KINETIC ANALYSIS
OF
GLUCOSE-6-PHOSPHATE BRANCH POINT
IN *Saccharomyces cerevisiae*

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Approval of the Graduate School of Natural and Applied Sciences.

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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ABSTRACT

KINETIC ANALYSIS OF GLUCOSE-6-PHOSPHATE BRANCH POINT IN *Saccharomyces cerevisiae*

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Glycolysis is the main metabolic route in *Saccharomyces cerevisiae* and it is the sequence of enzyme catalyzed reactions that oxidatively convert glucose to pyruvic acid in the yeast cytoplasm. In addition to the basic steps, glycolysis involves branch points providing the intermediary building blocks of the cell (i.e amino acids and nucleotides). One of these pathways is glucose-6-phosphate branch point which is a junction of glycolytic pathway and pentose phosphate pathway. At this point glucose-6-phosphate can be converted to fructose-6-phosphate a metabolite of glycolytic pathway by phosphoglucoisomerase or it can be dehydrogenated to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase which is the first enzyme of the pentose phosphate pathway.

In this study, the influence of different nitrogen sources on the flux distribution through the pentose phosphate pathway and glycolysis in *Saccharomyces cerevisiae* was examined. For this purpose, four different compositions of nitrogen sources were used in growth media. The growth medium contained one of the following composition of nitrogen sources; only ammonium sulfate, only yeast nitrogen base, ammonium sulfate and histidine, yeast nitrogen base and histidine. Histidine was added because its synthesis branches from pentose phosphate pathway. In order to analyse the effect of the different compositions of nitrogen sources on the physiology of the yeast, specific activities of hexokinase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzymes were measured in the crude extracts of the biomass samples taken in the late exponential phase of the cultures.

Addition of histidine caused an increase in the specific activities of all the enzymes analysed in medium containing ammonium sulfate. The specific activity of hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase in medium containing yeast nitrogen base and histidine were higher than medium containing yeast nitrogen base. However, the specific activity of 6-phosphogluconate dehydrogenase decreased 3.1% in medium containing yeast nitrogen base and histidine medium with respect to medium with only yeast nitrogen base.

The OD value and dry weight in the culture containing histidine aminoacid was higher than the cultures contaning only ammonium sulfate and only yeast nitrogen base. Also the period of the exponential phase was shorter in medium containing ammonium sulfate and histidine and yeast nitrogen base and histidine than medium only ammonium sulfate and only yeast nitrogen base.

Keywords: Glucose-6-phoshate branch point, *Saccharomyces cerevisiae*, nitrogen source, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase

ÖZ

*Saccharomyces cerevisiae'*DA GLİKOZ-6-FOSFAT DAĞILIM NOKTASININ KİNETİK ANALİZİ

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Glikoliz, *Saccharomyces cerevisiae*'nın temel metabolik yoludur ve mayanın sitoplazmasında glikozu piruvata dönüştüren reaksiyonları katalizleyen enzimler dizisidir.

Glikoliz temel basamakların yanında, hücrenin temel yapıtaşlarının oluşumunu sağlayan dağılım noktaları içerir (amino asitler, nükleotitler gibi). Bu dağılım noktalarından biri glikolitik yolun pentoz fosfat yolu ile birleştiği glikoz-6-fosfat dağılım noktasıdır. Bu noktada glikoz-6-fosfat fosfoglikoz izomeraz enzimi ile fruktoz-6-fosfata dönüşebilir ya da pentoz fosfat yolunun ilk enzimi olan glikoz-6-fosfat dehidrogenaz enzimi ile 6-fosfoglukanolaktona dönüştürülür.

Bu çalışmada, *Saccharomyces cerevisiae* da farklı nitrojen kaynaklarının pentoz fosfat yolu ile glikolitik izyolu arasındaki akış dağılımına etkisi çalışılmıştır. Bu amaçla, büyüme ortamında dört farklı nitrojen kaynağı bileşimi kullanılmıştır. Büyüme ortamına eklenen nitrojen kaynakları, sadece amonyum sulfat, sadece yeast nitrogen base, amonyum sulfat ve histidin ya da yeast nitrogen base ve histidin karışımıdır. Histidin eklenmiştir çünkü histidin pentoz fosfat yolundan sentezlenen bir amino asittir. Farklı nitrojen kaynaklarının mayanın fizyolojisindeki etkisini

analiz etmek için kültürlerin geç logaritmik büyüme fazlarında alınan biyokütle örneklerinin kaba özütlerinde hekzokinaz, fosfoglikoz izomeraz, glikoz-6-fosfat dehidrogenaz ve 6-fosfoglukonat dehidrogenaz enzimlerinin spesifik aktiviteleri ölçülmüştür.

Amonyum sülfat içeren ortama histidin eklenmesi analiz edilen tüm enzimlerin spesifik aktivitelerinde artışa neden olmuştur. Yeast nitrogen base ve histidin içeren ortamda hekzokinaz, fosfoglikoz izomeraz ve glikoz-6-fosfat dehidrogenaz enzimlerinin spesifik aktivitesi sadece yeast nitrogen base içeren ortama göre daha yüksektir. Fakat 6-fosfoglukonat dehidrogenaz enziminin aktivitesi yeast nitrogen base ve histidin içeren ortamda 3.1% düşmüştür.

Histidin eklenen ortamlarda kültürlerin OD değerleri ve kuru ağırlıkları sadece amonyum sülfat ya da sadece yeast nitrogen base içeren ortamlara göre daha yüksektir. Ayrıca logaritmik büyüme fazının süresi histidin eklenen ortamlarda sadece amonyum sülfat ya da sadece yeast nitrogen base içeren ortamlara göre daha kısa sürmüştür.

Anahtar kelimeler: Glikoz-6-fosfat dağılım noktası, *Saccharomyces cerevisiae*, nitrojen kaynağı. glikoz-6-fosfat dehidrogenaz, fosfoglikoz izomeraz

To my family…

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LIST OF ABBREVIATIONS

AS: Ammonium sulfate

G6PDH: Glucose-6-phosphate dehydrogenase

HIS: Histidine

HXK: Hexokinase

$NADP^+$: Nicotinamide adenine dinucleotide phosphate, oxidized

NADPH: Nicotinamide adenine dinucleotide phosphate, reduced

6PGDH: 6-Phosphogluconate dehydrogenase

PGI: Phosphoglucose isomerase

PPP: Pentose phosphate pathway

YNB: Yeast Nitrogen Base

CHAPTER 1

INTRODUCTION

1.1. The Yeast *Saccharomyces cerevisiae*

1.1.1. The History of Baker's Yeast

The yeast *Saccharomyces cerevisiae* has played a central role in the evolution of microbiology, biochemistry, and genetics, in addition to its use as a technical microbe for the production of alcoholic beverages and leavening of dough. During the past 25 years, *Saccharomyces cerevisiae* has become one of the model organisms for molecular genetics and cell biology, which culminated on 24th April, 1996, with the release of the first complete nuclear genome sequence of a eukaryote (Dujon, 1996; Johnston, 1996; Hieter et al., 1996).

In the nineteenth century, the yeast *Saccharomyces cerevisiae* was first recognized as living organisms responsible for fermenting sugar to ethanol in wine making and in brewing, but few of the 600 or so yeast species accepted today were described until the twentieth century. In the late eighteenth and early nineteenth centuries, yeast was not considered to be a living organism. The first scientific research on yeast was done not by biologists but almost exclusively by chemists, who were investigating alcoholic fermentation.

The first written record of the actual existence of bread dates to around 2600 B.C. in Babylonia. The discovery of the leavened bread was generally attributed to ancient Egyptians by 3000 B.C. Bread making and brewing of beer are assumed to be closely allied arts. Mixing of a fermented beer with the wheat flour lead to the development of a sour-dough process. The practice of sour-dough fermentation continued into the 19th century, when the commercial bakers obtained their yeast supplies from local breweries. Due to its bitter taste and variable fermentation

activity, brewer's yeast was gradually replaced by distiller's yeast which in turn was replaced by baker's yeast.

1.1.2. Classification

The word 'yeast' is not easy defined, but basically yeasts are recognized as being unicellular fungi. More definitively: 'Yeasts are ascomycetous or basidiomycetous fungi that form sexual states which are not enclosed in a fruiting body'.

The subdivisions are based on aspects of yeasts' sexuality (Ascomycotina or Basidiomycotina) or lack of it (Deuteromycotina) and the lower taxonomic categories are based on various morphological, physiological and genetic characteristics.

Yeast classification is based on the following hierarchical system: (Walker, 1998)

Subdivision: Ascomycotina
Family: Saccharomycetaceae
Subfamily: Saccharomycetoideae
Genera: Saccharomyces
Species: cerevisiae

1.1.3. *Saccharomyces cerevisiae* in Biotechnology

Yeasts are of major economic, social and health significance in human culture. They have often been described as mankind's oldest 'domesticated' organisms, having been used to produce alcoholic beverages and leaven bread dough for millennia. In fact, the brewing of beer probably represented the world's first biotechnology.

In modern times, yeasts have found numerous other roles besides traditional food fermentations. In particular, genetically manipulated yeasts can now be exploited to produce many different biopharmaceutical agents for preventing and treating human disease.

Emerging yeast technologies especially evident in the enviromental and health-care sectors of biotechnology. In the former, yeasts are likely to play increasing roles in areas such as the bioremediation of industrial wastes and in biological control of fungal pests in agriculture. In several aspects of health-care biotechnology, yeasts are having a great impact. For example, in the prevention and treatment of several human diseases, recombinant yeast products are already playing important medical roles. Many new therapeutic proteins are under development and await clinical use.

In the future, yeast exploitation is likely to make significant impacts in relation to renewable energy supply, environmental biotechnology including biological control and in health-care issues particulary the study of human genetic disorders and cancer (Figure 1.1).

Andre Goffeau and his co-workers started to Yeast Genome Project under the scientific programmes of European Commission in 1980s and 6000 genes of *Saccharomyces cerevisiae* were identified. But a significant proportion of them (around 50 %) are of unknown functions (Dujon, 1996). International effort is now underway to analyse functionally the yeast genome in order not only to reveal the full biotechnological potential of yeasts, but also to achieve an understanding of how simple eukaryotic cells work.

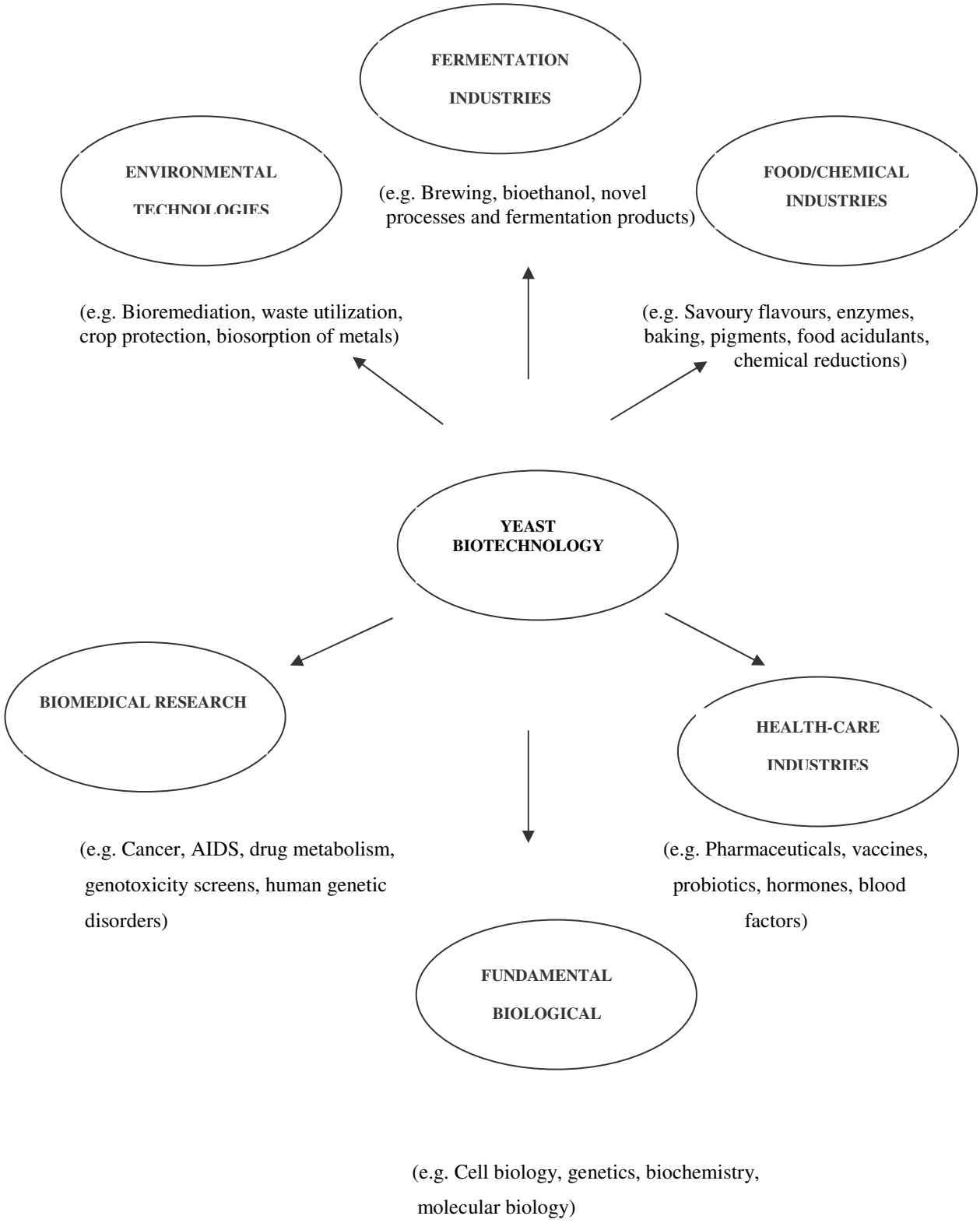


Figure 1.1. Diversity of outlets involving yeast biotechnology (Walker, 1998)

1.2 Glycolysis and Glucose-6-phosphate Branch Point

Glycolysis is the main metabolic route in *S. cerevisiae* and it is the sequence of enzyme catalyzed reactions that oxidatively convert glucose to pyruvic acid in the yeast cytoplasm.

Glycolytic pathway lies at the centre of the energy metabolism and the intermediates of glycolytic pathway are chanelled to different pathways like lactate production, ethanol production in different organisms which are industrially important. Glycolytic pathway is composed of 10 basic reactions that goes from glucose to pyruvate. In addition to these basic steps, glycolysis involves branch points providing the intermediary building blocks of the cell (i.e amino acids and nucleotides) (Figure 1.2).

In organisms most of the glucose, which is one of the common monosaccharide in nature, is catabolized via glycolysis to pyruvate, which in turn is oxidized in the citric acid cycle. The main function of glucose catabolism by this route is to generate ATP, which is the "energy currency" of the cells. There are, however, other metabolic pathways taken by glucose, which leads to special products needed by the cell. These pathways constitute part of the secondary metabolism of glucose (Lehninger, 1993).

One of these pathways is glucose-6-phosphate branch point which is the first branch point of the glycolytic pathway (Figure 1.3). In glucose-6-phosphate branch point glucose-6-phosphate can be converted to fructose-6-phosphate a metabolite of glycolytic pathway by phosphoglucose isomerase or it can be dehydrogenated to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase in the pentose phosphate pathway (PPP).

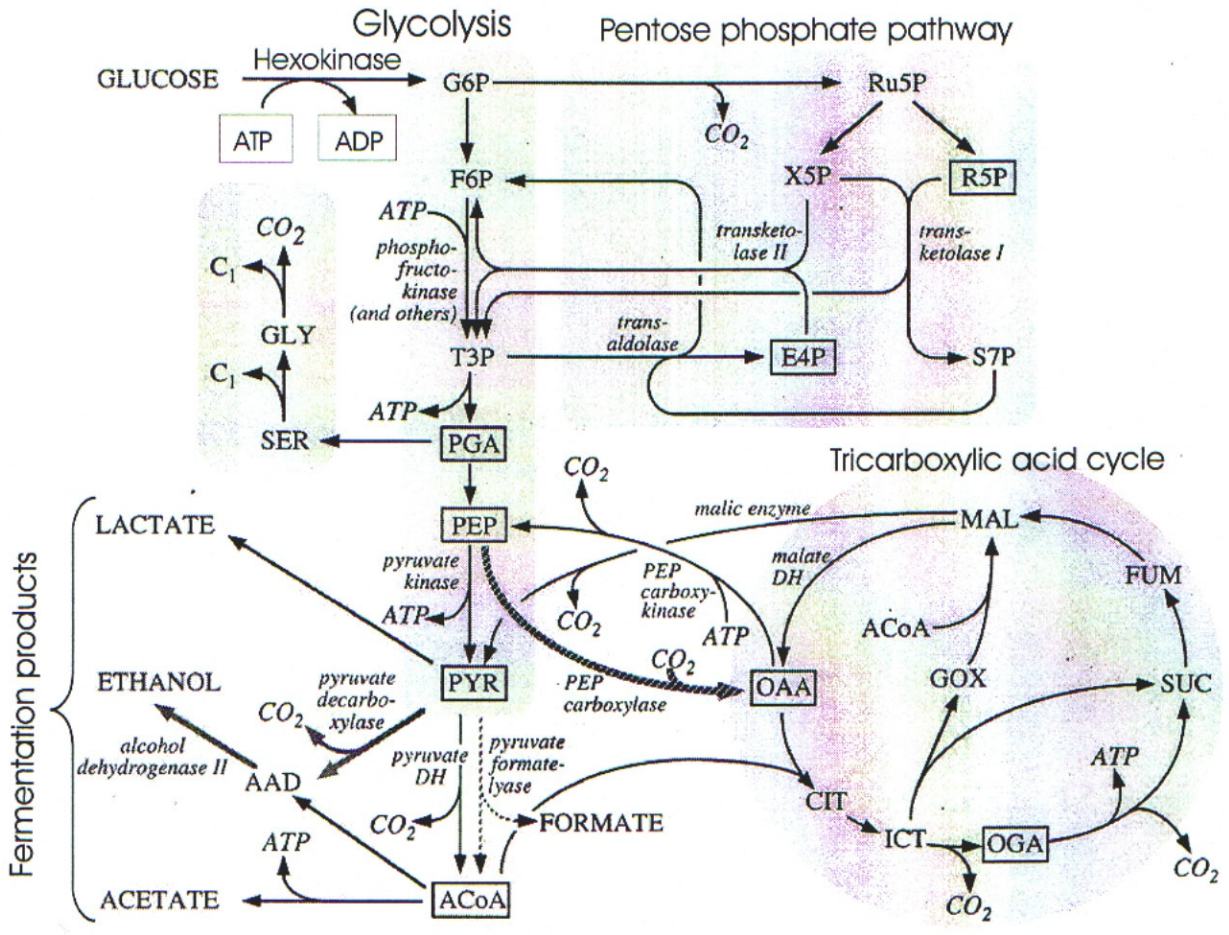


Figure 1.2: Glycolysis and some of the related pathways

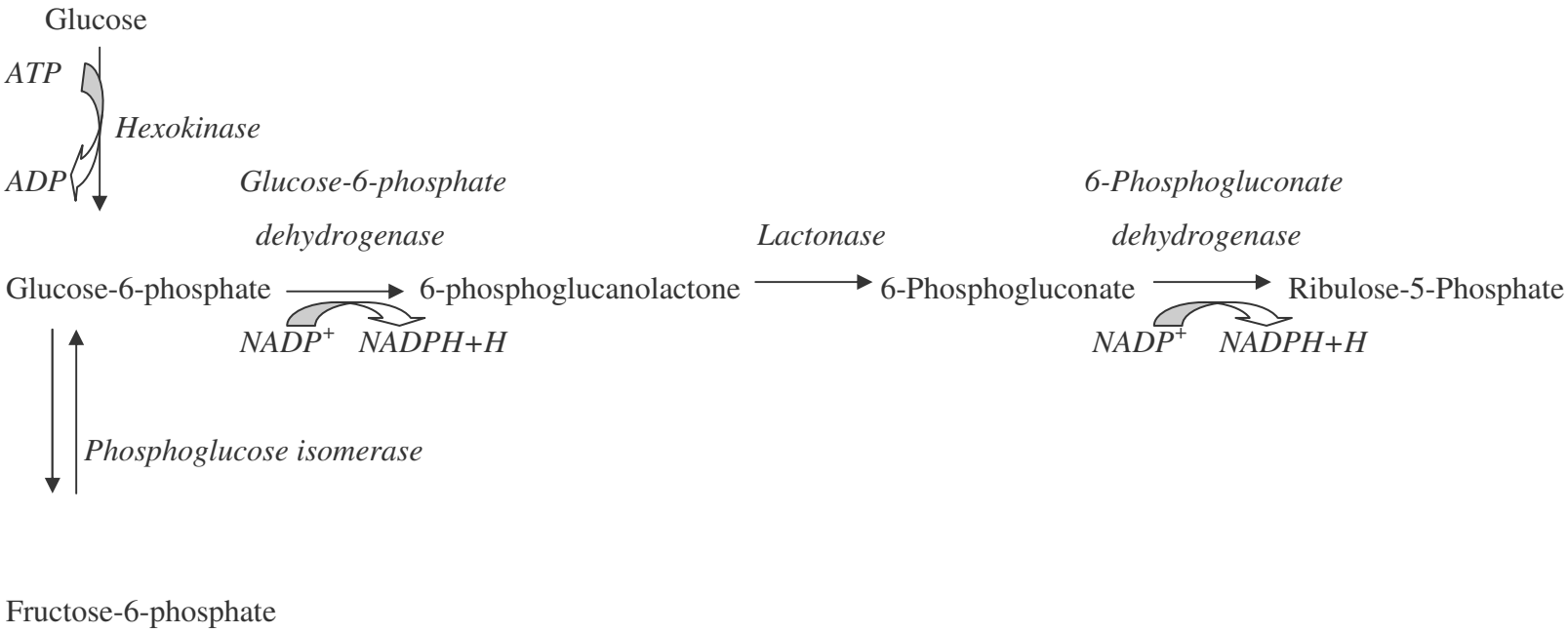


Figure 1.3: *Glucose-6-phosphate branch point*

1.3. The Pentose Phosphate Pathway

The first evidence of this pathway's existence was obtained in the 1930s by Otto Warburg who discovered $NADP^+$ through his studies on the oxidation of glucose-6-phosphate to 6-phosphogluconate. It was not until the 1950s, however, that the pentose phosphate pathway was elucidated by Frank Dickens, Bernard Horecker, Fritz Lipmann, and Efraim Racker (Voet and Voet, 1990).

The pentose phosphate pathway (PPP) serves several purposes, including synthesis and degradation of sugars other than hexoses, particularly pentoses necessary for nucleotides and nucleic acids, and other glycolytic intermediates. Most important is the ability to synthesize NADPH, which has a unique role in biosynthetic reactions. The direction of flow and path taken by G6P after entry into the pathway is determined largely by the needs of the cell for NADPH or sugar intermediates. When more NADPH than ribose-5-phosphate is required, the pathway leads to complete oxidation of G6P to CO_2 and resynthesis of G6P from ribulose-5-phosphate. Alternatively, if more ribose-5-phosphate than NADPH is required, G6P is converted to fructose-6-phosphate and glyceraldehyde-3-phosphate by the glycolytic pathway. Two molecules of fructose-6-phosphate and one molecule of glyceraldehyde-3-phosphate are converted into three molecules of ribose-5-phosphate by reversal of the transaldolase and transketolase reactions (Devlin,1997).

The pathway can be divided into two parts (Figure 4). (i) Oxidative (ii) Non-oxidative. In the oxidative, irreversible part, glucose-6-phosphate is oxidized to ribulose-5-phosphate and CO_2. NADPH, which is required for several reductive biosyntheses, is generated by the two dehydrogenase enzymes involved. Since no transhydrogenase activity could be detected in *Saccharomyces cerevisiae* (Bruinenberg et al., 1983), no direct interconversion of NADH and NADPH can occur. The oxidative part of the PPP was therefore thought to be the major source of NADPH in S. cerevisiae.

In the nonoxidative, reversible part of the pathway, ribulose-5-phosphate is converted to fructose-6-phosphate and glyceraldehyde-3-phosphate. Since glucose-6-phosphate, fructose-6-phosphate, and glyceraldehyde-3-phosphate are also glycolytic intermediates, these two pathways are in close contact. Intermediates of the nonoxidative part of the PPP are required for the biosynthesis of several molecules. Ribose-5-phosphate is the precursor of phosphoribosyl pyrophosphate, which is required for the biosynthesis of purine and pyrimidine nucleotides, nucleic acids, several coenzymes, and the amino acids histidine and tryptophan. Erythrose-4-phosphate, another intermediate of the pathway, is a precursor for the synthesis of the aromatic amino acids tryptophan, phenylalanine, and tyrosine and for the biosynthesis of the *p*-aminobenzoate and *p*-hydroxybenzoate. (Zimmerman and Entian, 1997)

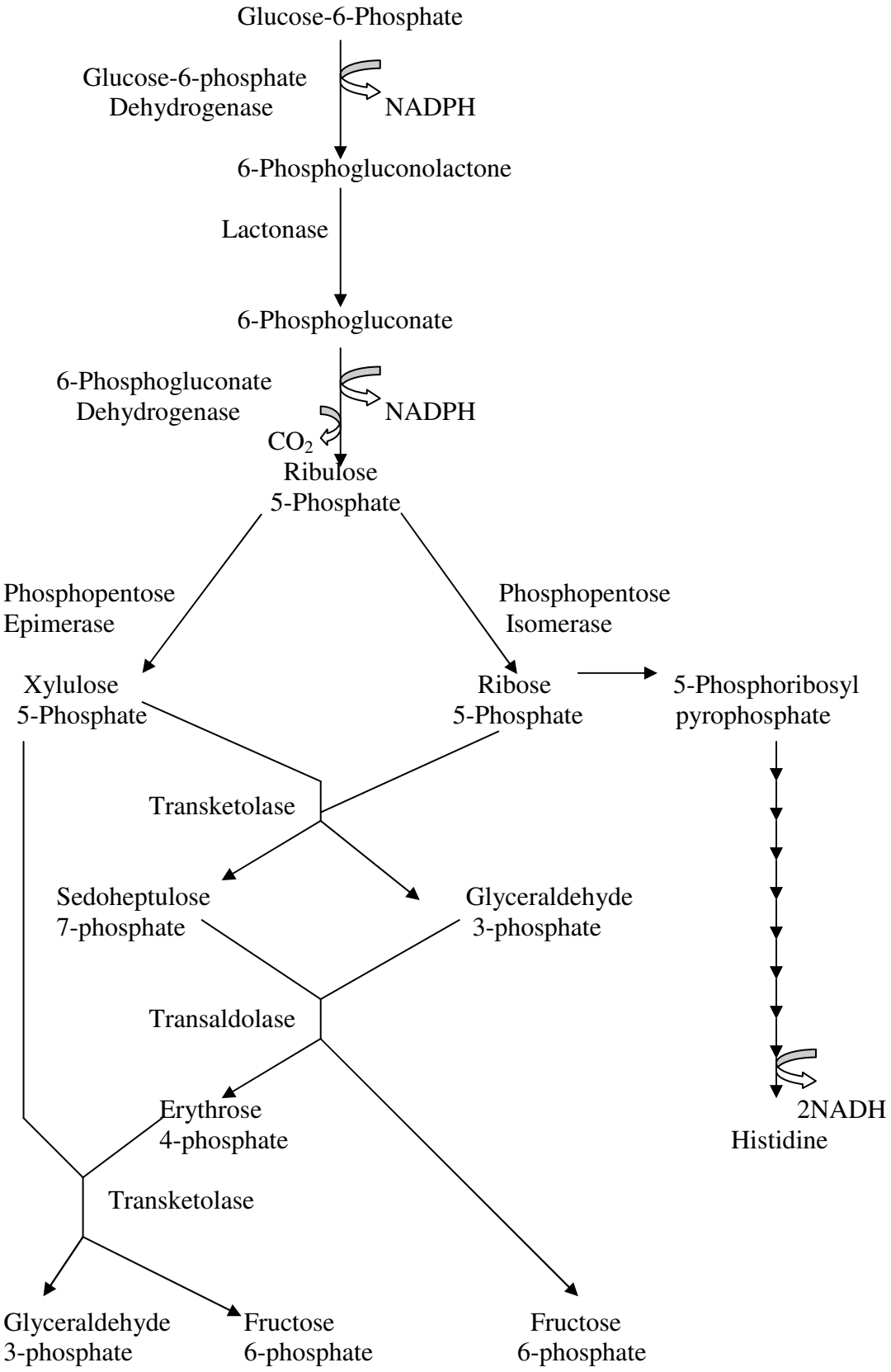


Figure 1.4: Pentose phosphate pathway

1.3.1 Reactions of oxidative parts

Glucose-6-phosphate dehydrogenase catalyzes net transfer of a hydride ion to $NADP^+$ from C(1) of G6P to form 6-phosphogluconolactone. G6P, a cyclic hemiacetal with C(1) in the aldehyde oxidation state, is thereby oxidized to a cyclic ester (lactone) (Figure 1.5).

The second reaction involves the hydrolysis of 6-phosphogluconolactone to 6-phosphogluconate, the subtsrate of the next oxidative enzyme in the pathway. Because the lactone produced by the G6PDH reaction is very unstable, it has been uncertain whether the enzyme that hydrolyzes it, 6-phosphogluconolactonase is required for functioning of the PPP. Although the nonenzymatic reaction occurs at a significant rate , 6-phosphogluconolactonase is required when the rate of oxidation of NADPH is accelerated.

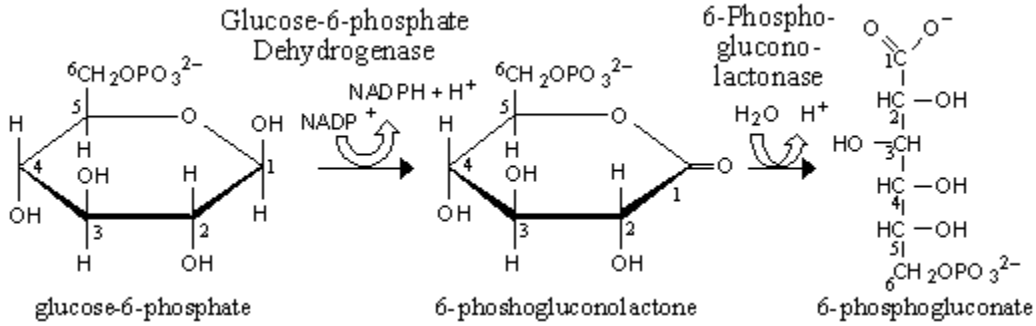


Figure 1.5: The glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase reactions

Phosphogluconate dehydrogenase catalyzes oxidative decarboxylation of 6-phosphogluconate, to yield the 5-C ketose ribulose-5-phosphate. The hydroxyl at C3 (C2 of the product) is oxidized to a ketone. This promotes loss of the carboxyl at C1 as CO_2. $NADP^+$ again serves as oxidant (electron acceptor) (Figure 1.6).

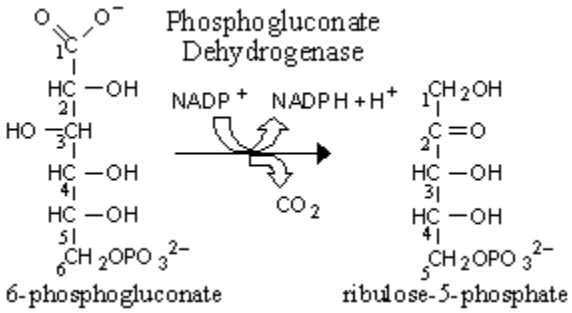


Figure 1.6: The phosphogluconate dehydrogenase reaction

1.3.2. Reactions of nonoxidative parts

Epimerase interconverts the stereoisomers ribulose-5-phosphate and xylulose-5-phosphate. Isomerase converts the ketose ribulose-5-phosphate to the aldose ribose-5-phosphate. (Figure 1.7).

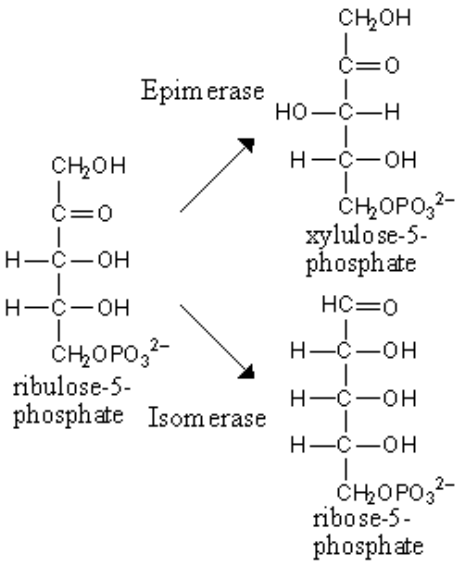


Figure 1.7: The ribulose-5-phosphate isomerase and ribulose-5-phosphate epimerase reactions

Transketolase catalyzes the transfer of a C2 unit from Xu5P to R5P yielding GAP and sedoheptulose-7-phosphate (Figure 1.8).

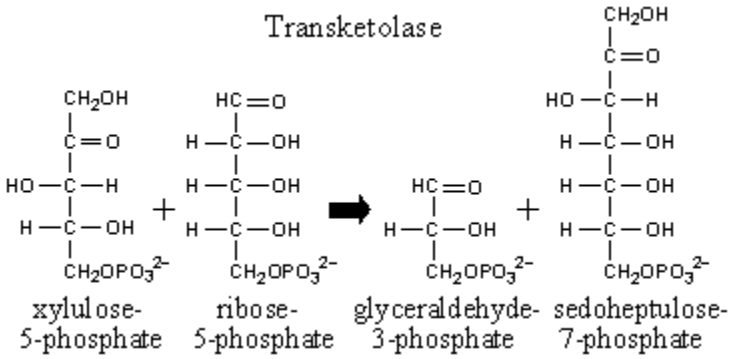


Figure 1.8: The transketolase reaction

Transaldolase catalyzes the transfer of a C3 unit from S7P to GAP yielding erythrose-4-phosphate (E4P) and F6P (Figure 1.9). In the second transketolase reaction, a C2 unit is transferred from a second molecule of Xu5P and E4P to form GAP and another molecule of F6P.

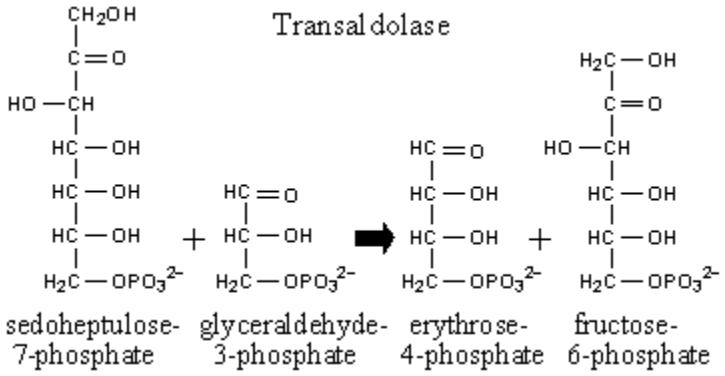


Figure 1.9: The transaldolase reaction

1.3.4. Regulation of PPP

Glucose-6-phosphate Dehydrogenase is the committed step of the Pentose Phosphate Pathway. This enzyme is regulated by availability of the substrate $NADP^+$. As NADPH is utilized in reductive synthetic pathways, the increasing concentration of $NADP^+$ stimulates the Pentose Phosphate Pathway, to replenish NADPH. Cells normally maintain their $[NAD^+]$/[NADH] ratio, near 1000, which favor metabolite oxidation, while keeping their [$NADP^+$]/[NADPH] ratio near 0.01, which favor metabolite reduction. The first two enzymes of this pathway, G6PDH and 6-PGD are almost completely inhibited at the physiological free NADPH/ $NADP^+$ ratio, in the order of 100:1 in the rat liver. Thus, the studies suggest that NADPH/ $NADP^+$ ratio is important in the regulation of the enzymes of the pathway.

Depending on relative needs of a cell for ribose-5-phosphate, NADPH, and ATP, the Pentose Phosphate Pathway can operate in various modes, to maximize different products.

There are three major scenarios:

- Ribulose-5-phosphate may be converted to ribose-5-phosphate, a substrate for synthesis of nucleotides and nucleic acids. The pathway also produces some NADPH.

- Glyceraldehyde-3-phosphate and fructose-6-phosphate, formed from the 5-carbon sugar phosphates, may be converted to glucose-6-phosphate for reentry into the linear portion of the Pentose Phosphate Pathway, maximizing formation of NADPH

- Glyceraldehyde-3-phosphate and fructose-6-phosphate, formed from the 5-carbon sugar phosphates, may enter glycolysis, for synthesis of ATP.

1.4. Phosphoglucose Isomerase (EC 5.3.9.1)

Phosphoglucose isomerase (PGI) opens the way for entry of glucose-6-phosphate into the glycolytic pathway. The enzyme is an aldose-ketose isomerase, which catalyzes the reversible interconversion of D-glucopyranose-6-phosphate and D-fructofuranose-6-phosphate by promoting the intramolecular transfer of a proton between C-1 and C-2 (Achari et.al, 1981) (Figure 1.10).

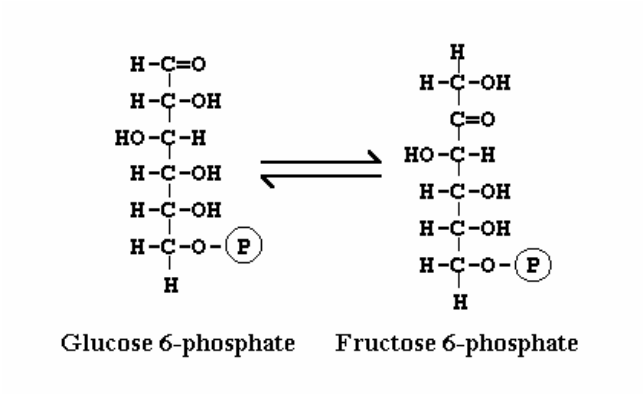


Figure 1.10: The phosphoglucose isomerase reaction

PGI has a strategic position in metabolism as several metabolic pathways branch off from the glycolytic/gluconeogenic pathway both at G6P (biosynthesis of cell wall components, oxidative part of pentose phosphate pathway) and F6P (mannitol biosynthesis, nonoxidative part of pentose phosphate pathway).

Yeast PGI is a dimeric enzyme with a subunit molecular mass of about 60kDa (Aguilera and Zimmermann, 1986). PGI from most sources is inhibited by several compounds which are intermediates of central metabolism (e.g.6-phosphogluconate, erythrose-4-P, ATP) In particular inhibition by erythrose 4-P (E4P) is strong, e.g., the K_i value for yeast PGI is 2 μM. The physiological significance of inhibition by E4P is, however, questionable, since E4P has never been detected unequivocally in metabolite extracts. Paoletti developed a sensitive assay for determination of E4P, but failed to detect it in tissue extracts. One explanation could be that the level of E4P in cells is too low to be detected by this method (Ruijter and Visser, 1998).

1.5. Glucose-6-phosphate-dehydrogenase (EC 1.1.1.49)

Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the oxidation of glucose-6-phosphate to 6-phospho-gluconolactone. (Figure1.11). It catalyzes net transfer of a hydride ion to $NADP^+$ from C(1) of G6P to form 6-phospho-glucanolactone. G6P, a cyclic hemiacetal with C(1) in the aldehyde oxidation state, is thereby oxidized to a cyclic ester (lactone). This important reaction is often considered to be irreversible. However, its apparent irreversibility is caused by the rapid removal of the immediate product, 6-phospho-gluconolactone (Beutler and Kuhl, 1986; Levy,1979). The enzyme is a specific for $NADP^+$ and is strongly inhibited by NADPH (Voet and Voet, 1990).

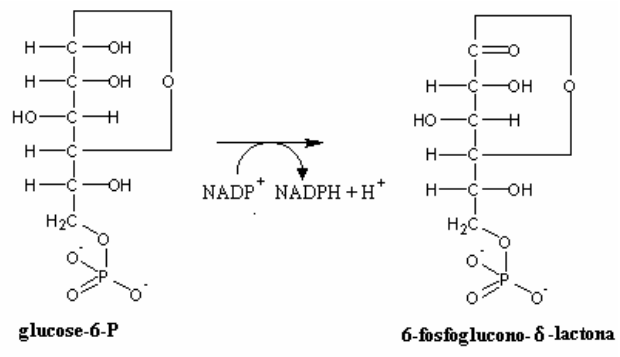


Figure 1.11: The glucose-6-phosphate dehydrogenase reaction

G6PDH was discovered by Warburg and Christian in 1931. An active preparation was obtained then from horse erythrocytes and in 1932 from Lebedev juice made from brewers' yeast (Glaser and Brown, 1955). G6PDH protein was totaly sequenced in 1969 (Zaitseva, Chuckhrai and Poltorak, 2000).

It is an oligomeric protein. The subunits are identical and the molecular weight differs among organisms.Yeast glucose-6-phosphate dehydrogenase in solutions exists as the equilibrium mixture of dimers and tetramers. Only dimers are active. The molecular mass of the dimer is 102kD (Zaitseva, Chuckhrai and Poltorak, 2000).

The gene (*ZWF1*) codes for a protein with 505 amino acids and a derived molecular weight of 57.4 kDa. Thomas et al. (1991) cloned the gene (*MET19=ZWF1*) by complementation of the methionine auxotrophic mutant *met19*. Nogae and Johnston (1990) cloned the same gene (*ZWF1*) and found that mutants deleted for this gene are slightly more sensitive against oxidizing agents (Zimmerman and Entian, 1997).

In *Schizosaccharomyces pombe* G6PDH catalyzes $NADP^+$-linked oxidation of D-glucose-6-phosphate by the ordered Bi Bi mechanism with $NADP^+$ as the leading reactant. High $NADP^+$ concentration inhibits the enzyme by forming the dead end ternary complex . In addition, it is subjected to product inhibition by NADPH and noncompetitive inhibition by A(G)TP (Tsai and Chen, 1998).

1.6. 6-Phosphogluconate Dehydrogenase (EC 1.1.1.44)

6-Phosphogluconate dehydrogenase catalyzes the oxidation of 6-phosphogluconate to ribulose-5-phosphate and CO_2 (Figure 1.6).

The enzyme from yeast was purified by Horecker and Smyrniotis (1951). A crystalline enzyme preparation was further analyzed by Pontremoli (1961), who determined the Michaelis constants (K_m) for 6-phosphogluconate (160 μM) and for $NADP^+$ (260 μM).

In *Corynebacterium glutamicum* the 6-Phosphogluconate dehydrogenase was found to operate according to a Theorell-Chance ordered bi-ter mechanism. The enzyme was inhibited by NADPH, ATP, fructose 1,6-bisphosphate, D-glyceraldehyde 3-phosphate (Gra3P), erythrose 4-phosphate and ribulose 5-phosphate. The inhibition by NADPH was considered to be the most important, with inhibition constants of around 25 μM for the enzyme (Moritz et.al, 2000).

The enzyme differs from the known 6-phosphogluconate dehydrogenases of other sources in that the Schizosaccharomyces enzyme is tetrameric having a subunit mass of 38 kDa that it requires $NADP^+$ obligatorily for activity, and that it can be activated by divalent metal ions such as Co^{2+} and Mn^{2+}. Initial rate and product inhibition results suggest that 6-phosphogluconate dehydrogenase from *Schizosaccharomyces pombe* catalyzes $NADP^+$ linked oxidative decarboxylation of 6-phosphogluconate by an equilibrium random mechanism with two independent binding sides, namely one site for the nicotinamide coenzyme, $NADP^+$/NADPH, another site for 6-phosphogluconate- d-ribulose-5-phosphate and for CO_2. (Tsai and Chen, 1998)

S. cerevisiae can use gluconolactone as a poor carbon source. It is metabolized by 6-phosphoglucono-lactonase to gluconate, which can be phosphorylated by a gluconate kinase (EC 2.7.1.12) to 6-phosphogluconate. Both enzymes, in addition to 6-phosphogluconate dehydrogenase, are induced during growth on gluconolactone (Sinha and Maitra, 1992).

1.6. The Aim of the study:

The aim of the study is to investigate the influence of different nitrogen sources on the flux distribution through the pentose phosphate pathway and glycolysis in *Saccharomyces cerevisiae*. For this purpose, in the growth medium four different nitrogen source were used; ammonium sulfate , yeast nitrogen base and the amino acid histidine, which is synthesized from pentose phosphate pathway, were added to ammonium sulfate and yeast nitrogen base. The specific activities of the enzymes hexokinase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were measured at late exponential phase of the cultures.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Yeast Strain

The laboratory strains *Saccharomyces cerevisiae* CEN.PK 113.7D (MATa MAL2-8^c SUC2) was used in this study. It was obtained from Peter Kötter (Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany).

2.1.2. Chemicals

$NADP^+$, ATP, glucose-6-phosphate, fructose-6-phosphate, 6-phosphogluconate, glucose-6-phosphate dehydrogenase, were obtained from Sigma Chem. Ltd.

2.2. METHODS

2.2.1. Storage of The Yeast Strain

The yeast strain was inoculated in 0.5 ml 20 % w/w glycerol solution in eppendorf tubes and kept at –80 ^oC for the storage. During the routine experiments, yeast strain were kept on agar plates containing 2 % w/v bacto-agar, 2 % w/v glucose, 1 % w/v bacto-peptone and 1 % w/v yeast extract at 4 ^oC. This stock was used in all inoculations of the pre-cultures.

2.2.2. Medium Composition

The inoculum and experimental cultures were grown in the same defined medium. The medium composition is given in Appendix A (Albers et.al., 1996)

The influence of different nitrogen sources on the flux distribution through the pentose phosphate pathway and glycolysis was examined. For this purpose, four different nitrogen sources were used; (i) ammonium sulfate (7.5 g of $(NH_4)_2SO_4$ per liter), (ii) yeast nitrogen base (9.92 g of YNB per liter) (iii)AS+Histidine (7.5 g of $(NH_4)_2SO_4$ and 36.9 mg histidine ($C_6H_9N_3O_2$) per liter) (iv) YNB+Histidine (9.92 g of $(NH_4)_2SO_4$ and 36.9 mg histidine per liter). The composition of Yeast nitrogen base is given in Appendix B.

For optimization studies, *Saccharomyces cerevisiae* were grown in rich medium (Appendix C)

The mineral components and glucose were autoclaved separately at 121 ^oC for 15 min. The solutions of vitamins and histidine were filter sterilized.

2.2.3. Culture Conditions

The all experiments were carried out in batch cultures in stirring flasks containing the medium in 1/4 portion of the total volume. Incubation was carried out at 30 ^oC and 160 rpm in an incubator shaker. Pre-cultures were grown aerobically in an incubator shaker at 30 ^oC and 160 rpm and inoculated to the fresh medium in the exponential phase in 1/15 portion.

2.2.4. Growth Rate Determination

Growth was followed by measurement of the optical density at 600 nm and culture samples were diluted in the same range such that the measured optical density is equal or lower than 0.600. Dilution was done by water against water as blank.

Specific growth rate was calculated according to equation given below:

$$\frac{1}{x} * \frac{dx}{dt} = \mu$$

$$\frac{dx}{x} = \mu * dt$$

$$\ln(x_2/x_1) = \mu * (t_2 - t_1)$$

$$\mu = \ln (x_2/x_1) / (t_2 - t_1)$$

x: Concentration of cells (OD_{600})
t = time
μ = Specific growth rate (h^{-1})

The part that $\ln (x_2/x_1)$ value stayed at a stable level was taken as the exponential phase of the corresponding culture. Pre-cultures were grown aerobically at 30 ^{o}C and 160 rpm in 250 ml Erlenmeyer flasks and inoculated to the fresh medium in the late exponential phase in 1/15 portion. The main cultures were grown at 30 ^{o}C and 160 rpm in 1 L Erlenmeyer flasks. The biomass was harvested for enzyme assays in the late exponenetial phase of the cultures.

2.2.5. Dry Weight Determination

Dry weights were determined by using a standart curve. For dry weight measurements preweighed nitrocellulose filters (pore size: 0.45 µm) were used. The medium were diluted to various OD_{600} values ranging from 0.1 to 0.6. After filtration of 15 ml each of these media, the filters were washed with demineralized water and dried in an microwave oven for 20 min. These samples were weighed again and the difference between the preweighed filter and these samples gave the weight (dry cell weight) of the yeast samples at different OD_{600} values. The OD versus dry weight standart graph was prepared according to the findings.

2.2.6. Preparation of Crude Extract

All the steps were carried out at 0-4 ^{o}C. Culture samples were harvested at late exponenetial phase by centrifugation (10,000 rpm for 30 minutes) in Sorvall centrifuge and washed with extraction buffer by centrifugated again at 10,000 rpm for 30 minutes. Wet yeast cells in %4 of the extraction buffer were sonicated at 10μ amplitude (Soniprep 150) gently for 1 minute with 10 seconds intervals. The supernatant obtained by centrifugation of the cell homogenate at 10,000 rpm for 30 min at 4 ^{o}C in Sorvall RC5C with GSA 10 type rotor is referred to as crude extract.

2.2.7.Enzyme Assays

Enzyme activities were assayed spectrophotometrically by following NADPH formation at 340 nm . The cell of the spectrophotometer was kept at constant temperature (30^{o}C) by using a circulatory water bath .

Average OD_{340} change per minute is calculated and the unit of enzyme is calculated using this value and ε = 6.22 mM^{-1} cm^{-1} for NADPH according to equation given below.

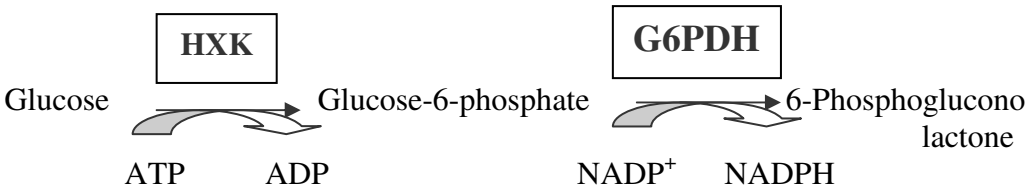
$$\text{Enzyme activity}(U.ml^{-1}) = \frac{\Delta OD_{340}/min}{\varepsilon} \text{ x dilution factor(s) x } 10^{3}$$

One unit of enzyme activity is described as the amount of enzyme that catalyzes the conversion of 1 μmole of substrate per minute under assayed condition. The specific activity of the enzyme will be given as units per mg of protein

2.2.7.1. Hexokinase Assay

Hexokinase enzyme activity was measured by a coupled assay system taken from Bergmeyer (1967) with some modifications.

The schematic representation of the assay is given below:



In the assay glucose-6-phosphate formation is measured by coupling it with a further reaction in which glucose-6-phosphate is converted into 6-phosphogluconolactone in the presence of glucose-6-phosphate dehydrogenase which simultaneously catalyzes the reduction of $NADP^+$ to NADPH. Formation of NADPH is measured spectrophotometrically at 340 nm.

Reaction mixture (600µl) was contained 100mM Tris-HCI pH 7.70, 1.7 mM EDTA, 10 mM $MgCl_2$, 5mM ATP, 0.5mM $NADP^+$, 3.5 U/ml glucose-6-phosphate dehydrogenase, 10mM glucose. Buffer, distilled water and enzyme (enzyme was incubated for 5 min) were incubated at 30 ^oC before the reaction was started. The reaction was initiated by addition of $NADP^+$.

The reaction rate was measured as the increase in the absorbance of NADPH formed at 340 nm with 10 seconds intervals for 3 minutes in spectrophotometer (Shimadzu UV-1202). The slope of this curve gives the reaction rate. One unit of hexokinase was defined as the amount of enzyme, which catalyzes the formation of 1 µmole of glucose-6-phosphate per minute under assayed conditions.

2.2.7.2. Glucose-6-phosphate Dehydrogenase Assay

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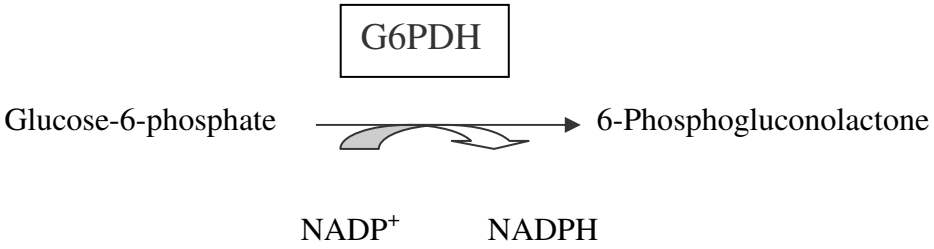
Glucose-6-phosphate dehydrogenase enzyme activity was measured by an assay system taken from Postma (Postma et.al., 1988). The schematic representation of the assay is given below:



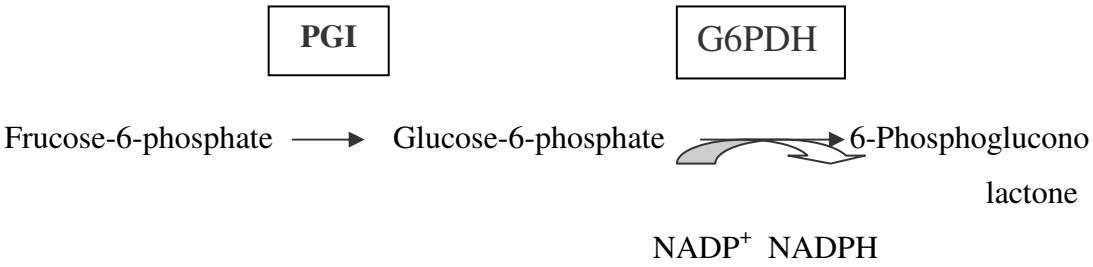
The assay mixture contained 0.4 mM $NADP^+$, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$, and tris-HCI buffer, pH 8. Buffer, distilled water and enzyme (enzyme were incubated for 5 min) were incubated at 30 ^oC before the reaction was started.

The reaction was started by the addition of $NADP^+$. The reaction rate is measured as the increase in the absorbance at 340 nm at 10 seconds intervals for 3 minutes. The slope of this curve is proportional to the reaction rate. One unit of glucose-6-phosphate dehydrogenase activity is defined as the amount of enzyme that causes 1 µmole of $NADP^+$ per minute under assayed conditions.

2.2.7.3. Phosphoglucose isomerase Assay

Phosphoglucose isomerase enzyme activity was measured by a coupled assay system. In the assay, glucose-6-phosphate formation was measured by coupling it with a further reaction catalyzed by glucose-6-phosphate dehydrogenase. In this reaction glucose-6-phosphate was converted into 6-phosphogluconolactone in the presence of glucose-6-phosphate dehydrogenase which simultaneously catalyzed the reduction of $NADP^+$ to NADPH. Formation of NADPH was measured spectrophotometrically at 340 nm.

The reaction scheme is given below:

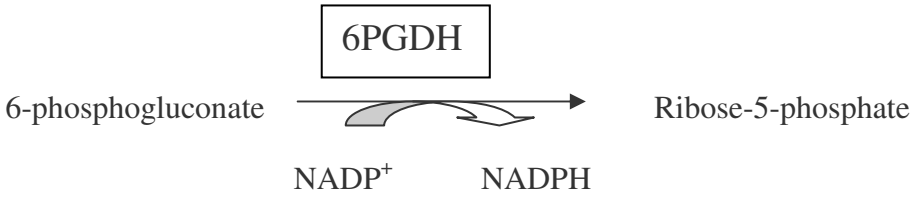


Reaction mixture contained Bis-Tris buffer pH 8.0, 5 mM $MgCl_2$, 0.5mM $NADP^+$, 2 U/ml glucose-6-phosphate dehydrogenase, 1 mM fructose-6-phosphate. The reaction was started by the addition of $NADP^+$and followed at 340 nm with 10 seconds intervals up to 3 minutes.

The slope of this increase is proportional to the reaction rate. One unit of phosphoglucose isomerase activity is defined as the amount of enzyme catalyzing the formation of 1 µmole of glucose-6-phosphate from 1 µmole of fructose-6-phosphate per minute under assayed conditions.

2.2.7.4. 6-Phosphogluconate Dehydrogenase Assay

6-phosphogluconate dehydrogenase enzyme activity was measured by an assay system taken from Moritz (Moritz et.al., 2000). The schematic representation of the assay is given below:



The assay mixture contained 1 mM $NADP^+$, 1 mM 6-phosphogluconate, 10 mM $MgCl_2$, and 50 mM Tris-HCI buffer, pH 7.5. Buffer, distilled water and enzyme (enzyme was incubated for 5 min) were incubated at 30 ^oC before the reaction was started.

The reaction was started by the addition of $NADP^+$. The reaction rate was measured as the increasae in the absorbance at 340 nm at 10 seconds intervals for 3 minutes. The slope of this curve is proportional to the reaction rate. One unit of glucose-6-phosphate dehydrogenase activity is defined as the amount of enzyme that causes 1 µmole of $NADP^+$ per minute under assayed conditions.

2.2.8.Protein Determination

Protein concentration was performed according to the Bradford (1980) using bovine serum albumin (BSA) as standard. The composition of the reagents and preparation of the standard curve is given in Appendix D.

All experiments were carried out in three parallel runs.

CHAPTER 3

RESULTS

3.1. Effects of different nitrogen source on growth of *Saccharomyces cerevisiae*

For all growth media, pre-cultures were grown aerobically at 30 ^{o}C and 160 rpm in 250 ml Erlenmeyer flasks and inoculated to the fresh medium in the exponential phase in 1/15 portion. Figures 3.1, 3.3, 3.5 and 3.7 show the absorbance at 600 nm versus time graphs of precultures of *S. cerevisiae* on growth medium containing one of the following nitrogen source; i) ammonium sulfate ii) yeast nitrogen base, iii) ammonium sulfate + histidine iv) yeast nitrogen base + histidine. The specific growth rate versus time graphs of precultures of *S. cerevisiae* of the above mentioned cultures are shown in Figures 3.2, 3.4, 3.6, 3.8 respectively.

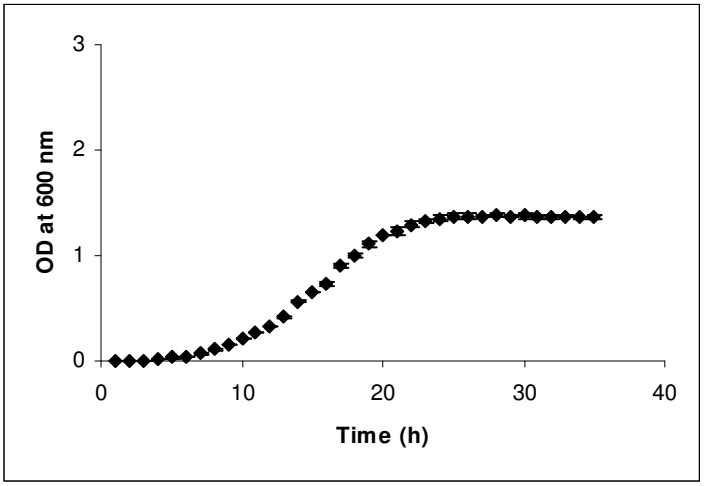


Figure 3.1: Growth of preculture of *S. cerevisiae* on growth media containing ammonium sulfate as nitrogen source

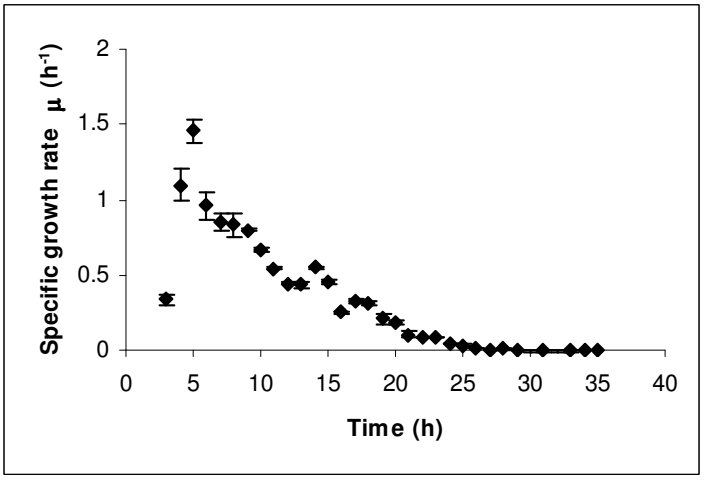


Figure 3.2: Specific growth rate of preculture of *S. cerevisiae* on media containing ammonium sulfate as nitrogen source

Figure 3.2 shows the specific growth rate versus time graph of *S. cerevisiae* on growth media containing ammonium sulfate as nitrogen source. From Figure 3.2 between the 5th and 9th hour the organism is in exponential phase. Although there are some errors in the 5th and 6th hour samples, at 7th, 8th and 9th hours of growth specific growth rate stayed at a stable level. This part was taken as the exponential phase of the corresponding culture.

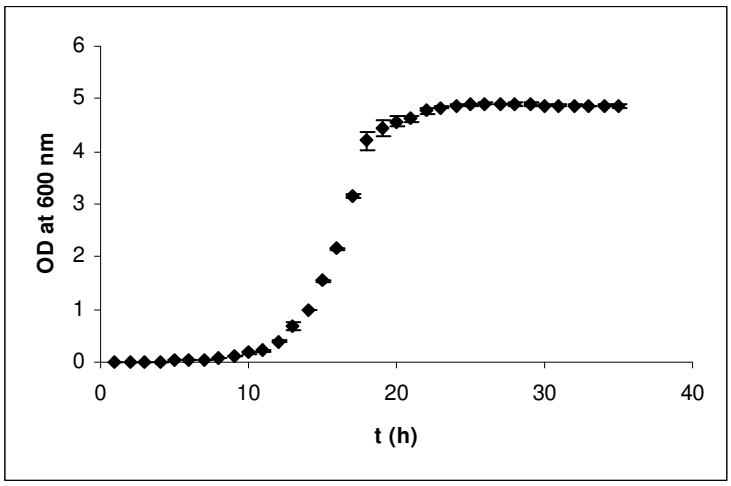


Figure 3.3 Growth of preculture of *S. cerevisiae* on growth media containing yeast nitrogen base as nitrogen source

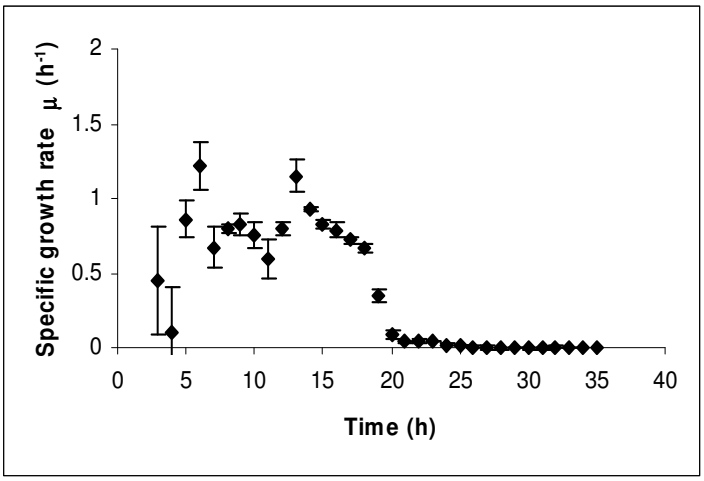


Figure 3.4: Specific growth rate of preculture of *S. cerevisiae* on media containing yeast nitrogen base as nitrogen source

Figure 3.4 shows the specific growth rate ln (n+1)/n versus time graph of *S. cerevisiae* on growth media containing yeast nitrogen base as nitrogen source. The organism is in exponential phase between the 5th and 12th hour . Although there are some errors in the 6th, 7th and 11th hour samples, at 5th, 8th, 9th, 10th and 12th hours of growth the specific growth rate stayed at a stable level. This part was taken as the exponential phase of the corresponding culture. The preculture was transferred to main culture at its 10th hour in late exponential phase.

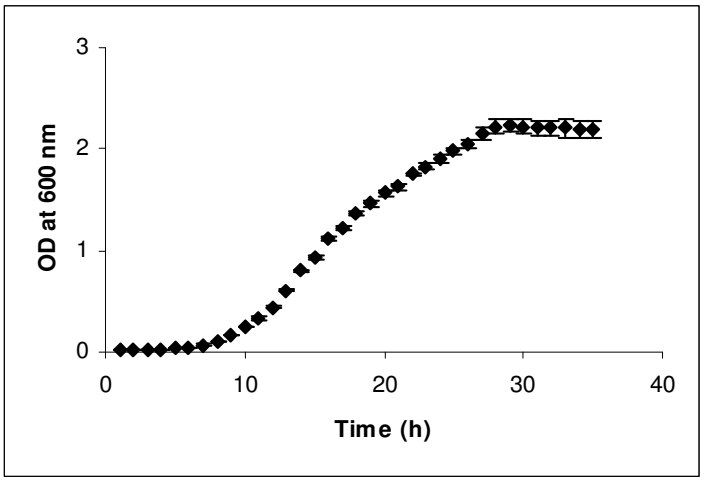


Figure 3.5: Growth of preculture of *S. cerevisiae* on media containing ammonium sulfate + histidine as nitrogen source

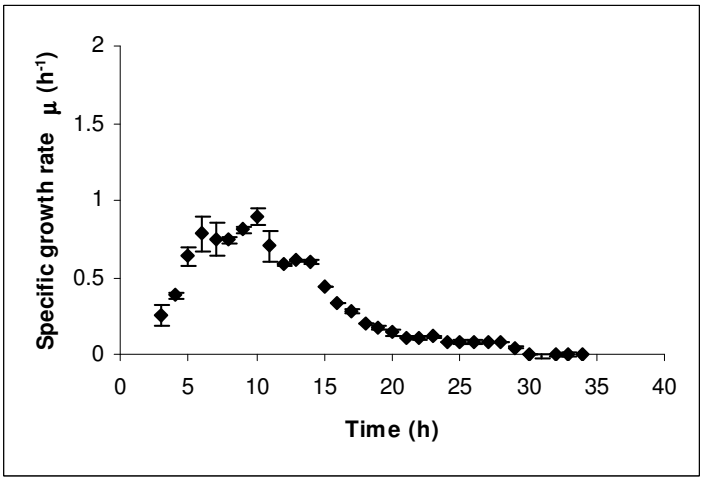


Figure 3.6: Specific growth rate of preculture of *S. cerevisiae* on growth media containing ammonium sulfate + histidine as nitrogen source

The preculture of *S. cerevisiae* on growth medium containing ammonium sulfate + histidine as nitrogen source was transferred to main culture at its 10^{th} hour in late exponential phase. As seen in Figure 3.6 between the 6th and 10th hour the organism was in exponential phase.

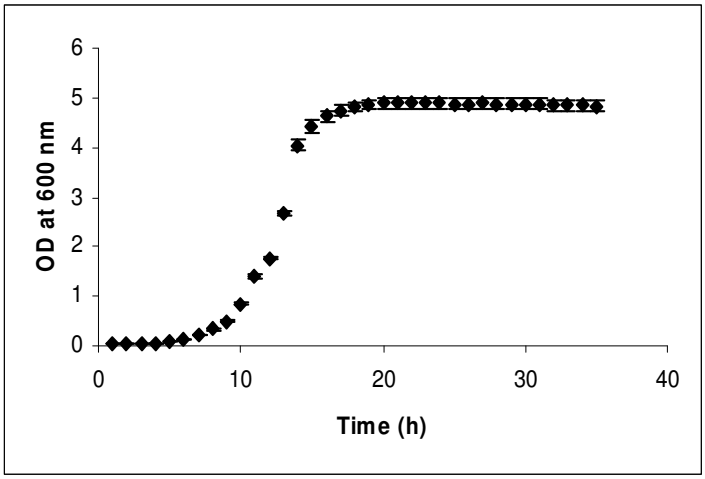


Figure 3.7: Growth of preculture of *S. cerevisiae* on growth media containing yeast nitrogen base + histidine as nitrogen source

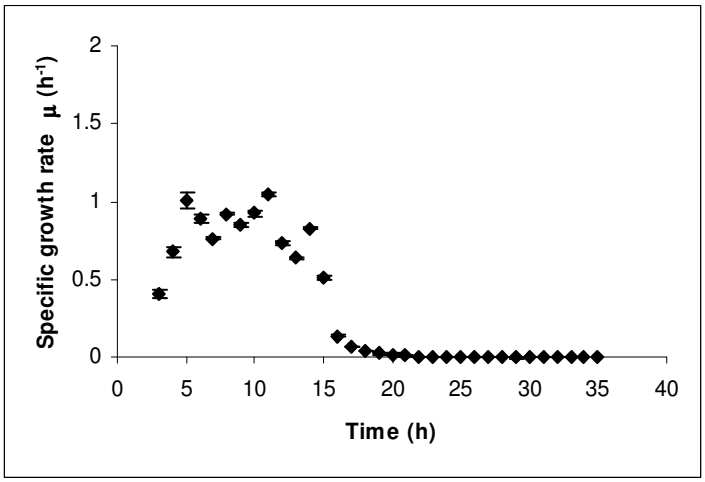


Figure 3.8: Specific growth rate of preculture of *S. cerevisiae* on growth media containing yeast nitrogen base + histidine as nitrogen source

Figure 3.8 shows the specific growth rate versus time graph of *S. cerevisiae* on growth media containing YNB + histidine as nitrogen source. Since the specific growth rate a stable level between the 5th and 11th hours, this part was taken as the exponential phase of the corresponding culture. The preculture was transferred to main culture at its 10 th hour in late exponential phase.

The main cultures were grown at 30 ^{o}C and 160 rpm in 1 L Erlenmeyer flasks. Pre-cultures were grown aerobically at 30 ^{o}C and 160 rpm in 250 ml Erlenmeyer flasks and inoculated to the fresh medium in the exponential phase in 1/15 portion. Preculture of *S. cerevisiae* on growth medium containing different nitrogen source inoculated to fresh medium at different time . For growth on medium containing ammonium sulfate as nitrogen source inoculated to fresh medium after eight hour in the exponential phase, for other nitrogen source precultures were transferred after 10 hours while they were still in exponential phase. Figures 3.9, 3.11, 3.13 and 3.15 show the OD_{600}- t graphs of main cultures of *S. cerevisiae* on growth medium containing one of the following nitrogen source ammonium sulfate, yeast nitrogen base, ammonium sulfate + histidine and yeast nitrogen base + histidine. The specific growth rate-t graphs of *S. cerevisiae* on growth medium containing one of the following nitrogen source ammonium sulfate, yeast nitrogen base, ammonium sulfate + histidine and yeast nitrogen base + histidine are shown in Figure 3.10, 3.12, 3.14, 3.16 respectively.

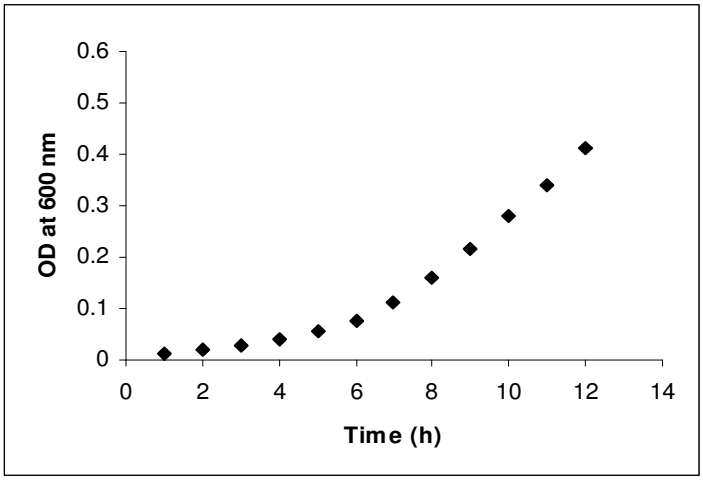


Figure 3.9: Growth of *S. cerevisiae* on media containing ammonium sulfate as nitrogen source

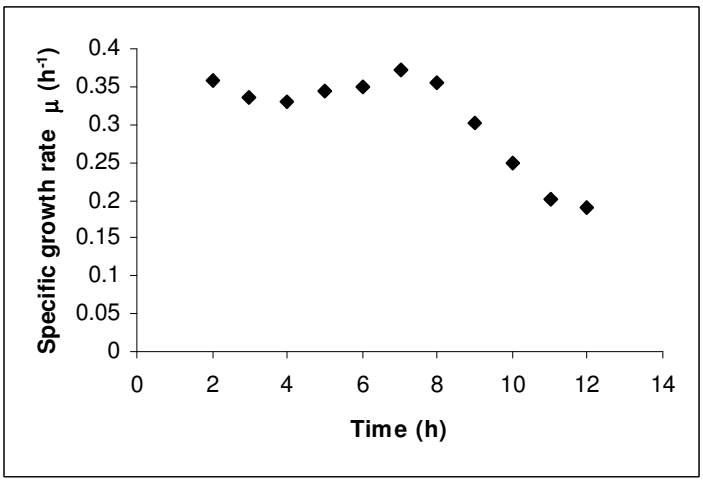


Figure 3.10: Specific growth rate of *S.cerevisiae* on growth media containing ammonium sulfate as nitrogen source

The transferred culture were still in exponential phase for 7-8 hours of growth (Figure 3.10). After 8 hours of growth the stationary phase began as characterized by a decrease in specific growth rate. The biomass for enzyme activity measurements were harvested at 6^{th} hours culture.

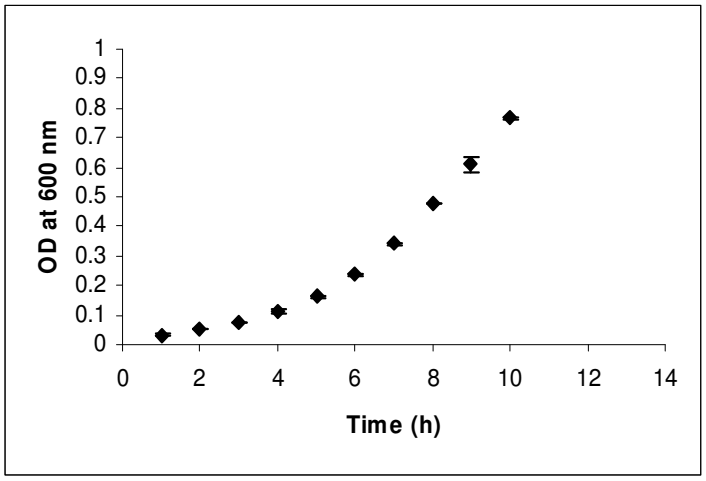


Figure 3.11: Growth of *S. cerevisiae* on media containing yeast nitrogen base as nitrogen source

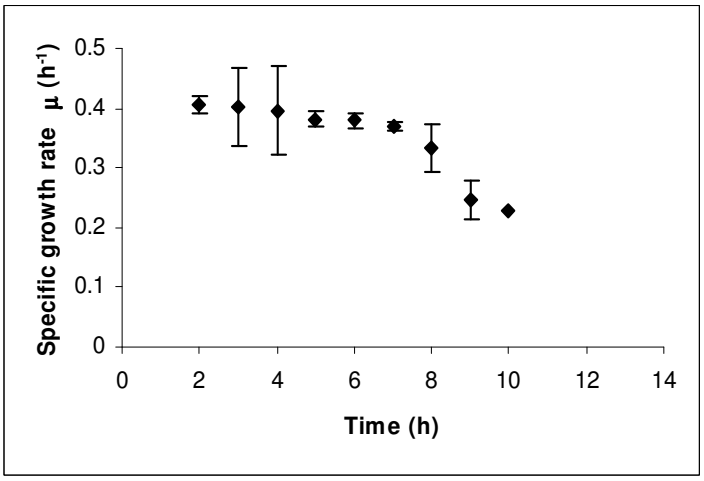


Figure 3.12: Specific growth rate of *S .cerevisiae* on growth media containing yeast nitrogen base as nitrogen source

As seen in Figure 3.12 the exponential phase of *S.cerevisiae* on growth medium containing yeast nitrogen base as nitrogen source continued nearly 7 hour. In the 5^{th} hours of culture the biomass was harvested for enzyme activity measurements

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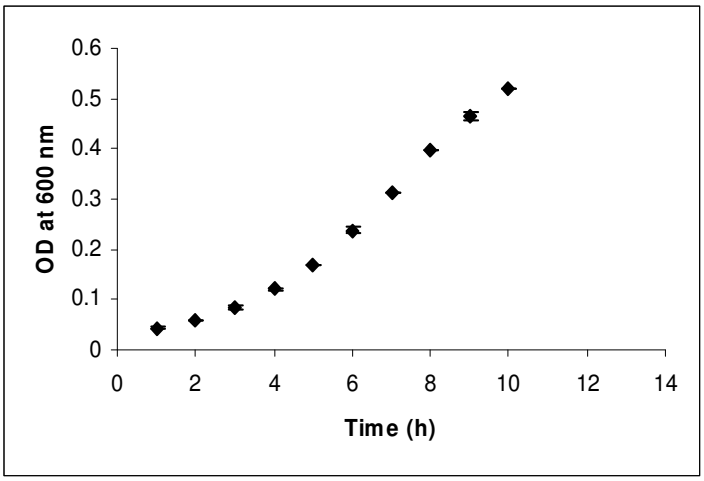

Figure 3.13: Growth of *S. cerevisiae* on media containing ammonium sulfate + histidine as nitrogen source

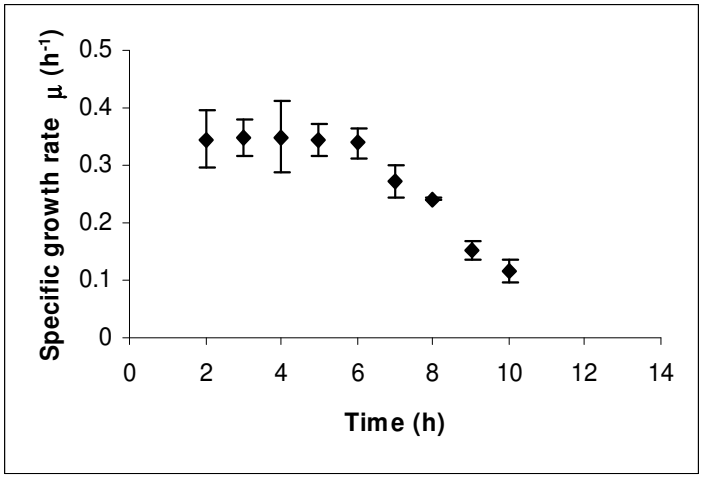

Figure 3.14: Specific growth rate of *S.cerevisiae* on growth media containing ammonium sulfate + histidine as nitrogen source

The growth curve of *S.cerevisiae* on growth medium containing ammonium sulfate + Histidine as nitrogen source is illustrated in Figure 3.14. It was seen that the organism was in exponential phase until 7th hour. The biomass was harvested at its 5th hour for enzyme activity measurements.

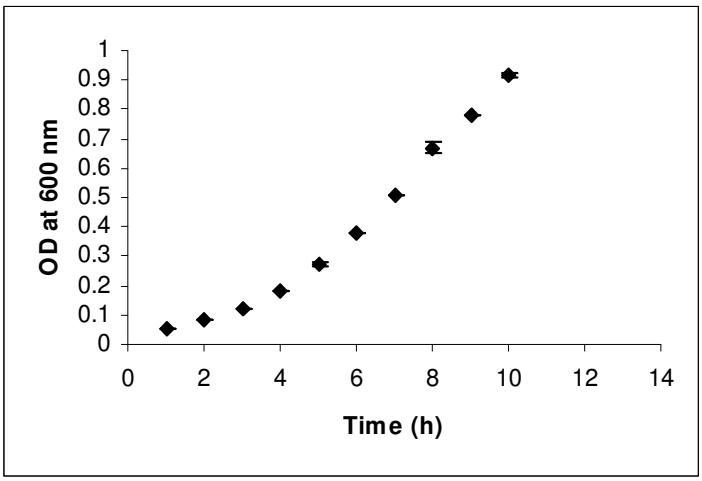

Figure 3.15: Growth of *S.cerevisiae* on media containing yeast nitrogen base + histidine as nitrogen source

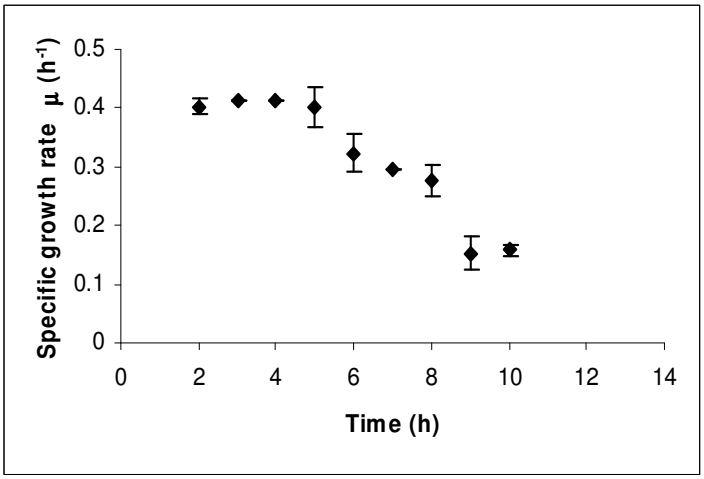

Figure 3.16: Specific growth rate of *S.cerevisiae* on growth media containing yeast nitrogen base + histidine as nitrogen source

Figure 3.16 shows the growth curve of *S.cerevisiae* on growth medium containing the yeast nitrogen base + Histidine as nitrogen source. The organism was grown exponential phase up to 6^{th} . The biomass was harvested at its 4^{th} hour for enzyme activity measurements.

As seen Figure 3.10, 3.12, 3.14 and 3.16 , the specific growth rate is 0.4 in YNB and YNB+histidine containing media and 0.35 in ammonium sulfate and ammonium sulfate+histidine containing media. In growth medium containing ammonium sulfate + histidine and YNB+histidine the period of exponential phase is shorter than in growth medium containing ammonium sulfate and YNB.

3.2. Dry Cell Weight Determination

3.2.1. Standart curve for dry cell weight determination

Dry cell weights were determined by using a standart curve (Figure 3.17). For dry cell weight measurements preweighed nitrocellulose filters (pore size: 0.45 μm) were used. The medium were diluted to various OD_{600} values ranging from 0.1 to 0.6. After filtration of 15 ml of each of these media , the filters were washed with demineralized water and dried in an microwave oven for 20 min. These samples were weighed again and the difference between the preweighed filter and these samples gave the weight (dry weight) of the yeast samples at different OD_{600} values.

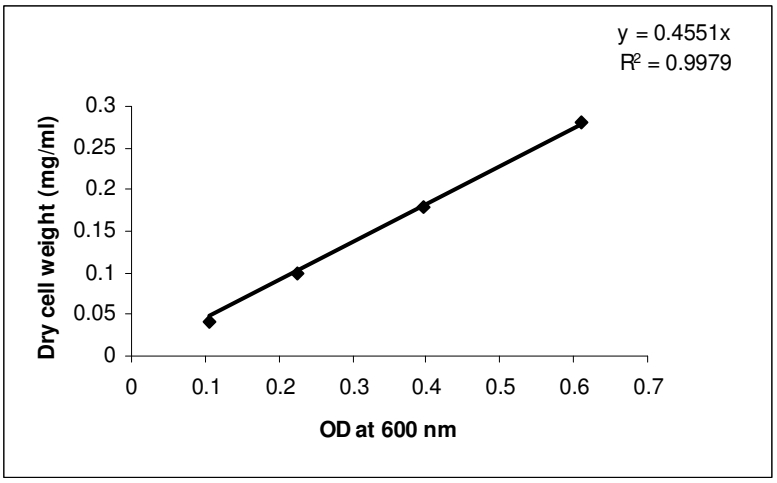

Figure 3.17: Standart curve for dry cell weight of *S. cerevisiae*

3.2.2. Dry cell weight determination of *Saccharomyces cerevisiae* on growth media containing different nitrogen source

Using the dry cell weigt versus OD at 600 nm standard curve (Figure 3.17) the previous graphs showing the OD at 600 nm versus time graps (Figure 3.9, 3.11, 3.13, 3.15) were converted to dry weight versus time graphs (Figure 3.18, 3.19, 3.20, 3.21). Namely these graphs are dry weight versus time graphs of S. cerevisiae on growth media containing ammonium sulfate, yeast nitrogen base, ammonium sulfate + histidine and yeast nitrogen base + histidine as nitrogen source.

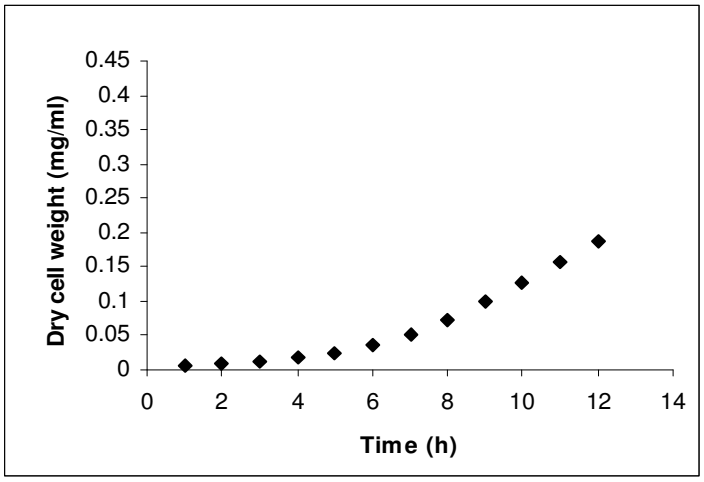

Figure 3.18: Dry cell weight versus time graph of *S.cerevisiae* on growth media containing ammonium sulfate as nitrogen source

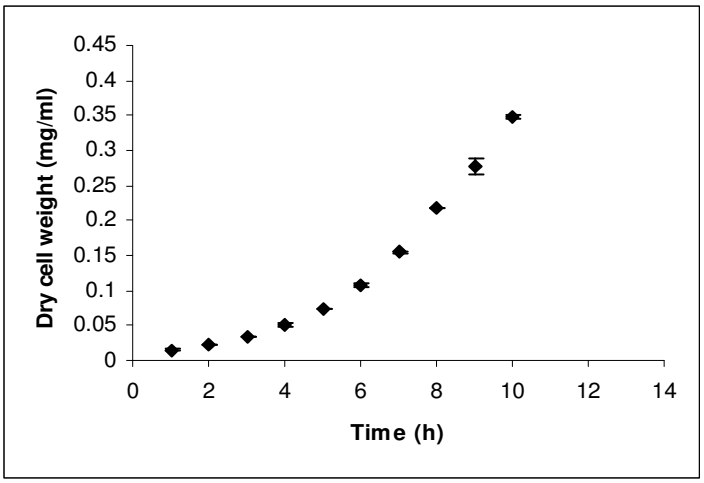

Figure 3.19: Dry cell weight versus time graph of *S.cerevisiae* on growth media containing yeast nitrogen base as nitrogen source

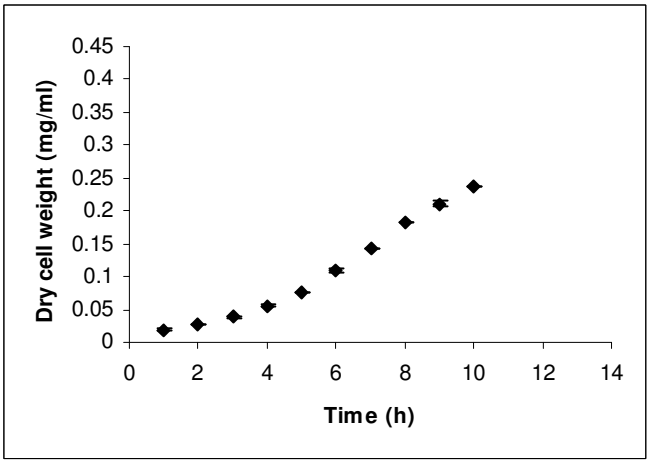

Figure 3.20: Dry cell weight versus time graph of *S.cerevisiae* on growth media containing ammonium sulfate+histidine as nitrogen sources

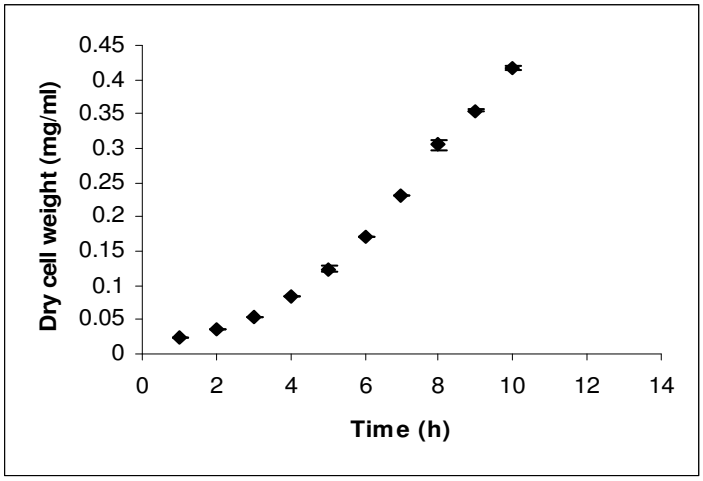

Figure 3.21: Dry cell weight versus time graph of *S.cerevisiae* on growth media containing yeast nitrogen base+histidine as nitrogen sources

3.3 Preparation of crude extract

The cell crude extracts were prepared as explained in Chapter 2. The cell distruption was performed with sonicator (Soniprep 150). Since the volume of biomass suspension was small, causing the denaturation of the enzymes due to be heated up in a short time. The sonication time was optimised. For optimization, the same volume of sample (2.5 ml) was sonicated separately for 1, 2 and 3 minutes and crude extracts were prepared by centrifugation of the sonicated material. The specific activity of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase were determined in these crude extracts. The results are shown in Figures 3.22 and 3.23.

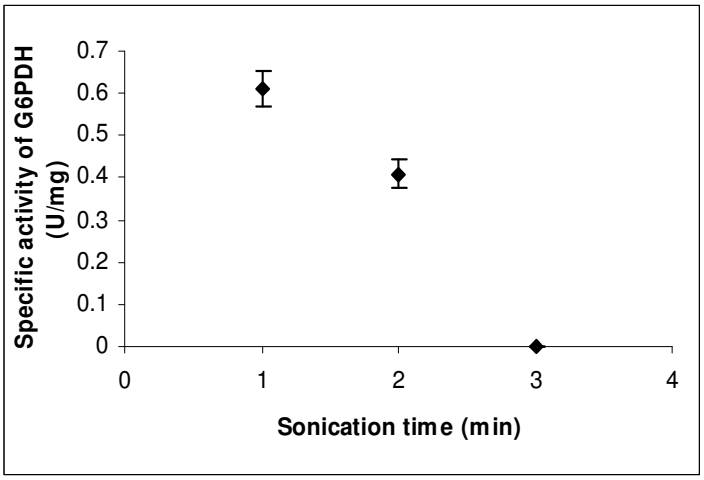

Figure 3.22 : Effect of sonication time on G6PDH activity

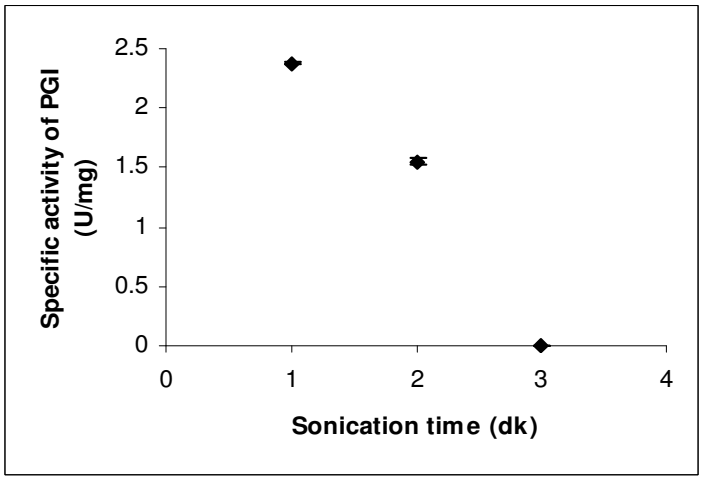

Figure 3.23 : Effect of sonication time on PGI activity

As seen from Figure 3.22 and 3.23 enzyme activity could not be determined in 3 minutes sonicated samples. The specific activity of both enzymes decreased when the sonication time increased from 1 minute to 2 minutes

Depending on the results obtained in Figure 3.22 and 3.23 further optimisation with sonication times less than 1 minute were tested. An equal volume of sample (2.5 ml) was sonicated for 20, 30, 45 and 60 seconds and the specific activities of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase were determined. The results showed that 1 minute of sonication is the optimum time for 2.5 ml 4% yeast biomass suspensions. (Figure 3.24 and 3.25)

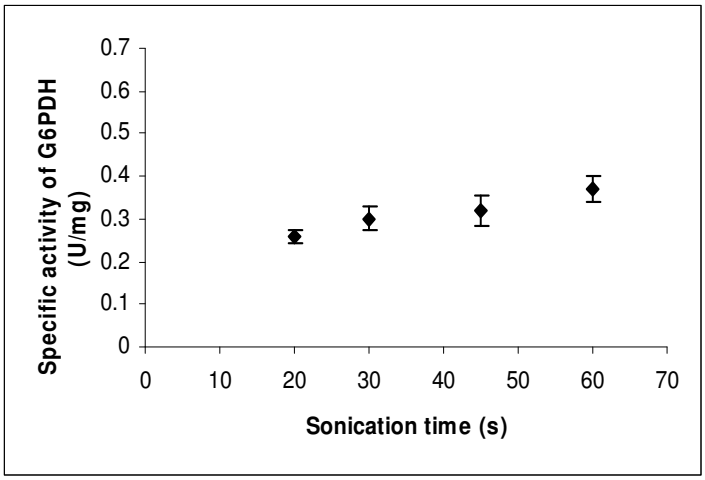

Figure 3.24 : Effect of sonication time on G6PDH activity

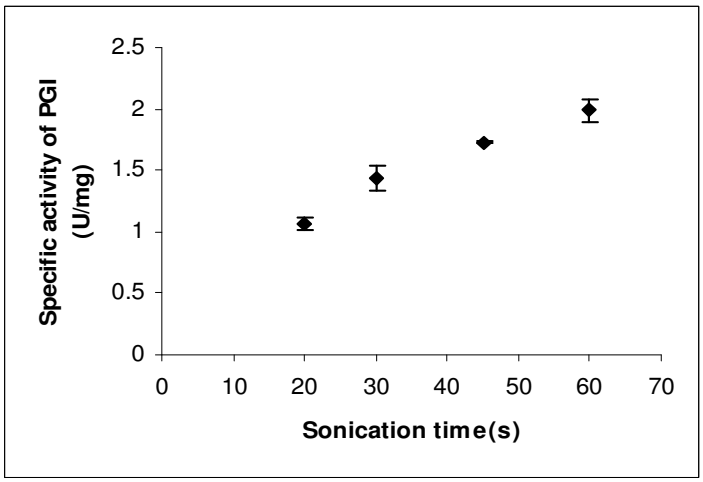

Figure 3.25 : Effect of sonication time on PGI activity

Figure 3.24 and Figure 3.25 shows the increase in specific activities of G6PDH and PGI respectively. The increase in specific activity with time of sonication is much more obvious for PGI activity. The specific activity obtained at 60 sec of sonication is 88 % more than obtained at 20 sec of sonication.

According to the optimised conditions, wet yeast cells in 4% of the extraction buffer were sonicated at 10 μ amplitude gently for 1 minute with 10 seconds intervals in this study.

3.4. Optimization of Enzyme Assays

For optimization studies, *Saccharomyces cerevisiae* were grown in rich medium and the enzyme activities were measured in crude extracts. Enzyme activities were assayed spectrophotometrically by following NADPH formation at 340 nm. Sensitive and reliable activity measurement of enzymes is important in this study. For this purpose, different spectroscopic enzyme assays were tested and chosen the most suitable ones. The chosen assays were optimized both for pH and temperature. The results of optimization was shown that the chosen enzyme assays were suitable for this study.

3.4.1. Effect of Temperature on G6PDH and PGI Activity

Determination of the effect of temperature on the glucose-6-phosphatedehydrogenase and phosphoglucose isomerase activities were carried out in crude extract. The cell of the spectrophotometer was kept at constant temperature at the indicated temperatures by using circulatory water bath. Buffer, distilled water and enzyme (enzyme was incubated not more than 3 min) were incubated at constant temperature before the reaction is started.

Figure 3.26 shows the activity of glucose-6-phosphate dehydrogenase at 25 ^{o}C, 30 ^{o}C, 35 ^{o}C .The activity of G6PDH was found to be the highest at 35 ^{o}C among the three different temperatures tested (Figure 3.26). For further G6PDH activity assays 30 ^{o}C was used.

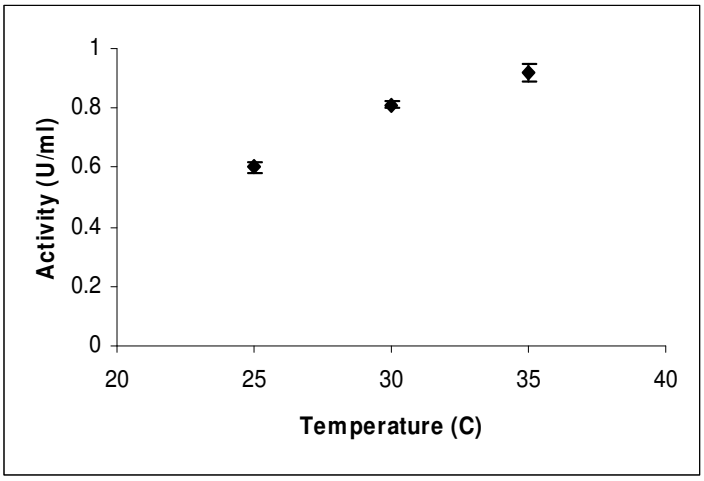

Figure 3.26: Effect of temperature on glucose-6-phosphate dehydrogenase enzyme activity

Four different assay temperatures were tested for the activity of phosphoglucose isomerase . As seen in Figure 3.27, the activity of PGI increased going up from 25 ^{o}C to 30 ^{o}C and 35 ^{o}C. The activity stayed constant at 30 ^{o}C and 35 ^{o}C while it decreased at 40 ^{o}C. The optimum temperature was determined as 30-35 ^{o}C. For further activity assays 30 ^{o}C was used as the assay temperature.

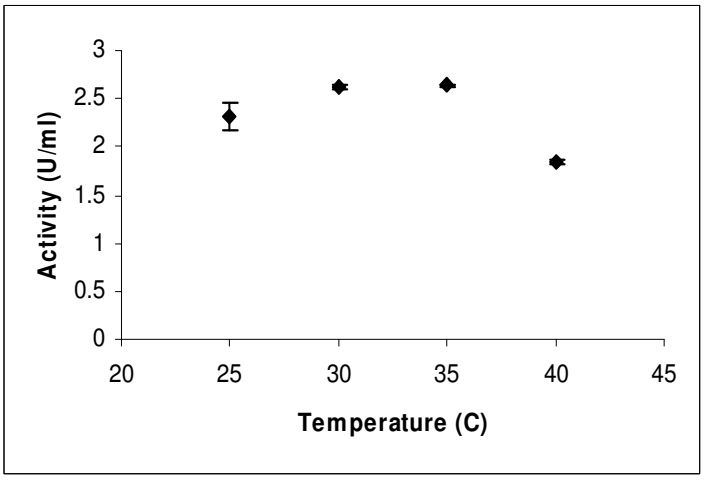

Figure 3.27: Effect of temperature on phosphoglucose isomerase enzyme activity

3.4.2. Effect of pH on G6PDH and PGI Activity

In determination of effect of pH on glucose-6-phosphate dehydrogenase and phosphoglucose isomerase activity , different buffer systems were used and reactions were carried out as described in "Material & Methods".

The pH of the assay mixture was varied between 6 and 8.5 for glucose-6-phosphate dehydrogenase assay and between 6 and 9 for phosphoglucose isomerase assay. The change in the enzyme activities at various pH are shown in Figure 3.28 and 3.29.

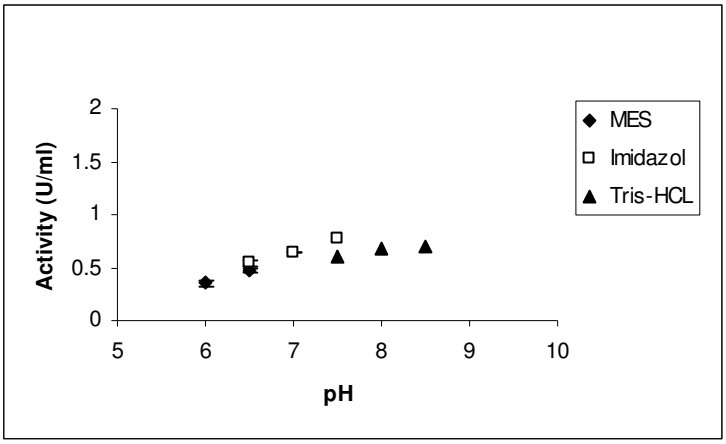

Figure 3.28: Effect of pH on glucose-6-phosphate dehydrogenase enzyme activity. The pH values and buffers used are; 100 mM MES (pH 6 and 6.5), 100 mM Imidazol-HCI (pH 6.5, 7 and 7.5), 100 mM Tris-HCI (pH 7.5, 8 and 8.5)

Figure 3.28 shows the activity of glucose-6-phosphate dehydrogenase at different pH values. Three different buffer systems were used to find the optimum pH of G6PDH. The highest G6PDH activity for this enzyme was obtained at pH 7.5 using imidazol.

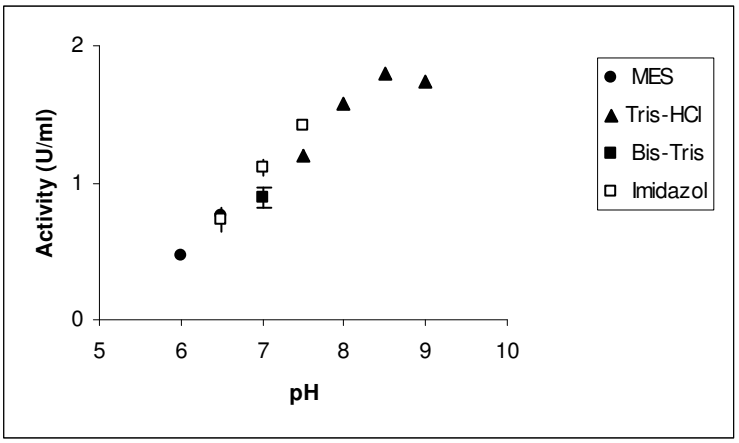

Figure 3.29: Effect of pH on phosphoglucose isomerase enzyme activity. The pH values and buffers used are; 100 mM MES (pH 6 and 6.5), 100mM Imidazol-HCI (pH 6.5, 7 and 7.5), 100 mM Bis-Tris (pH 7), 100 mM Tris-HCI (pH 7.5, 8, 8.5 and 9)

As indicated in Figure 3.29 four different buffer systems were used to find the optimum pH of PGI. The highest activity for this enzyme was obtained at pH 8.5 using Tris-HCI. This pH value were used as the optimum pH for activity measurements of PGI.

3.5. Effect of Different Nitrogen Sources on Glucose-6-phophate Branch Enzyme Activity

The influence of different nitrogen sources on the flux distribution through the pentose phosphate pathway and glycolysis was examined. For this purpose, four different nitrogen source were used and specific activities of hexokinase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were measured in the late exponential phase.

Enzyme activities were assayed spectrophotometrically as described in "Materials and Methods"

As shown in Table 3.1. when the specific activities of enzymes were compared in medium containing only AS and AS+Histidine, the specific activities of all enzymes were increased in medium containing AS+Histidine. The increase in the specific activities of enzymes was 31.7% for hexokinase, 60.38% for phosphoglucose isomerase, 20% for glucose-6-phosphate dehydrogenase and 52.98% for 6-phosphogluconolactone dehydrogenase.

When the increase in the hexokinase activity take into consideration, the ratios of the other enzymes to hexokinase were 1.70 for phosphoglucose isomerase, 0.40 for glucose-6-phosphate dehydrogenase and 0.27 for 6-phosphogluconate dehydrogenase in medium containing AS. For medium containing AS +Histidine the ratio of enzymes were, 2.1 for phosphoglucose isomerase, 0.40 for glucose-6-phosphate dehydrogenase and 0.31 for 6-phosphogluconate dehydrogenase (Table 3.2).

Like medium containing only AS and AS+Histidine, the specific activity of hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase were higher in medium containing YNB+Histidine than YNB. The increase in the specific activities of enzymes was 61.99% for hexokinase, 40.87% for phosphoglucose isomerase, 53.06% for glucose-6-phosphate dehydrogenase. However in contrast to medium containing AS and AS+Histidine the specific activity of 6-phosphogluconate dehydrogenase decreased 3.1% in YNB+Histidine medium.

As seen in Table 3.2, the ratio of the enzymes to hexokinase were 1.80 for phosphoglucose isomerase, 0.30 for glucose-6-phosphate dehydrogenase and 0.35 for 6-phosphogluconate dehydrogenase in medium containing YNB. In medium containing YNB +Histidine the ratios were, 1.56 for phosphoglucose isomerase, 0.29 for glucose-6-phosphate dehydrogenase and 0.21 for 6-phosphogluconate dehydrogenase.

Table 3.1: Specific activities of the glucose-6-phosphate branch point enzymes. hexokinase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

Nitrogen Source	μ (h^{-1})	HXK	PGI	G6PDH	6PGDH
		Sp. Act.(U/mg)	Sp. Act.(U/mg)	Sp. Act.(U/mg)	Sp.Act.(U/mg)
AS	0.35	0.624±0.028	1.060±0.153	0.250±0.014	0.168±0.004
AS+His	0.35	0.822±0.061	1.700±0.073	0.300±0.027	0.257±0.023
YNB	0.40	0.642±0.036	1.150±0.104	0.196±0.016	0.226±0.014
YNB+His	0.40	1.040±0.021	1.620±0.087	0.300±0.020	0.219±0.004

Table 3.2: Ratio of the specific activities of glucose-6-phosphate branch point enzymes, hexokinase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzymes

Ratio of the specific activities of the enzymes	Nitrogen Source			
	AS	AS+Histidine	YNB	YNB+Histidine
HXK / G6PDH	2500±0.144	2.700±0.201	3.280±0.075	3.460±1.978
HXK / PGI	0.590±0.089	0.500±0.029	0.560±0.044	0.640±0.358
HXK/6PGDH	3.710±0.245	3.198±0.344	2.841±0.312	4.749±2.699
G6PDH/ HXK	0.400±0.023	0.365±0.026	0.305±0.007	0.288±0.170
PGI / HXK	1.700±0.254	2.100±0.126	1.791±0.146	1.558±0.932
6PGDH/HXK	0.269±0.018	0.313±0.032	0.352±0.037	0.211±0.124
G6PDH/PGI	0.236±0.045	0.176±0.020	0.170±0.015	0.185±0.012
PGI/G6PDH	4250±0.849	5.670±0.605	5.870±0.539	5.400±0.354
6PGDH/PGI	0.158±0.020	0.151±0.008	0.197±0.019	0.135±0.004
PGI/6PGDH	6.310±0.842	6.615±0.349	5.088±0.482	7.397±0.241
6PGDH/G6PDH	0.672±0.050	0.857±0.135	1.153±0.145	0.730±0.047
G6PDH/6PGDH	1.488±0.114	1.167±0.192	0.867±0.116	1.370±0.089

According to these findings, addition of histidine caused an increase in the specific activities of all the enzymes analysed. However, the specific activity of 6-phosphogluconate dehydrogenase decreased 3.1% in YNB+Histidine medium. As suggested by the ratios of the specific activities of the enzymes according to each other the flux showed a considerable increase at the 6-phosphogluconate dehydrogenase point and in the phosphoglucose isomerase point in medium containing AS+Histidine but a decrease in YNB+Histidine. It might be caused for the content of the YNB. There are 10 mg histidine / 6.7gr YNB in the YNB medium and it could be enough to effect the activity of 6-phosphogluconate dehydrogenase in YNB.

CHAPTER 4

DISCUSSION

The influence of different nitrogen sources on the flux distribution through the pentose phosphate pathway and glycolysis in *Saccharomyces cerevisiae* was examined in this study. For this purpose, four different nitrogen sources were used and the activities of enzymes in glucose-6-phosphate branch point were measured in the late exponential phase. The growth medium was contained one of the following nitrogen source ammonium sulfate, yeast nitrogen base and histidine aminoacid, which is synthesis from pentose phosphate pathway, were added to ammonium sulfate and yeast nitrogen base.

Enzyme activities were assayed spectrophotometrically by following NADPH formation at 340 nm. For sensitive and reliable activity measurement of enzymes, different spectroscopic enzyme assays were tested and chosen the most suitable ones. The chosen assays were optimized both for pH and temperature. The results of optimization was shown that the chosen enzyme assays were suitable for this study. Determination of the optimum temperature and pH of the enzymes was carried out in the crude extract.

For glucose-6-phosphate dehydrogenase the optimum temperature and pH were found as 35 ^{o}C and 7.5, respectively. Glaser and Brown had determined the optimum pH as 8.5 for glucose-6-phosphate dehydrogenase (Glaser and Brown, 1955).

For phosphoglucose isomerase the optimum temperature was determined as 35 ^{o}C and the optimum pH was determined as 8.5. In *Aspergillus niger* the activity of phosphoglucose isomerase was found to be maximal between pH 7.5 and 10, whereas it decreased below pH 7.5 (Ruijter and Visser, 1998).

The OD value and dry weight in the culture containing histidine is higher than the cultures contaning only AS and only YNB. The synthesis of yeast biomass is clearly dependent on the quality and quantity of nitrogen source in the growth medium, since the proportion of nitrogenous compounds in yeast cells is about 50% (by weight) (Torija et.al., 2003). The biomass yield on glucose was 1.5 times higher with the mixture of aminoacids than it was with either glutamic acid and ammonium as the nitrogen source (Albers et.al., 1996).

As shown in Figures 3.10., 3.12., 3.14., 3.16. period of the exponential phase is shorter in medium containing histidine in addition to AS or YNB than in medium containing only AS and only YNB. In addition, the specific activities of all the enzymes analysed were higher in cultures containing histidine than cultures containing only AS or only YNB. According to these results, although no differences were determined in the specific growth rates of both AS and AS+Histidine and YNB and YNB+Histidine, in the cultures adding histidine to ammonium sulfate and YNB the flux into the biomass formation was more than cultures containing only AS or only YNB. In the study of Albers et. al. ammonium salt, glutamic acid or a mixture of amino acids was used as nitrogen source and the shortest cultivation time was obtained for growth on a mixture of aminoacids. (Albers et.al., 1996). They thought that this result may be explained by the decrease in need of amino acid synthesis in this case. (Albers et.al., 1996). In our study only histidine amino acid was added to medium and period of the exponential phase was shorter in medium containing histidine in addition to AS or YNB. It might be also due to the decrease in need of amino acid synthesis. 9.1% of the cellular protein is histidine in *S. cerevisiae* (Stephanopoulos, Aristidou & Nielsen, 1998). Also high biomass was observed in histidine containing mediums might be the result of utilization of intermediatery components in purin and pyrimidin biosynthesis.

The positive effect of aminoacids on the growth and fermentation rates has previously been noted by Thomas and Ingledew (Thomas & Ingledew;1990). Some studies have shown that a mixed source (ammonia and amino acids) is more effective for promoting yeast growth and fermentation rate (Ribereau-Gayon et al., 2000).

For determination of difference of specific growth rate more exactly, the experiments could be done in continues culture in fermentor.

The enzymes analysed have many activators and inhibitors. The pentose phosphate pathway intermediates 6-phosphogluconate and erythrose 4- phosphate were competitive inhibitors of PGI (Ruijter and Visser, 1998). Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were inhibited by NADPH and the 6-Phosphogluconate dehydrogenase additionally by ATP, fructose 1,6-bisphosphate, D-glyceraldehyde 3-phosphate, erythrose 4-phsphate and ribulose 5-phosphate. (Moritz et.al., 2000; Tsai&Chen, 1998). Therefore, the concentration of these metabolites and other intracellular metabolites should be analysed in these medium. For further understanding of histidine flux the enzyme in the histidine route has to be analysed.

CHAPTER 5

CONCLUSION

In this study, the influence of different nitrogen sources on the flux distribution through the pentose phosphate pathway and glycolysis was examined in *Saccharomyces cerevisiae*.

Four different compositions of nitrogen sources were used. The growth medium was contained one of the following composition of nitrogen sources; only ammonium sulfate, only yeast nitrogen base, ammonium sulfate and histidine, yeast nitrogen base and histidine. Histidine was added because its synthesis branches from pentose phosphate pathway. In order to analyse the effect of the different compositions of nitrogen sources on the physiology of the yeast specific activities of hexokinase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzymes were measured in the crude extracts of the biomass samples taken in the late exponential phase of the cultures.

The OD_{600} value and dry cell weight in the culture containing histidine amino acid were higher than the cultures contaning AS and YNB. The specific growth rate was 0.4 in YNB and YNB+histidine containing media and 0.35 in ammonium sulfate and ammonium sulfate+histidine containing media. Also. period of the exponential phase was shorter in medium containing AS and histidine and, YNB and histidine than medium containing only AS and only YNB.

When the specific activities of enzymes were compared in medium containing only AS and AS+Histidine, the specific activities of all enzymes were increased in medium containing AS+Histidine. The specific activity of hexokinase,

phosphoglucose isomerase and glucose-6-phosphate dehydrogenase were higher in medium containing YNB+Histidine than medium containing only YNB. The specific activity of 6-phosphogluconate dehydrogenase decreased 3.1% in YNB+Histidine medium.

According to these findings, although no differences were observed in the specific growth rate of both AS and AS+Histidine and YNB and YNB+Histidine, in the cultures containing histidine in addition to ammonium sulfate and YNB the flux into the biomass formation was more than cultures containing AS or YNB only. For determination of difference of specific growth rate more exactly the experiments should be done in continuous culture in fermentors.

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APPENDIX A

MEDIUM COMPOSITION FOR GROWTH OF *Saccharomyces cerevisiae*

Component	Amount/liter
Mineals	
KH_2PO_4	3,0 g
$MgSO_4$	1.0 g
Trace elements	
EDTA	30 mg
$CaCl_2.7H_2O$	9 mg
$ZnSO_4.7H_2O$	9 mg
$FeSO_4.7H_2O$	6 mg
H_3BO_3	2 mg
$MnCl_2.7H_2O$	1.7 mg
$Na_2MoO_4.7H_2O$	0.8 mg
$CoCl_2.7H_2O$	0.6 mg
$CuSO_4.7H_2O$	0.6 mg
KI	0.2 mg
Carbon and energy source	
glucose	20 g
vitamins	
D-Biotin	0.05 mg
p-Aminobenzoic acid	0.2 mg
Nicotinic acid	1 mg
Calcium pantothonate	1 mg
Pyridoxine HCI	1 mg
Thiamine HCI	1 mg
m-inositol	25 mg

nitrogen sources	concentration
ammonium sulfate	7.5 g/L
yeast nitrogen base	6.7 g/L
Histidine	36.9 mg/L

APPENDIX B

COMPOSITION OF YEAST NITROGEN BASE

Formula **Ingredients per liter**	**Bacto** **Yeast Nitrogen Base**
Nitrogen sources	
Ammonium sulfate	5 g
Amino acids	
l-Histidine monohydrochloride	10 mg
dl-Methionine	20 mg
dl-Tryptophan	20 mg
Vitamins	
Biotin	2 mcg
Calcium Pantothenate	400 mcg
Folic acid	2 mcg
Inositol	2000 mcg
Niacin	400 mcg
p-Aminobenzoic Acid, Difco	200 mcg
Pyrodoxine Hydrochloride	400 mcg
Riboflavin	200 mcg
Thiamine Hydrochloride	400 mcg
Compounds supplying trace elements	
Boric acid	500 mcg
Copper Sulfate	40 mcg
Potassium Iodide	100 mcg
Ferric Chloride	200 mcg
Manganase Sulfate	400 mcg
Sodium Molybdate	200 mcg
Zinc Sulfate	400 mcg
Salts	
Potassium Phosphate Monobasic	1 g
Magnesium Sulfate	0.5 g
Sodium Chloride	0.1 g
Calcium Chloride	0.1 g

APPENDIX C

RICH MEDIUM COMPOSITION FOR GROWTH OF *Saccharomyces cerevisiae*

Chemical	**Amount (g/L)**
Glucose	20 g/L
$(NH_4)_2SO_4$	1.2 g/L
KH_2PO_4	0.3 g/L
yeast extract (Difco)	1 g/L
$CaCl_2$	0.25 g/L
$MgSO_4.7H_2O$	0.25 g/L

APPENDIX D

COMPOSITION OF THE BRADFORD'S REAGENT AND STANDART CURVE PREPARATION TABLE

5X stock solution:

500 mg Brillant Blue G
250 ml 95 % Ethanol
500 ml 85 % Phosphoric acid

Complete the volume to 1000 ml with distilled water and store at 4 ^{o}C. When to use dilute stock 1:4 with distilled water and filter through normal filter paper. Wait for at least 24 hours at 25 ^{o}C before use. Since the reagent is sensitive to light, store it in a dark bottle or cover the transparent bottle with aliminum foil.

Standart Curve Preparation:

Prepare a stock solution of 1 mg/ml BSA.

Tube #	BSA (µl)	dH_2O	Bradford's reagent (ml)
1	0	500	5
2	5	495	5
3	10	490	5
4	15	485	5
5	20	480	5
6	25	475	5

After addition of reagents vortex and wait for 10 min. Read OD at 595 nm.