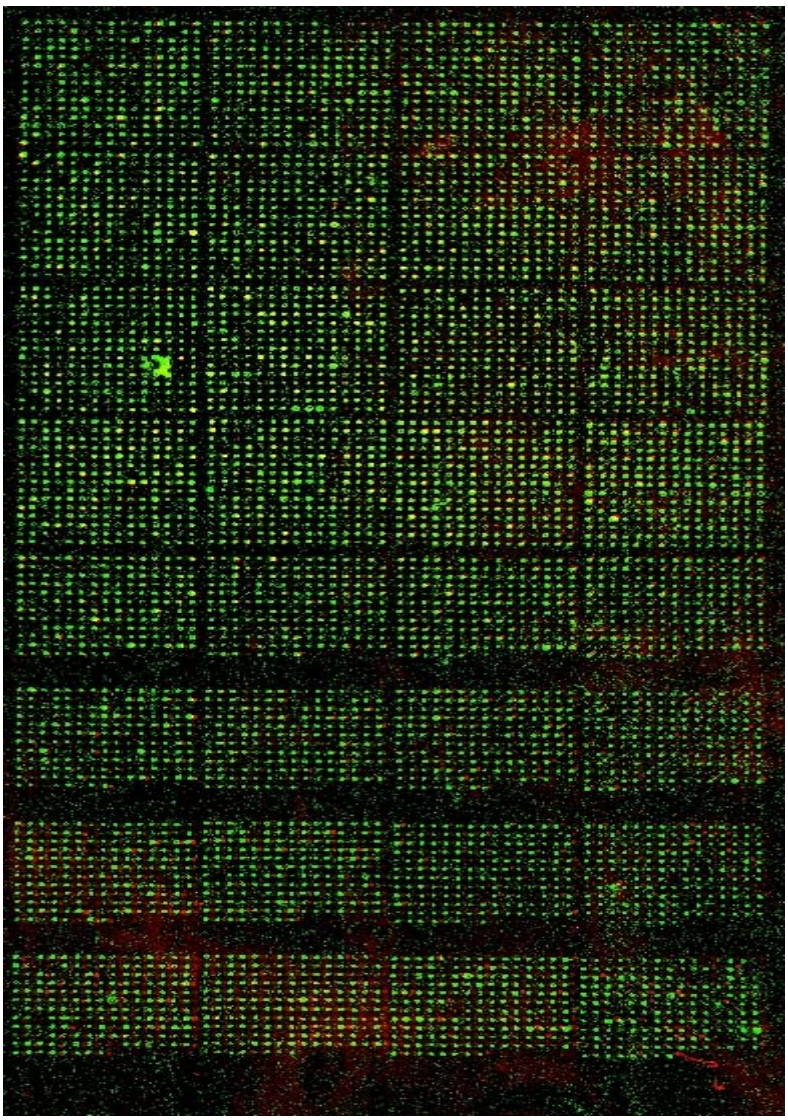


Figure (4.25): Yeast (*S. Cerevisiae*, wild type, H620) whole genome expression for cells in S-phase which were labelled with cy3 and cy5, the cells was taken from 2 L- bioreactor continiuous culture.

The yeast whole genome chip consists of 6250 oligonucleotides of specific genes. The above experiment was carried out twice. The image processing and analysis were performed with commerial software Imagene (by Dr Klockow, during her Ph.D-work in Institute of Technical Chemistry, Hanover University). 5062 genes were analyzed with the program to examine the regulatory behaviour, the rest of genes were either empty places or the software was not able to analyze them. In the first experiment 1364 genes showed a regulatory behaviour, of which 433 genes were up regulated and 931 genes were down regulated when G1-phase was analyzed against S-phase.

In the second repeated experiment, 1935 gene showed a regulatory behaviour, of which 502 genes were up regulated and 1433 were down regulated. It was found that 532 genes were reproduced in both experiments as regulatory genes, of which 130 genes were up-regulated and 402 were down regulated. Spellman *et al*., 1998 created a comprehensive catalogue of 800 cell cycle regulated genes which constituted 10% of all protein-coding genes in yeast genome. The transcription level of these genes varied periodically within the cell cycle as detected with using microarray hybridization technology. 90 genes of these 800 genes were identified also during this research as regulatory genes among the 532 reproducible genes. Depending on these results, these genes were used on further spotting of low density microarray for further cell cycle regulatory behaviour monitoring.

Table (3): A List of regulated genes during G1 and S-phases produced using yeast whole genome microarray analysis for a synchronous continuious culture of *S. Cerevisiae*

Systemic Name/ Standard Name	Gene description	Ratio G1/S (first experiment)	Ratio G1/S (second experiment)
Metabolic genes			
YBL064C/PRX1	Mitochondrial peroxiredoxin	2.29	3.03
YBL098W/BNA4	Kynurenine 3-mono oxygenase	-2.44	-2.66
YBR256C/RIB5	Riboflavin synthase	2.22	4.41
YDL093W/PMT5	Protein O-mannosyltransferase	-3.83	-2.25
YDR400W/URH1	Uridine nucleosidase	-2.67	-3.07
YGR055W/MUP1	High affinity methionine permease	-8.97	-3.53
YGR234W/YHB1	Nitric oxide oxidoreductase	-3.61	-6.00
YKR069W/MET1	S-adenosyl L-methionine urophyringen III transmethylase	-3.18	-4.59
YLR180W/SAM1	S-adenosylmethionine synthetase	-3.55	-1.69
YLR214W/FRE1	Ferric reductase and cupric reductase	-2.43	-3.44
YML125C/PGA3	Essential protein required for maturation of Gas1p and Pho8p and protein trafficking	-2.04	-2.40
YOL101C/IZH4	Membrane protein involved in zinc metabolism	-2.33	-3.85
YOR074C/CDC21	Thymidylate synthase	-2.43	-2.75
YOR049C/RSB1	Suppressor of sphinogolipid long chain base sensitivity	-2.34	-4.62
YPL095C/EEB1	Acyl-coenzymeA:ethanol O-acyltransferase	-4.19	-2.44
Cell wall organization and biosynthesis genes			
YBR067C/TIP1	Major cell wall mannoprotein	2.26	8.39
YDR055W/PST1	Cell wall protein that contains GPI attachment site	2.12	3.82
YBR158W/AMN1	Protein required for chromosome stability and daughter cell separation	2.36	3.54

YFL039C/ACT1	Actin	1.55	1.84
YHR029C/YHI9	Protein involved in a membrane regulation metabolic pathway	2.79	4.30
YKL096W/CWP1	Cell wall mannoprotein	3.80	3.18
YLR343W/GAS2	1,3-beta-glucanosyltransferase	-2.63	-2.98
YLR286C/CTS1	Endochitinase	4.58	4.09
YLR380W/CSR1	Phosphatidylinositol transfer protein with a potential role in lipid turnover	1.19	1.59
YNL160W/YGP1	Cell wall related secretary glycoprotein	13.84	8.89
Vacuole related gene			
YCL063W/VAC17	Protein involved in vacuole inheritance	-3.74	-3.05
Transcriptional genes			
YCR040W/MATALPHA1	Transcriptional co-activator	-3.78	-2.11
YDR451C/YHP1	One of two homeobox transcriptional repressors	-2.58	-2.53
YJL201W/ECM25	Unkown protein ; promoter contains a binding sequence for global transcription factor (Abf1p)	-2.27	-3.67
YNR009W/NRM1	Transcriptional co-repressor of MBF (MCB binding factor)-regulated gene expression	-3.44	-5.83
YOL017W/ESC8	Protein involved in telomeric and mating-type locus silencing in addition to interaction with a component of the RNA pol II mediator complex	-2.16	-5.18
Mitotic genes			
YDL003W/MCD1	Essential protein required for sister chromatid cohesion	-2.61	-2.89
YLR286C/CTS1	Homolog of nuclear distribution factor NudE, NUDEL	2.44	2.99
Protein kinases genes			
YDR247W/VHS1	Cytoplasmic serine/threonine protein kinase that plays a role in G1/S progression	2.36	4.52
YDR507C/GIN4	Protein kinase involved in bud growth	-2.13	-4,01
YPL209C/IPL1	Aurora kinase involved in regulating kinetochore- microtubule attachments to chromosomes	-3.06	-3.17
DNA replication genes			
YGR109C/CLB6	B-type cyclin involved in DNA replication during S phase	-2.27	-3.93
YJR043C/POL32	Third subunit of DNA polymerase, involved in chromosomal DNA replication	-2.22	-3.06
YKL108W/SLD2	Protein required for DNA replication, phosphorylated in S-phase by Cdks	-3.2	-2.48
YPR120C/CLB5	ß-type cyclin involved in DNA replication during S-phase. Activates Cdc28p to promote initation of DNA synthesis	-3.46	-10.61
Chromatin assembly gene			
YPR018W/RLF2	Largest subunit (p90) of the chromatin assembly complex	-5.21	-4.01
Cdc28p substrate			
YGR014W/MSB2	Mucin family member	-2.34	-2.85
YGR035C	potential Cdc28p substrate	-2.81	-2.04

YML119W	Potential Cdc28p substrate	-2.16	-2.43
YKL185W/ASH1	Zinc finger inhibitor of HO transcription; potential Cdc28p substrate	3.90	6.16
YOR188W/MSB1	Protein involved in positive regulation of 1,3 beta glucan synthesis, potential Cdc 28p substrate	-2.43	-7.73
Cell cycle progression genes			
YGR108W/CLB1	B-type cyclin involved in cell cycle progression	-4.87	-5.92
YHR127W	Uncharacterized protein, localizes to the nucleus	-2.41	-3.96
YHR152W/SPO12	Nucleolar protein of unknown function, positive regulator of exit from mitosis	2.23	2.02
YPR119W/CLB2	ß-type cyclin involved in cell cycle progression	-4.05	-10.56
DNA repair genes			
YDR545W/YFR1	Helicase encoded by the subtelomeric regions	3.75	3.28
YHR154W/RTT107	Protein implicated in Mms22-dependent DNA repair during S phase	-2.24	-4.04
YJL092W/SRS2	DNA helicase and DNA-dependent ATPase involved in DNA repair	-2.80	-2.29
YHR173C	Dubious ORF unlikely to encode a functional protein	-5.98	-4.12
YIL121W/QDR2	Multidrug transporter, plays a role in potassium uptake	-3.32	-2.20
YML021C/UNG1	Uracil-DNA glycosylase, required for repair of uracil in DNA	-3.01	-2.21
YMR078C/CTF18	Subunit of a complex with Ctf8p that shares some subunits with Replication Factor C and play a role in DNA damage replication checkpoint	-2.12	-2.53
YNL273W/TOF1	Subunit of a replication-pausing checkpoint complex (Tof1p-Mrc1p-Csm3p)	-2.11	-2.80
RNA synthesis			
YPR187W/ RPO26	RNA polymerase, core subunit	-3.54	-4.50
Spindle pole genes			
YJL018W/MPS3	Essential integral membrane protein required for spindle pole body duplication	-9.99	-3.07
YNL126W/SPC98	Component of the microtubule-nucleating Tub4p (gamma-tubulin) complex	-2.39	-2.50
YOR129C	Putative component of the outer plaque of the spindle pole body	-2.98	-4.44
YPL124W/SPC29	Inner plaque spindle pole body component	-4.69	-2.39
YPL255W/BBP1	Protein required for spindle pole duplication	-2.85	-2.68
Protein folding genes			
YOL088C/MPD2	Member of the protein disulfide isomerase family , exhibits chaperone activity	-2.01	-2,23
YOR391C/HSP33	Possible chaperone and cysteine protease	2.93	1.17
Proteolytic gene			
YFL006W/BLM10	Proteosome activator subunit	3.30	3.12

YLR373C/VID22	Glycosylated integral membrane protein	-2.08	-3.01
Histone modification genes			
YOR025w/HST3	Member of the Sir2 family ofNAD$^+$ dependent protein deacetylases	-3.67	-3.09
YPL116W/HOS3	Trichostatin A-insensitive homodimeric histone deacetylase with specificity for histones	-2.12	-4.84
Pathogen related genes			
YJL079C/PRY1	Pathogen related protein	2.50	2.53
YKR013W/PRY2	Pathogen related protein	-2.26	-3.06
Genes of Uncharacterized proteins			
YEL068C	Hypothetical protein	-5.88	-2.32
YIL025C	Hypothetical protein	-2.90	-2.84
YJL181W	Putative protein of unknown function; expression is cell-cycle regulated	-3.78	-2.59
YKL183W/LOT5	Protein of unkown function, its gene expression increases at lower temperature	-2.04	-3.05
YKR012C	Hypothetical protein	-2.29	-3.18
YKR013W/PRY2	Pathogen related protein	-2.26	-3.06
YKR046C/PET10	Protein co-purifies with lipid particles and suggests a role in respiratory growth	2.14	3.69
YLR040C	Hypothetical Protein	-20.23	-7.68
YLR169W	Hypothetical Protein	-4.59	-9.96
YLR326W	Hypothetical protein	-3.61	-2.54
YLR413W	Putative protein of unknown function	-20.56	-3.56
YLR465C/BSC3	Dubious open reading frame	-2.04	-4.19
YML034C	Dubious ORF	-3.90	-6.81
YMR031C	Non-tagged protein is detected in a phosphorylated state in highly purified mitochondria	2.78	2.88
YMR144W	Hpothetical protein	-2.47	-4.12
YOL114	Hypothetical protein	-3.52	-3.69
YOR052C	Hypothetical protein	2.79	2.09
YPR013C	Hypothetical protein	-7.60	-5.10

+ Value means up-regulated in G1, i.e down-regulated in S-phase
- Value means down-regulated in G1, i.e up-regulated in S-phase

In yeast cells, mechanisms ensure cell-cycle events of S-phase and DNA synthesis are initiated only after the activation of events normally restricted to the G1 phase of the cell cycle. The yeast cells must reach a critical size during the G1-phase before entering the synthesis phase. The functional classes of genes that showed periodic fluctuation in their regulatory behaviour, as shown in the above table, mirrored the events that occurring throughout the regulated *S. cerevisiae* cell cycle including cell wall biogenesis, cell separation, DNA synthesis/ folding and repair, bud emergence and growth, spindle pole body duplication, and vacuole inheritence.

4.4.1.1 Yeast cell wall biogenesis

Yeast *Saccharomyces cerevisiae* has a rigid cell wall outside its cell membrane. In *S. cerevisiae*, the cell wall makes 30% of the dry weight of the cell and 25 to 50% of the volume based on calculations from electron micrographs (Orlean, 1997). The cell cycle was arrested for a while at the G1 phase and cell wall synthesis was promoted (Mera *et al.*, 2004). During cell division in yeast, cell surface expansion occurs asymmetrically before bud emergence since it should be remodeled in response to the new different physiological states (e.g., budding, mating, and sporulation). The monitoring of offline results of *S. cerevisiae* biomass (cell dry weight) during this research work showed the maximum values during G1-phase, which can be explained to be due to the up regulation of most of genes of cell wall biosynthesis in G1. It was found in the above Table (3) that genes of major cell wall mannoproteins (YBR067) (YKL096W), cell wall protein that contain glycosylphospatidyl inositol attachment site (YDR055W), cell wall secretory glycoprotein (YNL160W) were up regulated during G1 phase to ensure that yeast cells are grown enough and protected from any further mechanical injury, hypotonic lysis, or chemical substances that could damage the cells. Phosphotidylinsotitol transfer protein (YPL380W) was seen also to be up-regulated in G1-phase since it plays a role in cell wall organization and biogenesis. Actin (YFL039C) showed aslo the same expression pattern since it provides the structural basis for cell polarity in *S.cerevisiae* (Pruyne & Bretscher 2000). Actin patches are implicated in maintaining cell wall integrity and endocytotic internalization (Kaksonen *et al.*, 2003).

4.4.1.2 Yeast cell separation

For cell separation, the chitinous primary septum synthesized by Chs2p has to be dissolved. Yeast possesses a single endochitinase responsible for this process. Since excessive chitinase activity might perturb cell wall integrity, correct timing and localization of its activity are important. For correct timing of chitinase activity, transcriptional regulation is required. *CTS1*, the gene encoding chitinase, is transcribed very early in the G1 phase of the cell cycle, an action controlled by the transcription factor Ace2p (Smits et al. 2001). For this reason endochitinase (YLR286C) appeared to be up-regulated in G1-phase in comparison with S-phase in the above results.

YBR158W is a protein required for the optimal growth of yeast cells since it checks accuretly the daughter cell separation and chromosome stability during G1-phase before entering the S-phase, it was normally to be found up-regulated during G1 in the above results.

4.4.1.3 Yeast DNA replication

During S-phase, the deoxynucleotides required for DNA synthesis can either be made de novo, or they can be salvaged from endogenous degradation processes. *Saccharomyces cerevisiae* contains three enzymes that are able to salvage deoxycytidine in vitro. However of these three enzymes, uridine nucleosidases (YDR400W/UHR1) has been shown to metabolize deoxycytidine in vivo (Kurtz *et al.*, 2002) and showed up-regulation during S-phase in Table (3) When yeast cells were grown in media containing ethanol instead of glucose, Uridine nucleosidase specific activity increased ten times its original value (Mangi, 1977). Ethanol was detected only in this research work closely to the beginnig of the S-phase in the mean time of the uridine nucleosidase up-regulation.

The ß-type cyclins (YGR109C and YPR120C) involved in DNA replication during S-phase showed also down-regulation behaviour during G1-phase since they activate Cdc28, the main yeast cell cycle kinase, to promote initation of DNA synthesis.

YKL108W / SLD2 is a Protein required for chromosomal DNA replication in S. *cerevisiae*, which is phosphorylated in S-phase by S-phase cyclin-dependent kinases, phosphorylation is essential for DNA replication. It was meaningful to be up-regulated in S-phase in this research results to form Dpb11-Sld2 complex in an important step close to the initiation of DNA replication (Kamimura *et al.*, 1998).

4.4.1.4 Yeast DNA repair

Many DNA polymerases are able to correct mistakes in DNA by removing mismatched nucleotides. These polymerases have a distinct nuclease activity that allows them to excise incorrect bases. This nuclease activity contributes to remarkably high fidelity of DNA replication, which has an error rate of less than 10^{-8} per base pair (Berg *et al*., 2006). Of these polymerases, the third subunit of DNA polymerase delta (YJR043C/POL32), which involves in chromosomal DNA replication, showed a down regulation behaviour during G1-phase (up-regulated during S-phase) in the above results.

Mms22p is a protein which protects *Saccharomyces cerevisiae* from DNA damage induced by topoisomerase II (Baldwin *et al*., 2005). The protein (YHR154W/RTT107) which interacts with Mms22p and is implicated in Mms22-dependent DNA repair during S-phase was down-regulated in G1-phase and in turn up-regulated in S-phase, as it is clear in the above table. This ensures the presence of the repairing mechanism in the mean time of the

DNA synthesis during the S-phase, since also uracil-DNA glycosylase (YML021C/UNG1), which is required for repairing uracil in DNA was up-regulated during S-phase, moreover, (YNL273W/TOF1), a subunit of a replication-pausing checkpoint complex (Tof1p-Mrc1p-Csm3p) showed also the same expression behaviour.

DNA helicases are enzymes that unwind a region of duplex DNA through the disruption of the hydrogen bonds that hold the two strands of the duplex together. These enzymes usually act in concert with other enzymes in DNA metabolic activities such as replication, repair, or recombination and are thought to open the DNA helix in preparation for the DNA replication or repair complex to act on regions of single stranded DNA (Matson and Kaiser-Rogers, 1990; Matson, 1991). It was logic depending on these obervations to find the yeast DNA helicase (YJL092W/ SRS2) down-regulated during G1-phase (up-regulated during S-phase) in the data of the above table while another helicase (YDR545W/ YFR1) expressed an opposite behaviour to ensure that the repair system of DNA was functionally active either in G1 or S-phase.

4.4.1.5 Yeast kinases

S. cerevisiae protein kinases regulate all the steps of the cell cycle and its progression precisely. Out of these kinases, in the above gene expression experiment, Cytoplasmic serine/threonine protein kinase was up-regulated during G1 to facilitate the progression of the cells into S-phase after the processes of G1 were completely finished.

Since bud growth and emergence take place during S-phase. It was of significance that protein kinase involved in bud growth (YDR507C/GIN4) to be found down-regulated during G1-phase.

Aurora kinase showed also the same behaviour of up regulation during S-phase in the above results, since its function began to take place after the chromosomal replication through regulating the process of attachment of microtubules to the newly formed chromosomes via kinetochores, a key step before chromosomal segregation (Buvelot *et al*., 2003).

4.4.1.6 Histones and DNA folding

Histones are the chief protein components of chromatin.They act as spools around which DNA winds and they play a role in gene regulation. Without histones, the unwound DNA in chromosomes would be very long. Largest subunit (p90) of the Chromatin Assembly

Complex (YPR018W/RLF2) that assembles newly synthesized histones onto recently replicated DNA showed the down-regulation behaviour during G1-phase and the up-regulated one during S-phase. This is an essential process during the DNA synthesis. Histone modifications act in diverse biological processes such as gene regulation, DNA repair and chromosome condensation (Strahl and Allis, 2007). One of these modifications is deacetylation, which was shown though histone deacetylase (YPL116 W /HOS3) expression in the above microarrays to be up-regulated during S-phase because histone acetylation weakens the association of histones with DNA, thereby altering nucleosomal conformation and stability (Struhl, 1998).

4.4.1.7 Yeast DNA methylation

Many genes involved in methionine biosynthesis are cell cycle regulated (Spellmann *et al.*, 1998). Indeed, Unger and Hartwell (1976) noted that starvation for sulfur or for methionine effectively causes G1 arrest, suggesting that cell cycle progression is particularly sensitive to the availability of methionine. High affinity methionine permease (YGR055/ MUP1) showed up-regulated behaviour during S-phase, as shown in the above results. S-adenosylmethionine (SAM) is the main donor of methyl groups for methylation of nucleic acids which is synthesized in S-phase (Patton *et al*., 2000), so the down-regulation behaviour of SAM synthetase (YLR180W/SAM1) during G1 phase was of significance in the above Table (3) , since the nucleic acid synthesis process was begun only during S-phase.

4.4.1.8 Spindle pole duplication

The spindle pole body (SPB) duplication, which is essential for nuclear division, is a controversial issue in *S. cerevisiae* concerning its timing. In these experiments, all the genes concerned with SPB duplication (YJL018W, YNL126W, YOR129C, YPL124W, and YPL255W), were down-regulated during G1 which means that the process of SPB duplication was promoted in the mean time of DNA duplication (S-phase). Byers and Goetsch, 1975 reached to a finding through electron microscopy analysis that SPB duplication was completed during S-phase. This conclusion was ascertained again by Adams and Kilmartin, 1999. In 2002, Horak *et al.,* used the microarray hybridization technology and stated that SPB duplication was concomitant with G1/S transition stage, on the other hand Spellmann *et al*., 1998 noticed that the process of SPB duplication began during G1 where six known components of SPB reached peak expression during G1 and only one component peaked during S-phase. Depending on these results, it can be concluded that the SPB duplication process may take place at extreme boarder line between the two phases to the

extent that its gene pattern of expression and regulation differ from one experiment to another depending on uncharacterized factors like the yeast cell size or its nutritional state which promote the beginning of the process either in late G1 or earlier S-phase

4.4.1.9 Vacuole inheritance

Organelle inheritance is tightly regulated during the cell cycle. Vacuole inheritance initiates early in the cell cycle and ends in G2 (Weisman, 2003). In early S-phase, the maternal vacuole projects a striking array of tubular-vesicular material, termed the segregation structure, into the bud (Weisman and Wickner, 1988). This vacuolar exchange continues until the onset of M-phase. The protein involved in vacuole inheritance (YCL063W/ VAC17), in the above table showed up-regulated behaviour during S-phase to accelerate the vacuole inheritance process.

The above results of gene expression using the yeast whole genome commercial microarry gave a real picture of G1 and S-phase events, through illustrating the regulatory behaviour of the expressed genes, their biological roles and the manner in which the encoded proteins carry out cellular processes which are of great importance in the control of cell cycle complicated pattern. More importantly, the above 90 genes have been used successfully in production of low denisty oligonucleotides microarray to monitor the gene expression and the regulatory pattern during the cultivation process of *Saccharomyces cerevisiae*.

4.5 Production of specific low density oligonucleotide microarrays

The oligonucleotides of about 90 cell cycle regulated genes in the previous Table (3), in addition to 50 genes (chosen in co-operation with department of environmental microbiology, Helmholtz center, Leipzig, Germany) concerned mainly with glucose metabolism, listed in Appendix (7.2), were used for this purpose. These 70-mer oligonucleotides were mixed with Arraylt Micro spotting plus buffer in a ratio of 1:1 and spotted in triplicates on the silylated glass slides using Affymetrix 427 arrayer, as shown in Figure (4.26). These biochips were ready for hybridization to follow up yeast gene expression in G1 and S-phase in addition to glucose metabolism (the main carbon and energy source in the chemostat during the *S. cerevisiae* cultivation.)

Figure (4.26): The Affymetrix 427 arrayer applies the ring and pin spot deposition technology whereby it is able to spot 42 slides at one time, with capacity up to 5000 spot per slides. The oligonucleotides are in 96 well microplates. The ring captures aliquots from the well and the pins deposit a fraction of fluid directly onto a slide surface (biochip).

4.5.1 Monitoring of gene expression and regulation of *S. cerevisiae* inside the 10L chemostat using the new spotted low denisty oligonucleotides microarray

The spotted low density oligonucleotide microarray monitored the gene expression and regulation during G1 and S-phase of three sequential cycles of *S.cerevisiae* cultivated continuously in 10 L chemostat. The samples were collected from inside the bioreactor after the stability of oscillation from 221.9 h till 228 h as shown in Figure (4.13). A total of 6 µg of RNA per sample were used for each analysis, RNA was reverse transcribed into cDNA in order to be hybridized directly onto the surface of the microarray. Indirect labelling by the tyramide signal amplification was used to increase the cy3 and cy5 signals on the microarray.

Differences in gene expression between G1 and S-phase were quantified by scanning the hybridized microarrays with Axon 4000B scanner and followed by image analysis using the GenePix pro 6.0 software.

4.5.1.1 The results of gene expression in the first examined cell cycle

The results of flow cytometric analysis of the collected samples of the first examined cycle showed that *S.cerevisiae* cells were in late G1 and these cells were in their way to S-phase which reached optimum value after 45 min (Figure 4.13). The sample which was taken from inside the bioreactor at 221.9 h from the beginning of the cultivation represented G1 (containing 77.5% cells in G1). The sample, which showed optimum percentage of cells in S-phase (53.9%), was collected from the bioreactor at 222.65 h and represented S-phase in the microarray analysis. The gene expression of cells in G1 was tested against S-phase using the self spotted low density oligonucleotide microarray as designed in Figure (4.27) and shown in Figure (4.28).

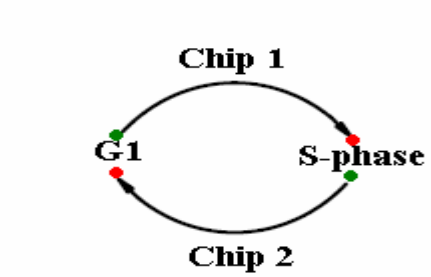


Figure (4.27): The design of the experiments involved 2 direct comparisons, where G1 and S-phase were hybridized together on the same slides with dying each phase with different dye in each chip. Green symbol denotes cy3 and the red one denotes cy5

Chip1 **Chip2**

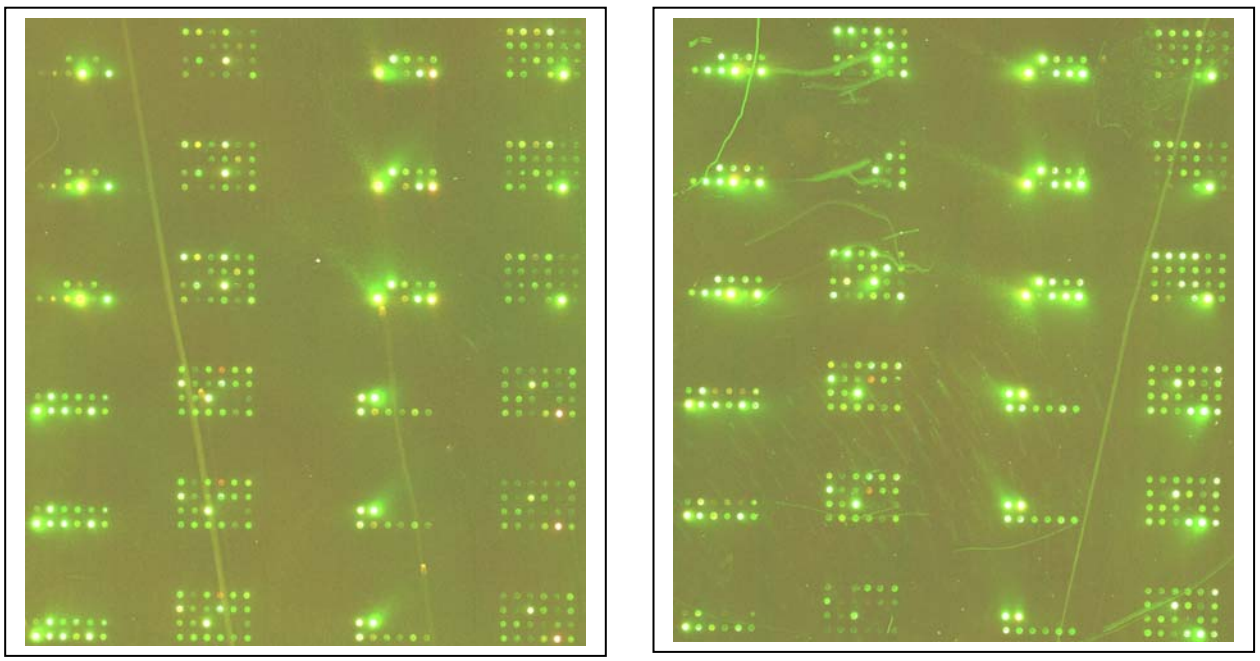


Figure (4.28): Microarray scan for the gene expression of the first examined yeast cell cycle during G1 and S-phases, chip 1 represents G1 dyed with flurosenated cy5 against S-phase dyed with biotinated cy3, chip 2 is vice versa. 6 µg of total RNA per sample was used. The signals were amplified with TSA method.

The results of the above presented experiment showed the expression of 96 genes, of which 10 genes showed a regulatory behaviour, as obtained from data analysis of gene expression of G1 against S-phase.

Table (4): The list of regulated genes during the first studied yeast cell cycle, cultivated in 10 L chemostat

Gene ID	Gene description	Type of regulation	Ratio (G1/S)
Metabolic genes			
YKL085W	Malate dehydrogenase	-	-2.15
YLR304C	Aconitase	-	-4.89
YFR053W	Hexokinase1	-	-3.88
Q0250	Cytochrome c oxidase subunit II	+	3.27
YDR001C	Trehalase	+	3.19
YH096C	Hexose transporter with moderate affinity to glucose	+	2.54
Cell wall biogenesis gene			
YLR343W	Putative 1,3 beta glucanosyltransferase	+	2.55
DNA repair gene			
YHR154W	Protein that interacts with Mms22p	+	4.03
Spindle pole body duplication gene			
YJL018W	Essential integral membrane protein for spindle pole duplication	+	1.63
YGR035C	**Uncharacterized protein**	+	4.92

+ Value means up-regulated in G1, i.e down-regulated in S-phase
- Value means down-regulated in G1, i.e up-regulated in S-phase

- **Metabolic gene behaviour**

As it was clear from Figures (4.12 & 4.13), that NADH was high in the mean time of collecting G1 sample. The rate of citric acid cycle is decreased in presence of high level of NADH, and this in turn, leads to down regulation of some enzymes of citric acid cycle like aconitase and malate dehydrogenase, as shown in Table (4). NADH is the most important electron donor in the respiratory chain reaction of yeast to generate ATP. Cytochrome c oxidase is one of three large protein complexes through which the electrons are transferred

from NADH to oxygen. It was of significance to find cytochrome c oxidase subunit II to be up regulated in G1 since the level of NADH was high in the mean time. As shown in Figure (4.11), the concentration of glucose began to increase to 35 mg/L in the cultivating media inside the bioreactor, in the mean time of collecting G1 sample. The reduced glucose consumption is obviously caused by cells without a bud. The reason might be the activation of internal storage carbohydrate like trehalose and glycogen (Muench *et al*., 1992 & strandinov *et al*., 1992). The trehalase (YDR001C) is the enzyme which catalyzes the conversion of trehalose to glucose, it was logic depending on these previous observations to find it up-regulated in late G1. The increased expenditure of trehalose could cause reduction on glucose consumption during certain periods of yeast cell cycle.

- **Cell wall maintenance**

1,3 beta glucanosyltransferase is involved in cell wall assembly and maintenance which take place completely during G1 phase. It was of significance to find the gene of this enzyme to be up regulated in the above results.

- **DNA repair**

Protein that interacts with Mms22p , play a role in DNA repair during S-phase, but in the above results, this protein showed an up regulatory behaviour during G1 which gives an indication that the yeast cells have a repair and check system for the DNA during all the phases of the cell cycle , not only during the DNA synthesis in S-phase.

- **Spindle pole duplication**

YJL019W, the integral membrane protein required for spindle pole duplication, showed up-regulatory behavior during G1 in the above results. This ensures that the spindle pole duplication process may begin in G1 and complete itself in the beginning of the S-phase.

4.5.1.2 The results of Gene expression in the second examined cell cycle

The sample which was taken from inside the bioreactor at 224.01 h from the beginning of the cultivation represented G1 (containing 89.72 % cells in G1). The sample, which showed optimum percent of cell in S-phase (62.5%) during this cycle, was collected from the bioreactor at 225.767 h and represented S-phase in the microarray analysis. The gene expression of cells in G1 was tested against S-phase using the self spotted low density oligonucleotide microarray as designed in Figure (4.27) and shown in Figure (7.1). The results of this experiment showed the expression of 94 genes, of which 22 genes showed a regulatory behaviour listed in Table (5).

Table (5): The list of regulated genes during the second studied yeast cell cycle, cultivated in 10 L chemostat

Gene ID	Gene description	Type of regulation	Ratio (G1/S)
Metabolic genes			
YGR240C	α-subunit of 6 phosphofructokinase	+	2.70
YJL155C	Fructose 2,6 bisphosphatase	+	3.49
Q0250	Cytochrome C oxidase	+	7.73
YMR105C	phosphoglucomutase	+	4.98
YLR258W	Glycogen synthase	+	7.77
YBR126C	Trehalose 6 phosphate synthase	+	6.78
YMR303C	Alcohol dehydrogenase II	-	-2.70
DNA replication			
YJR043C	Third subunit of DNA polymerase (delta)	-	-3.6
DNA repair			
YHR154W	Protein that interacts with Mms22p	-	-2.16
YNL273W	Subunit of a replication-pausing checkpoint complex (Tof1p-Mrc1p-Csm3p)	+	2.00
YJL092W	DNA helicase and DNA-dependent ATPase involved in DNA repair	-	-6.03
Chromatin assembly gene			
YPR018W	Largest subunit (p90) of the chromatin assembly complex	-	-2.18
RNA synthesis			
YPR187W	RNA polymerase subunit (part of central core)	-	-6.54
Proteolytic gene			
YLR373C	Glycosylated integral membrane protein	-	-4.12
Protein kinase			
YDR507C	Protein kinase involved in bud growth	-	-6.85
YJL164C	Putative cAMP dependent protein kinase catalytic subunit	+	2.76
YPL203W	cAMP dependent protein kinase catalytic subunit	-	-4.03

Cell cycle progression			
YGR108W	B-type cyclin involved in cell cycle progression	+	5.04
Spindle pole body duplication			
YPL255W	Protein required for spindle pole duplication	-	-4.76
YPL124W	Inner plaque spindle pole body component	+	5.32
Transcription regulatory genes			
YDR451C	One of two homeobox transcriptional repressors	+	3.51
Cell wall biogensis			
YLR380W	Phosphatidylinositol transfer protein with a potential role in lipid turnover	+	2.40

+ Value means up-regulated in G1, i.e down-regulated in S-phase
- Value means down-regulated in G1, i.e up-regulated in S-phase

- **Metabolic gene behaviour**

As it is clear from Figure (4.11), the glucose level during collecting G1 cells of the tested sample was near to the baseline, which gave an indication that the cells were mainly using glucose as its main energy source. In the above Table (5), α-subunit of 6 phosphofructokinase (YGR240C), a key enzyme of glycolysis that catalyzes the formation of fructose 1,6 bisphosphate from fructose-6-phosphate and ATP (Heinisch, 1986), showed up regulatory behaviour. The enzyme fructose 2,6 bisphosphatase (YJL155C), which is one of the key enzymes of glucose metabolism, was also clearly up regulated in G1 cells, since it regulates glycolysis and gluconeogenesis pathways according to the need of cells and the availability of glucose. The complete oxidation of glucose to produce ATP, needs NADH which is produced during different steps of glycolysis and Krebs cycle to pass through the respiratory chain reaction, so it was logic to find Cytochrome C oxidase, one of the main protein complexes in the chain, to be up regulated during G1.

In G1, yeast cells oxidize glucose to grow by respiration and they also store glucose as glycogen and trehalose, but in late G1, the cell's stores of glycogen and trehalose are suddenly liquidated to glucose (Futcher, 2006). In the above results the genes concerned with glycogen and trehalose synthesis (glycogen synthase (YLR258W) & Trehalose 6 phosphate synthase (YBR126C)) were up regulated which gave an indication that the cells were not in late G1-phase. Phosphoglucomutase (YMR105C) transfers phosphate group on glucose from the 1' to the 6' position in the forward direction or the 6' to the 1' position in the reverse, which is a very impotant step either in glycogen synthesis or degradation. This enzyme was up-regulated in G1, as clear in the above table.

Ethanol was detected mainly inside the bioreactor during S-phase, which ensured that the cells produced ethanol and used it in the same phase. The enzyme alcohol dehydrogenase (YMR303C) which catalyzes the initial step in the utilization of ethanol as a carbon source, showed down regulatory behavior during G1, i.e, it was up-regulated during S-phase in the mean time of ethanol presence inside the bioreactor.

● Nucleic acids synthesis and repair

The third subunit of DNA polymerase delta (YJR043C) which involves in chromosomal DNA replication, showed a down regulation behaviour during G1 (up-regulated during S-phase where the DNA synthesis takes place). Largest subunit (p90) of the Chromatin Assembly Complex (YPR018W) that assembles newly synthesized histones onto recently replicated DNA showed the same behaviour of up-regulation during S-phase.

The protein (YHR154W/RTT107) which interacts with Mms22p and is implicated in Mms22- dependent DNA repair during S-phase was down-regulated in G1-phase and in turn up-regulated during S-phase. The DNA helicase (YJL092C) involved in DNA repair was with the same expression pattern in the above table, on contrary to the subunit of replication-pausing checkpoint complex (Tof1p-Mrc1p-Csm3p), which facilitates gap repair of damaged DNA, showed up regulatory expression during G1. These results indicated the presence of repairing system during either G1 or S-phase to correct any mistakes in DNA.

The level of RNA was at its maximum level during S-phase as shown previously in Figure (4.23). The expression pattern of RNA polymerase subunit (YPR187W), part of central core, was up regulated during S-phase, since RNA polymerase synthesizes RNA chains from the newly replicated DNA in the same phase.

● Protein kinases

cAMP/dependent protein kinases regulate various processes involved in cell growth, energy metabolism, carbohydrate metabolism, cell cycle progression and bud site selection. It was meaningful to find in the above table one isoform of the catalytic subunit of cAMP-dependent protein kinase (YJL164C) to be up regulated during G1 and another isoform (YPL203W) to be up regulated during S-phase since these kinases are essential in the phosphorylation of various targets during the yeast cell cycle different phases.

Since bud growth and emergence take place during S-phase, it was of significance that protein kinase involved in bud growth (YDR507C) to be found down-regulated during G1 in the above results.

- **Spindle pole body duplication**

Spellmann *et al*., 1998 noticed that the process of SPB duplication began during G1, in the above results, the inner plaque of spindle pole body (SPB) component (YPL124W) showed up regulatory expression during G1, on contrary to, the protein (YPL 255W) required for the spindle pole body (SPB) duplication, localized at the central plaque showed up regulatory behavior during S-phase. This ensures again that the spindle pole duplication process may begin in G1 and complete itself in the beginning of the S-phase.

4.5.1.3 The results of Gene expression in the third examined cell cycle

The sample which was taken from inside the bioreactor at 227.28 h from the beginning of the cultivation process represented G1 (containing 80% cells in G1). The sample, which showed optimum percent of cells in S-phase (40.8%) during this cycle, was collected from the bioreactor at 228.723 h and represented S-phase in the microarray analysis. The S-phase did not reach its optimum peak when this sample was collected. The gene expression of cells in G1 was tested against S-phase using the self spotted low denisty oligonucleotides microarray as designed in Figure (4.27) and shown in Figure (7.2). The results of this experiment showed the expression of 105 genes, of which 6 genes showed a regulatory behaviour, as obtained from data analysis of gene expression of G1 against S-phase, listed in Table (6).

Table (6): The list of regulated genes during the third studied yeast cell cycle, cultivated in 10 L chemostat

Gene ID	Gene description	Type of regulation	Ratio (G1/S)
Metabolic genes			
YPR160W	Glycogen phosporylase	+	5.78
YMR303C	Alcohol dehydrogenase II	-	-6.88
DNA repair			
YNL273W	Subunit of a replication-pausing checkpoint complex (Tof1p-Mrc1p-Csm3p)	+	2.16
Protein Kinase			
YDR507C	Protein kinase involved in bud growth	-	-7.21

Mitotic gene			
YDL003W	Essential protein reqired for sister chromatid cohesion (subunit of the cohesion complex)	-	-2.94
YGR035C	**Uncharacterized protein**	+	4.35

+ Value means up-regulated in G1, i.e down-regulated in S-phase
- Value means down-regulated in G1, i.e up-regulated in S-phase

● Metabolic genes behaviour

It is likely that both the degradation and synthesis of glycogen occur simultaneously, since loss of glycogen phosphorylase causes hyperaccumulation of glycogen (Wang *et al*., 2001). Glycogen phosphorylase (YPR160W) was up regulated in G1 in the above table. In Figure 4.11, there was a small peak for glucose upon its normal level inside the bioreactor at 227.28 h (the same time of collecting G1 sample from inside the bioreactor). This can be explained by the up-regulatory behavior of glycogen phosphorylase at this time, since it breaks up glycogen into glucose subunits. The increased expenditure of glycogen could cause reduction in glucose consumption and increasing in its level inside the bioreactor.

The level of ethanol had reached 0.8 mg/L at 228.723 h inside the bioreactor during the mean time of collecting S-phase sample (Figure 4.11). It was of significance to find the enzyme alcohol dehydrogenase (YMR303C) which catalyzes the initial step in the utilization of ethanol to be up-regulated during S-phase in the mean time of ethanol presence inside the bioreactor.

● Mitotic gene behaviour

YDL003W, a subunit of cohesion complex, showed an up regulatory pattern of expression during S-phase in the above Table (6). Cohesins are chromosomal proteins that prevent the premature separation of sister chromatids (Michaelis *et al*., 1997). It was a normal behaviour for this protein to peak in S-phase to stop any incorrect separation from the sister chromatid. This protein functions directly on chromosomes morphogenesis from S-phase through mitosis (Guacci *et al*., 1997). The expression behaviours of DNA repair gene (YNL273W) and the Protein kinase involved in bud growth (YDR507C) in the above table were discussed before in details in the previous chips.

In conclusion these experiments demonstrated the potential of oligonucleotide based microarrays to assess the yeast continuous culture in correlation with the metabolic status of the cells. The expression of most genes coincided with the interval during which they functioned. Although it was currently not cost effective to array the whole yeast genome using MWG PAN yeast II array in order to study certain metabolic process during yeast cell cycle events. The results of these experiments showed a successful catalog of genes that can be used easily to generate low density arrays for monitoring, on the gene level, the metabolic activity of yeast cells using glucose as the main carbon source, in combination with cell cycle division inside the chemostat. .

5. Summary

Saccharomyces cerevisiae is often considered the most ideal model eukaryotic microorganism for the studies of genetics and molecular biology (in particular the cell cycle) because it is easy to be cultivated and manipulated under many different conditions, but as a eukaryote, it shares the complex internal cell structure of plants and animals. *Saccharomyces cerevisiae* was the first eukaryotic genome that was completely sequenced and released in electronic form. The yeast genome database is highly annotated and remains a very important tool for developing basic knowledge about the function and organization of eukaryotic cell physiology. Many yeast genes have homologues in human DNA, especially those involved in cell cycle control, meosis and DNA repair, so it has already provided biologists with a valuable resource for determining the function of individual human genes involved in medical problems, such as cancer, neurological disorders, aging and skeletal disorders.

This research study aimed to prepare different types of microarrays for the whole genome expression and specific low density oligonucleotides microarrays to follow up the differential gene expression and regulation for chemostat-cultivated yeast cells during G1 and S-phase events, which are important periods in *S. cerevisiae* cell cycle progression. The *S. cerevisiae* (H620) was cultivated for this aim continuously on the level of 2 liter bioreactor and the whole process was optimized to the level of 10 L.

The budding yeast *S.cerevisiae* exhibit autonomous oscillation, when grown aerobically in continuous culture, with glucose as the primary energy and carbon source, as observed during this study. The cultivation process on the level of 2 L was carried out at pH 5.5, dilution rate of 0.14 h^{-1} and at temperature of 30 °C. The samples collected from inside the bioreactor every 15 min after the stability of oscillation during time 188.5 h till 193.5 h from the beginning of the cultivation. The off-line determination of biomass (cell dry weight) showed a variation in its value between 12.3 g/L and 13.5 g/L, the carbon dioxide production rose up to its highest value in the mean time of the maximum value of biomass which occurred during G1, which gave a good indication for the synchronization of the cells in G1 during that time, since the yeast cells should grow well before the replication process in S-phase. As carbon dioxide production rate decreased to its minimum values, which was also an indicator for the low metabolic activity of the cells as this time, the biomass was also at its lower values (12.3 g/L and 12.7 g/L) where the cells was at G2/M phase. The profiles of carbon dioxide production rate, oxygen uptake rate and respiratory quotient (RQ) revealed

clearly the continuous oscillatory behaviour during the cultivation. RQ values were between 0.8 and 1.6. Glucose was oxidatively metabolized during G1 where RQ values were less than one. At the beginning of the budding process during S-phase, RQ values were increased to greater than one and then decreased again to less than one at the end of this phase, which indicated that the cells directed its metabolism for a short time to the oxidoreductive pathway. The measurement of NADH, FAD^+, and tryptophan dependent fluorescence, using BioView®-Sensor was an important tool for monitoring the cellular activity of yeast. The oscillation peaks of NADH were more indicative in its oscillation behaviour than those of tryptophan (protein) and flavine. The NADH dependent fluorescence began to increase during G1 and reached its maximum during S-phase which can be explained to be due to the increase of cellular activity. The flow cytometric analysis clarified the periodic changes of the cell cycle phases in the 2 L continuous culture of *S.cerevisiae*. The synchronization of the cells in G1 reached its maximum value of 82%, whereas the maximum synchronization percent of cells in S-phase was 53% and that of G2/M was 45%. The samples with maximum contents of G1 and S- phase were used in further production of the yeast whole genome expression chips.

The optimization of the whole process to the level of 10L was carried out under similar conditions (D 0.18 h^{-1} & T 30 °C), except for the pH that was changed to 4.5, which gave a higher stability for the oscillations till the end of the cultivation process. The batch culture of this cultivation showed its 2 characteristic peaks, which were corresponding to glucose and ethanol growth phases respectively. The samples were collected from inside the bioreactor during time 221.9 h till 228 h from the beginning of the cultivation. The biomass ranged between 11.3 g/L and 13.5 g/L and the respiratory quotients were between 0.6 and 1.3 during the cultivation process. The BioView®-Sensor revealed the continuous oscillation of NADH, protein and flavine. Ethanol concentration was determined using gas chromatography GC-14B. Ethanol is not detectable at all during certain periods of the cultivation, but simultaneously with decreasing the percent of dissolved oxygen inside the bioreactor, its concentration increased dramatically to reach sometimes a level of 0.8 g/L. It was evident that the fast production and consumption of ethanol was just in coincidence with the bud emergence, an event that was closely associated to the beginning of S-phase. This ensured that *S. cerevisiae* cells, grown in presence of glucose, retained an activity for the oxidation of ethanol. Moreover, the self sustained oscillations may be as a result of continual shifting between growth on glucose and growth on ethanol (as evidenced by sustained oscillations in RQ). The flow cytometric analysis of the cell cycle inside the 10 bioreactor clarified that the samples were collected from 3 sequential cell cycles. The results showed that the different

phases of the cell cycle were also in oscillation, the synchronization of cells in G1 reached its maximum values of 89.72 % and 80%, whereas the maximum synchronization percent of cells in S-phase were 53.9% and 62.1% and that of G2/M were 51.8% and 40.7%. The samples of highest percent of cells in G1 and S-phase, from this cultivation, were used further in the hybridization of the self spotted low density oligonucleotides microarrays to monitor the gene expression and regulation in the mean time of the cultivation process.

The total RNA was isolated from the samples to be used as a starting material in the hybridization protocol of the microarrays. The quality of the purified RNA was the single most important factor in determining the outcome of microarrays analysis. RNA was purified using hot phenol technique and lyticase lysis method of RNeasy Midi kit. Both methods were fast, suitable and reproducible although the Midi kit gave RNA samples with higher yield and better purity. The highest yeast RNA concentration obtained by Midi-kit, during the 2L cultivation, was 9.4 µg/µl and that of phenol was 7.2 µg/µl. OD 260/280 of the purified samples using Midi-kit were in between 1.9 and 2.1, whereas the purified samples using hot phenol were in range between 1.7 and 1.8. Depending on these results, Midi kit was the protocol of choice in preparing the required RNA for further microarray hybridizations. The quality of RNA was tested using Aliglent 2100 bioanalyzer and 1% agraose gel electrophoresis which ensured the presence of two intact bands at 18s and 28s without any degradation.

The relationship between the RNA concentration of the collected samples and the corresponding cell cycle phase was studied. During the 2 L cultivation, the highest concentration of RNA was in the mean time of S-phase (9.4 µg/µl), whereas the lowest value (3.4 µg/µl) was observed during G2/M. The cells in G1 reached a value of 6.4 µg/µl and 6.3 µg/µl during its 2 peaks respectively. During the 10 L cultivation, RNA concentrations were also at their maximum levels, in the same time of S-phase peaks; moreover, a doubling in the concentration of total RNA had seen once from 5.5 µg/µl during G1 to 10.5 µg/µl during S-phase in the same cycle. DNA synthesis, during S-phase, required the cells to be synthesizing RNA and the process of DNA synthesis is always initiated by the synthesis of short RNA chains by primase enzyme.

The whole yeast genome expression in G1 and S-phase was monitored using samples collected from 2 L continuous culture. The isolated RNA from 2 samples from the same cycle was used. The first sample, which contained 82% cells in G1, represented G1 phase and the

second sample, containing 53% of cells in S-phase, represented S-phase. cDNA was synthesized from 100 µg of total RNA using LabelStar reverse transcriptase and oligo (dt) primers. cDNA was labeled using cy3 or cy5 conjugated CTP. The purified cDNA was hybridized to the commercial whole genome chip (MWG PAN Yeast arrayII). This experiment repeated twice to ensure the reproducibility of the results. In the first time 1364 genes showed a regulatory behaviour, of which 433 genes were up regulated and 931 genes were down regulated, when G1 was analysed against S-phase. In the second repeated experiment, 1935 genes showed a regulatory behaviour, of which 502 genes were up regulated and 1433 genes were down regulated. 532 genes were reproduced in both experiments, of which 130 genes were up regulated and 402 genes were down regulated. 90 genes were selected to be used in further production of specific low denisity oligonucleotides microarrays. These genes concerned mainly with essential regulatory events which took place during cell cycle like cell wall organization and biogenesis, vacuole inheritance, Spindle pole body duplication, DNA replication, DNA repair, chromatin assembly, histone modification and cell cycle progression. These 90 genes were also among the comprehensive catalog of 800 cell cycle regulated genes produced by Spellmann *et al*., 1998, who stated that the transcriptional level of these genes varied periodically within the cell cycle.

70-mer oligonucleotides of these 90 cell cycle regulated genes, in addition to 50 genes (chosen by department of microbiology, Helmholtz center, Lepzig, Germany) concerned mainly with glucose metabolism (main carbon source during the cultivation), was used in the spotting of silylated glass slides using 417 Arrayer, Affymetrix. This self spotted low density olignucleotides microarray was used to monitor the metabolic activity of the synchronized yeast cells in close combination with cell cycle regulatory events. The samples, taken from 10L bioreactor, containing highest percent of G1 and S-phase cells, from three sequential cell cycles, were used to hybridize these self spotted microarray. 6 µg RNA of each sample was reverse transcribed into cDNA in order to be hybridized directly on the surface of microarray. Indirect labeling by tyramide signal amplification was used to increase the cy3 and cy5 signals on the microarray. The result of the first studied cell cycle showed the expression of 96 genes, of which 10 showed a regulatory behaviour, for citric acid cycle enzymes, storage carbohydrates biosynthesis, cell wall assembly and maintenance, spindle pole body duplication and DNA repair system. The results of the second studied cell cycle showed the expression of 94 genes, of which 22 genes showed a regulatory behaviour for the metabolic processes, cell wall biogenesis, DNA replication and repair, protein kinases, cell cycle progression, spindle pole duplication and RNA synthesis. Of great interest was the alcohol

dehydrogenase gene (YMR303C) which showed up regulation behaviour during S-phase, in the same time of ethanol detection inside the bioreactor, which ensured that the yeast cells produced ethanol and used it again during the same phase. The experimental results showed the highest concentration of total RNA inside the yeast cells during S-phase, this finding was also ascertained by the up regulatory behaviour of RNA polymerase core subunit gene (YPR187W) during the mean time of S-phase. The results of the third studied cell cycle showed the expression of 105 genes, of which 6 genes showed a regulatory behaviour concerning metabolic processes, DNA repair, protein kinase involved in bud growth, and finally mitotic process, which was controlled through the up-regulation of cohesion complex sunbunit (YDL003W) during S-phase to prevent any premature incorrect separation of sister chromatid. In conclusion the results demonstrated the potential of the spotted oligonucleotides based microarrays to assess the yeast continuous culture in correlation with the metabolic status of the cells during their division events especially G1 and S-phase. The expression of most genes coincided with the interval during which they functioned.

It is promising to make optimatization for the results of these spotted oligonucleotide microarray by adding more genes to this gene catalog to monitor easily on the gene level the whole cell cycle events during G1, S, and also G2/M. In addition, the seven different phases of batch cultre of *S.cerevisiae,* according to Locher *et al*., 1993, are in need for more further studies using the same technology, since the metabolic background of certain phases is still unclear. The optimization of the protocol of hybridization will be also the next near step by using higher different concentrations of RNA as a starting material, since in this research study, only 6 µg RNA was used and the results was really promising. .

6. References

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7. Appendix

7.1 Abbreviations

BSA	Bovine serum albumin
cDNA	Complementary DNA
cy3	Cyanine 3
cy5	Cyanine 5
D	Dilution rate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dTTP	Deoxy thymidine triphosphate
EDTA	Ethylene diamine tetraacetic acid
em	Emission
ex	Excitation
FAD^+	Flavine adenine dinucleotide (oxidized form)
$FADH_2$	Flavine adenine dinucleotide (reduced form)
g	Gram
h	Hour
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
L	Liter
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
NAD^+	Nicotine adenine dinucleotide (oxidized form)

NADH	Nicotine adenine dinucleotide (reduced form)
NaOH	Sodium hydroxide
nm	Nanometer
OD	Optical density
OUR	Oxygen uptake rate
PBS	Phosphate buffer saline
PI	Propidium iodide
PO_2%	Dissolved oxygen percent
RFI	Relative fluorescence inteni
RISP	Realtime integrating software platform
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolution per minute
RQ	Respiratory quotient
RT-PCR	Reverse transcription polymerase chain reaction
SPB	Spindle pole body
SDS	Sodium dodecyl sulphate
TNB	Tris sodium chloride buffer with blocking agent
TNB-G	TNB with goat serum
TSA	Tyramide signal amplification
U	Unit
µg	Microgram
µl	Microliter
UV	Ultra violet
V	Volt

7.2 List of genes concerned mainly with glucose metabolism *in S.cerevisiae* and used in the spotting of the low density oligonucleotide microarray.

This list of glucose metabolic genes in Table (7) was selected in co-operation with Department of Environmental Microbiology, UFZ, Helmholtz Center, Leipzig, Germany.

Table (7): Genes of metabolic behaviour during glucose metabolosm

Systemic Name/ Standard Name	Gene description	Ratio G1/S (first experiment)	Ratio G1/S (second experiment)
Metabolic genes			
YCL040W/GLK1	Glucokinase	1.05	3.40
YFR053C / HXK1	Hexokinase 1	1.08	-1.30
YGL 253W/ HXK2	Hexokinase 2	-1.28	1.44
YHR094C / HXT1	Low affinity glucose transporter	--	--
YMR011C / HXT2	High affinity glucose transporter	1.17	1.22
YDR345C / HXT3	Low affinity glucose transporter	1.67	1.10
YHR092C/ HXT4	High affinity glucose transporter	--	--
YHR096C/ HXT5	Hexose transporter with moderate affinity to glucose	2.22	6.50
YDR343C/HXT6	High affinity glucose transporter	--	--
YDR342C/HXT7	High affinity glucose transporter	1.02	2.27
YJL214W/HXT8	Hexose permease	-1.81	1.22
YJL219W/HXT9	Hexose permease	--	--
YFL011W/HXT10	High affinity hexose transporter	--	--
YOL156W / HXT11	Glucose permease	1.35	-2.13
YIL170W/HXT12	Putative hexose permease	--	--
YEL069C/HXT 13	High affinity hexose transporter	-9.30	-1.48
YNL318C/HXT14	Hexosetransporter	-5.79	-2.00
YDL245C/HXT15	Hexosetransporter	-8.34	-1.67
YJR158W/HXT16	Hexosepermease	--	--
YNR072W/HXT17	Hexosetransporter	-4.98	-5.78
YDL138W/RGT2	Low affinity glucose receptor	--	--
YDL194W/SNF3	High affinity glucose sensor	1.26	1.43
YLR081W/GAL2	Galactose permease	--	--
YMR105C/PGM2	Phosphoglucomutase	1.79	3.47
YIL107C/ PFK26	6-Phosphofructokinase-2-kinase	--	--
YGR240C/PFK1	α-Subunit of 6 phosphofructokinase	1.15	3.05
YMR205C/PFK2	β- Subunit of 6 phosphofructokinase	1.71	1.21
YLR377C/FBP1	Fructose 1, 6 bisphosphatase	3.46	-3.71
YJL155C/FBP26	Fructose 2, 6 bisphosphatase	1.87	1.26
YER178W/PDA1	α-Subunit of pyruvate dehydrogenase	1.11	2.78

YBR221C/PDB1	β- Subunit of pyruvate dehydrogenase	1.54	1.15
YLR304C/ACO1	Aconitase	1.16	2.35
YKL085W/MDH1	Malate dehydrogenase	2.12	1.05
Q0250	Cytochrome c oxidase subunit II	1.34	1.01
YOL086C/ADH1	Alcohol dehydrogenase I	--	--
YMR303C /ADH 2	Alcohol dehydrogenase II	--	--
YLR258W/GSY2	Glycogen synthase	4.26	1.09
YPR160W/GPH1	Glycogen phosphorylase	--	--
YPR184W/GDB1	Glycogen debranching enzyme	5.63	1.13
YDR001C/NTH1	Neutral trehalase	1.53	2.03
YPR026W/ATH1	Trehalase acid	2.52	2.34
YBR126C/TPS1	Trehalose 6 phosphate synthase	1.44	2.59
YDR074W/TPS1	Trehalose 6 phosphate phosphatase	1.27	1.86
YGL248W/PDE1	Low-affinity cyclic AMP phosphodiesterase	1.44	2.68
YOR360C/PDE2	High-affinity cyclic AMP phosphodiesterase	3.80	3.12
YHR205W/SCH9	Homolog of cAMP dependent protein kinase	1.34	1.01
YJL164C/TPK1	Putative cAMP dependent protein kinase catalytic subunit	3.19	1.01
YPL203W/TPK2	cAMP dependent protein kinase catalytic subunit	5.87	1.40
YKL166C/TPK3	cAMP dependent protein kinase catalytic subunit	-1.95	-4.97
YJL005W/CYR1	Adenylate cyclase	--	--

+ Value means up-regulated in G1, i.e down-regulated in S-phase
- Value means down-regulated in G1, i.e up-regulated in S-phase
--Unexpressed during the experiment according to the data analysis

7.3 The scan of specific low density oligonucleotide microarrays for gene expression of *S.cerevisiae* during G1 and S-phase in 10 L continuous culture

Chip 1 Chip 2

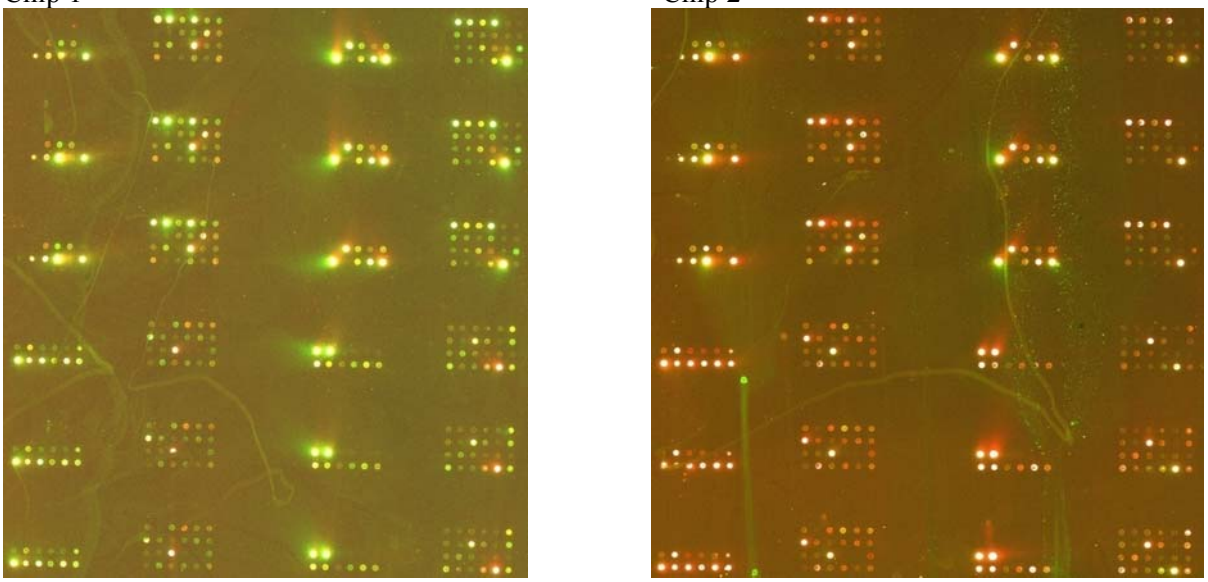

Figure (7.1): Microarray scan for the gene expression of the second examined yeast cell cycle during G1 and S-phase, chip 1 represents G1 dyed with flurosenated cy5 against S-phase dyed with biotinated cy3, chip 2 is vice versa. 6 ug of total RNA per sample were used. The signals were amplified with TSA method

Chip 1 Chip 2

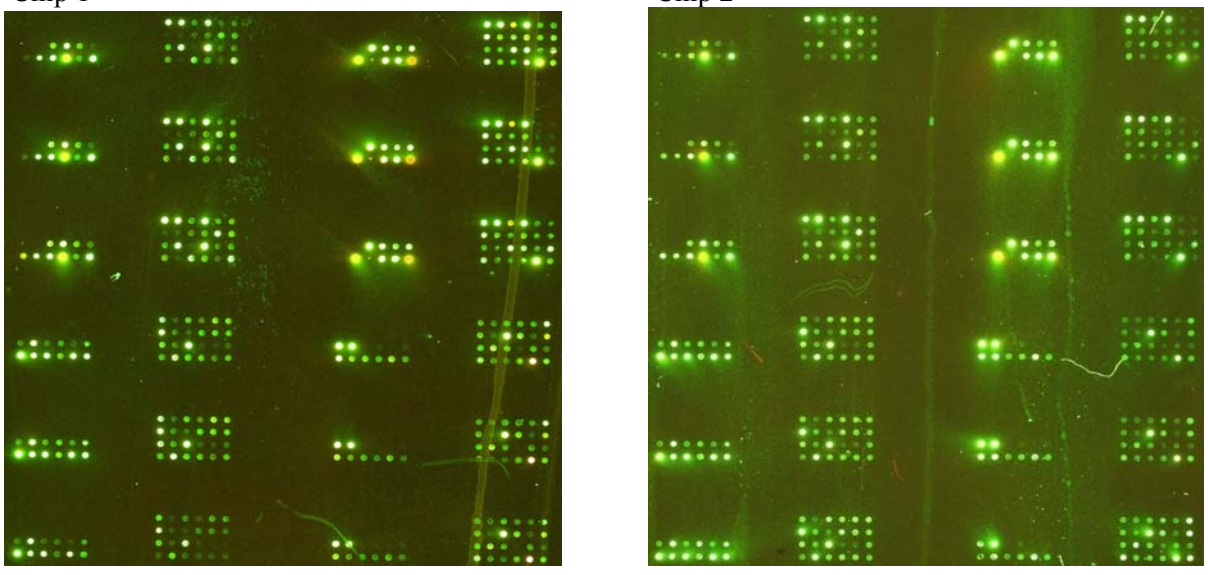

Figure (7.2): Microarray scan for the gene expression of the third examined yeast cell cycle during G1 and S-phase, chip 1 represents G1 dyed with flurosenated cy5 against S-phase dyed with biotinated cy3 , chip 2 is vice versa. 6 ug of total RNA per sample were used. The signals were amplified with TSA method

7.4 List of Figures

7.5 List of Tables

Lebenslauf

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Studium

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1998 - 2002	M.Sc Studium der Biochemie an der Naturwissenschaftlichen Fakultät, Ain-Shams Universität, Kairo, Ägypten, unter der leitung von Prof.Dr. Zeinab El Dardiri
2004 - 2008	Doktorand am Institut für Technische Chemie, Naturwissenschaftlichen Fakultät, Leibniz Universität Hannover, Deutschland, unter der leitung von Prof.Dr. Thomas Scheper

Berufstätigkeit

1996-1997	Wissenschaftlicher Mitarbeiter am Nationalen Institut für Kalibrierung und Standards (NIS), Giza, Ägypten
1997- 2008	Wissenschafticher Mitarbeiter am Insitut für Gentechnik und Biotechnologie, Mubarak city für Wissenschaft und Technologie, Alexandria, Ägypten

Veröffentlichungen

- Redwan, E. M., Khalil, A., El-Dardiri, Z. Z. (2003): Production and optimization of ovine antitetanus antibody (poster session). Human Antibodies 12, 37-50.

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