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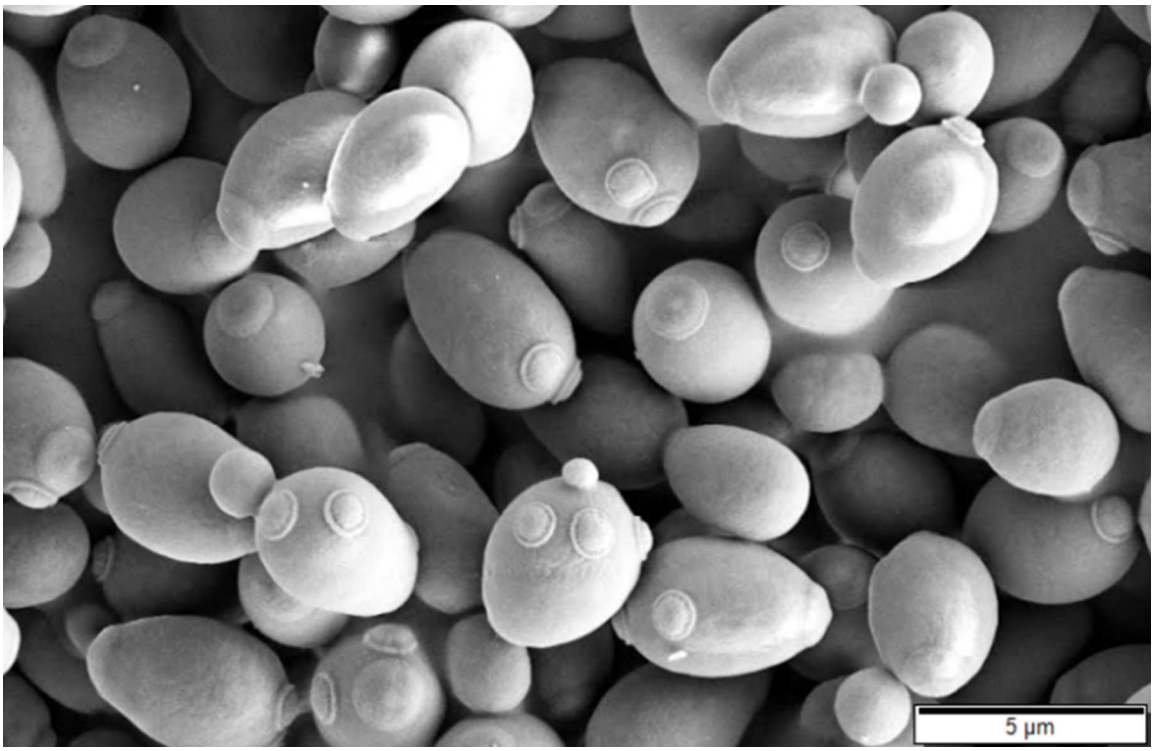
YEAST. MORPHOLOGY AND PHYSIOLOGY



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MINISTRY OF SCIENCE AND HIGHER EDUCATION

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ITMO UNIVERSITY

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YEAST. MORPHOLOGY AND PHYSIOLOGY

STUDY GUIDE

RECOMMENDED FOR USE AT ITMO UNIVERSITY
in the area of training 19.04.02, the discipline "Quality and Food Safety", as a study guide for the implementation of professional educational programs of higher education

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Reviewer: Barakova Nadezhda Vasilievna, PhD in Engineering, Associate Professor.

The textbook contains brief theoretical provisions concerning the morphology and physiology of yeast as the widely used microorganism in the fermentation industry, and discusses modern technological aspects of the yeast usage.

The study guide contains a description of the recommended laboratory and practical works. Laboratory works are devoted to the study of the yeast physiological activity. Each laboratory work contains brief theoretical provisions of the methods used, the equipment and materials used, the content and procedure for carrying out the work. Practical materials include examples of tasks for calculating the nutrient media components, assessing the usefulness of their chemical composition, as well as variants for individual tasks.

ITMO University is a leading Russian university in information and photonic technologies, one of the few Russian universities that received the status of a national research university in 2009. Since 2013, ITMO University has been a participant in the program to improve Russian universities' competitiveness among the world's leading research and educational centers, known as the "5 in 100" project. ITMO University's goal is to become a world-class research university, entrepreneurial in type, focused on the internationalization of all areas of activity.

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Introduction

The biosynthetic activity of yeast cells and their ability to adapt to the constantly changing environmental conditions during fermentation determine the fermentation capacity of yeast, its colloidal stability and biosynthesis of the beer's flavor components.

The biosynthetic activity of yeast cells depends on yeast nutrition, their age determined by the number of mitotic divisions of a yeast cell (which can be identified by a number of bud scars on the cell surface), as well as the physicochemical conditions of the environment. Cell activity changes in connection with osmotic, hydrostatic, alcoholic, temperature and mechanical stress, resistance to which depends both on strain characteristics of yeast and their physiological state.

The strain (from lat. *Stamm*, literally "trunk", "base") is a pure culture of viruses, bacteria and other microorganisms, or a cell culture isolated at a particular time and place.

To evaluate the biosynthetic activity of yeast the following indicators are used:

-viability or the number of living cells capable to form colonies, usually expressed in %;

- the content of dead cells, usually expressed in %;

-physiological activity ("vitality"), which is determined by the intensity of metabolic processes and is closely related to viability.

Viability is usually determined by methods based on multiplication of cells or dye staining methods.

When determining the viability of yeast by staining, various dyes are used, for example: methylene blue, Lugol's solution, safranin, magnesium salt of 1-anilino-8-naphthalene sulfonic acid (Mg-ANS), and dihydrorhodamine.

The physiological activity is identified by the content of reserve carbohydrates (glycogen and trehalose) in cells that are the source of endogenous glucose for the

biosynthesis of sterol and unsaturated fatty acids during the lag growth phase (adaptation period), with unsaturated fatty acids being the most important components of cellular membranes. In addition, endogenous glucose is involved in the metabolism of glycerol, an antistress cellular component that is synthesized when yeast is introduced into dense media.

The biosynthetic activity of cells can be determined by the rate of carbon dioxide release or the rate of oxygen consumption in the Warburg apparatus. The decrease in O_2 consumption rate indicates a slowdown in the metabolic processes associated with yeast multiplication.

Among the new methods for assessing the physiological state of yeast, the "acidification strength" test, measurement of the intracellular pH value (JCP method), and CO_2 emission method modified by Davydenko should be mentioned.

The choice of method assessing the physiological state of cells is determined by the tasks assigned to brewers. In any case, timely assessment of yeast biosynthetic activity is the basis for an efficient and successful process of fermentation and high-quality production.

1 Morphology of Yeast

Yeasts are unicellular organisms that belong to the kingdom of fungi and multiply by budding. As yeasts belong to facultative anaerobes, the catabolism of glucose in a cell takes place both in aerobic and anaerobic conditions.

Yeasts get the energy from organic compounds, which are most likely to be based on sugar. Thus, yeasts do not depend on sunlight for their energy needs and are often isolated from sugar-containing media.

Among the identified species widely used in the fermentation industry are the yeasts *Saccharomyces cerevisiae*.

S. cerevisiae is a small, unicellular organism. *S. cerevisiae* cells are round, ovoid in shape. Their size varies from 2.5 to 10 μm in diameter and from 4.5 to 21 μm in length. The size and shape of the cells belonging to the same strain vary within certain limits depending on the cultivation conditions. A yeast cell consists of macroscopic and submicroscopic components.

Macroscopic components include mitochondria, nucleus with nucleolus, ribosomes, endoplasmic reticulum, Golgi apparatus, cytoplasmic membrane, lysosomes and glycosomes. Submicroscopic components include fats, polyphosphates and glycogen. These components are non-permanent intracellular reserve substances.

The glycogen amount in a yeast cell features the physiological activity of yeast and varies depending on the yeasts growing conditions and their age. Its amount is determined by the method of staining with Lugol's solution, which stains glycogen in brown and the cells in yellow.

Yeasts are best cultivated in a neutral or slightly acidic medium with pH 4.8-5.0 under aerobic conditions with sufficient nutrition at optimal temperatures of 28-30 ºC.

All cellular organelles are surrounded by membranes. The composition of membranes includes a large number of phospholipids, and their content in both

quantitative and qualitative composition is determined by the nature of the organelles (Fig. 1.1).

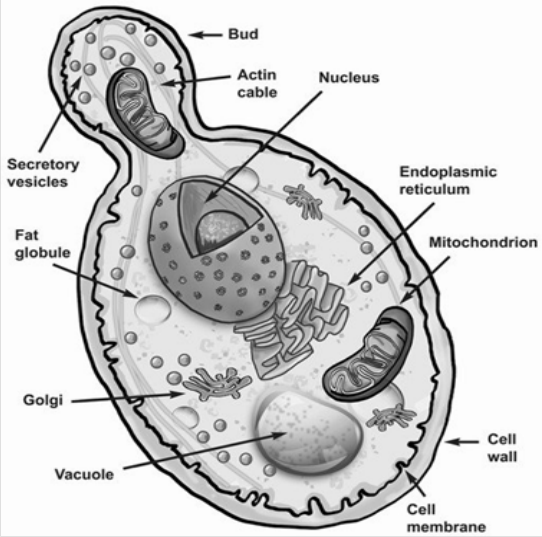

Figure 1.1 - Yeast cell structure

The plasma membrane separates the components of the cell from the external environment, while the nuclear membrane protects the genetic material. In addition, the mitochondrial membrane participates in metabolic energy generation, whereas the endoplasmic reticulum (ER) and the Golgi apparatus participate in the sorting and synthesis of proteins and lipids [1-4].

1.1 Cell wall

Each yeast cell is surrounded by a cell wall. The cell wall gives the cell a shape and provides mechanical and thermal protection from the environment. In addition, it acts as a screen from large molecules, which can damage the cell. This gives yeasts a much better chance of survival. Despite the harsh environmental conditions, the cell

wall also allows the cell to communicate with the environment. In addition, the cell wall participates in the multiplication of yeast (budding).

The cell wall is connected with the cell membrane through the periplasmic space by chains of glucan and chitin. It also contains various enzymes responsible for regulating yeast metabolism.

The wall of yeast cells contains 30-60 % of polysaccharides (β-1,3-glucan, β-1,6-glucan and mannan sugar polymers), 15-30 % of proteins, 5-20 % of lipids and a small amount of chitin (N-acetylglucosamine polymer).

The cell wall has two layers, the inner layer and the outer layer. The inner layer basically consists of a polysaccharide, whereas the outer layer consists mainly of glycoproteins (mannoprotein) bound to the inner layer. Depending on the growth conditions, an additional dark layer between the plasma membrane and the transparent inner layer of the cell wall can be observed. For this reason, the wall is sometimes called a three-layer structure.

Glucan is a glucose polymer, where the glucose molecules are connected between each other by β-1,6 and β-1,4 bonds. In S. cerevisiae there are three types of β-glucan polymers: alkali-soluble, alkali-acid-insoluble and alkali-acid soluble, and the ratio between fractions depends on the cultivation conditions. The mannan - branched polymer is composed of the residues of D-mannose. Mannan is part of the outer layers of the cell wall.

The main polysaccharide of the inner layer is branched β-1,3-glucan, which forms a continuous elastic network. This network is held together by hydrogen bonds between the chains of β-1,3-glucan and the cell wall protein. Most of the protein is connected with mannan-saccharide. This complex is called mannan-protein complex.

This network expands under normal osmotic conditions which explains why the cells shrink significantly when they die.

Proteins are directly associated with β-1,3-glucan network through the ester bond between the specific glutamine residues and the glucosyl hydroxyl group. In addition, the composition of the protein population in the cell wall strongly depends on the environmental conditions. Cell wall proteins can help the cell cope with various forms of stress.

In *S. cerevisiae*, the cell wall amounts to 15 % to 30 % of the dry mass depending on the cell growth conditions.

The chitin level in the cell wall is usually low. Some chitins are found in the side walls. In the maternal cell walls they are found in the areas of bud formation.

Chitin can be connected to the non-reducing ends of β-1,3-glucan and β-1,6-glucan chains.

Complex β-1,3-glucan-chitin is the main component of the inner wall. β-1,6-glucan binds the components of the inner and outer walls.

On the outer surface of the wall, there are mannoproteins, which are widely O- and N - glycosylated. They are tightly packed and limit the permeability of the wall for dissolution. The main components of *S. cerevisiae* cell wall are presented in Table 1 [4-7].

Table 1 - The main components of *S. cerevisiae* cell wall

Component (degree of polymerization)	(% of the wall weight)
β-1,3-glucan (1500)	50
β-1,6-glucan (150)	10
Mannoproteins	40
Chitin (120)	1-3

1.2 The cytoplasmic membrane

The cytoplasmic membrane separates the cell wall from the contents of the cell, participates in substance transportation, and creates an osmotic barrier for the entry and exit of substances into the cell, as it contains certain enzymes located on the outer surface of the membrane.

The cell membrane consists of three layers. The main components of the cell membrane are proteins, lipids and small amounts of carbohydrates (Fig. 1.2).

The protein membrane is about 50 % of the membrane weight. They can be either integral or peripheral.

Integral protein (transmembrane protein) participates in the construction of ion channels through the membrane and creates hydrophilic pores in the membrane for substance transportation. Most of them are glycoproteins. The carbohydrate part protrudes from the membrane and binds to lipids.

The peripheral protein (outer protein) lies outside the membrane and functions as enzymes.

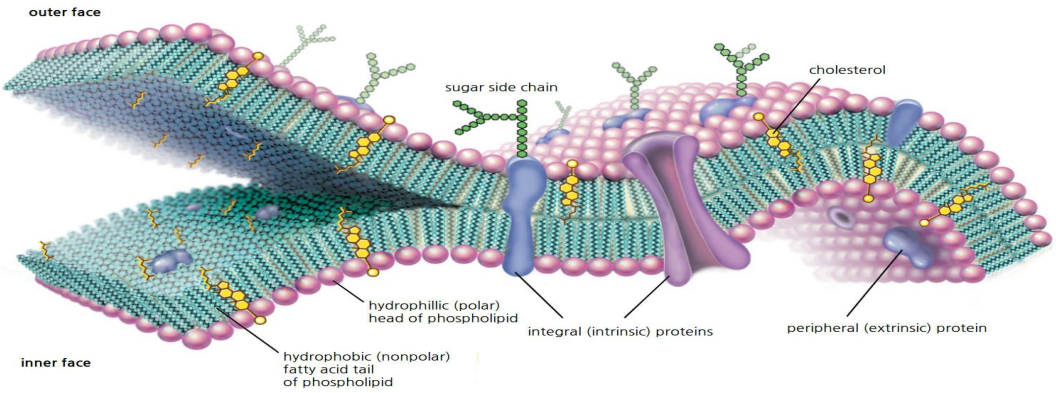

Figure 1.2 - Cell membrane structure

Lipids of the cell membrane are presented in the form of mono-, di- and triglycerides, phospholipids and sterols. Unlike higher eukaryotes, in which cholesterol is the most common sterol, yeasts contain mainly ergosterol (provitamin D_2).

Phospholipids included in the cell membrane are phosphoglycerides of fatty acid and consequently the composition of the cell membrane varies depending on the fatty acid composition. Each phospholipid molecule consists of a hydrophilic head and hydrophobic tails.

Esters and triacylglycerol (TAG) are the main storage lipids in eukaryotic cells. In *Saccharomyces cerevisiae*, triglycerides and sterols accumulate in the yeast membrane in the form of cellular molecules, which consist of 15 % triglycerides and 44 % sterols, and constitute up to 70 % of the total fat content. The amount of sterols in yeast is 0.1-2.5 % of dry weight. Ergosterol is the bulk sterol in yeast [1-4].

1.3 Core (nucleus)

The yeast nucleus (core) has a rounded shape, surrounded by two membranes dotted with pores. It plays genetic, information and metabolic functions. The main chemical component of the nucleus is DNA. When the cell is multiplied, the DNA is concentrated in chromosomes, where it stays in a bound state with proteins. In yeast, there are three types of RNA: matrix RNAs are formed in the nucleus and serve as matrices for protein synthesis, transport RNAs serve as aminoacid transporters and ribosomal RNA is the largest. The morphology of the nucleus varies depending on the conditions of cultivation. In aerobic conditions cells have larger nuclei, their location in the cell changes depending on the stage of yeast growth [1, 4].

1.4 Mitochondria

Shape, size, amount and internal structure of mitochondria vary depending on the cultivation conditions and yeast viability.

Mitochondria is surrounded by a double membrane, internal and external (Fig. 1.3). The inner membrane forms folds or cristae. These folds of the inner mitochondrial membrane increase the surface of the inner membrane, which allows it to localize a greater number of respiratory enzymes on it, thereby enhancing the efficiency of cellular respiration and the rate of ATP synthesis.

In aerobic conditions, the number of mitochondria in the cell increases and their size decreases. Inside the mitochondria there is a matrix in which the tricarboxylic acids cycle enzymes are located. The mitochondria contains DNA, where the mitochondrial DNA is about 15-23 % of the entire cell's DNA. The development of mitochondria is affected not only by the composition of the nutrient medium and the content of yeast growth factors in the medium, including the number of biotin, but also by physicochemical conditions of cultivation (temperature, oxygen concentration, pH, etc.) [1, 4].

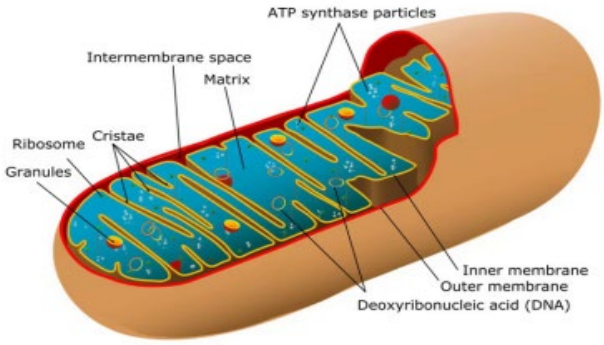

Figure 1.3 – The structure of the mitochondria

1.5 Endoplasmic reticulum

Endoplasmic reticulum (EPR) is a three-layer membrane with plenty of enzymes. The activity of some metabolic processes, like the biosynthesis of proteins, sugars, and fats, depends on the degree of network branching.

The conditions of cultivation affect the development of this structure. EPR is more developed in aerobic cultivated yeast than in anaerobic ones, and also more in young cells than in the old ones. The phases of yeast growth and their physiological state depend on EPR morphology.

This structure is connected to the nucleus and ribosomes via channels (Fig. 1.4). It is important for movement of various metabolites. Proteins are synthesized in ribosomes, transported into EPR channels and to the cell.

EPR in yeast is divided into smooth and rough reticulum. The rough part contains ribosomes, where protein synthesis takes place. The smooth one is formed from the rough and participates in the synthesis of lipids [1-4].

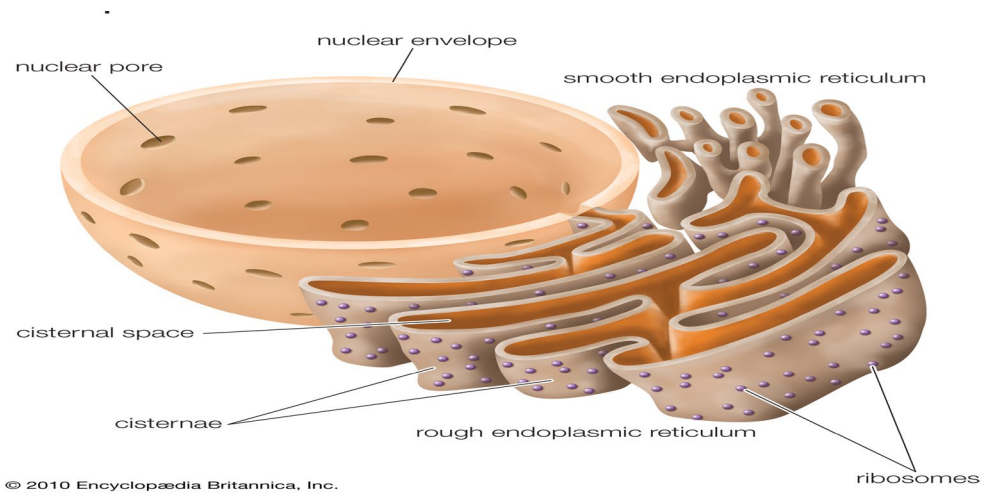

Figure 1.4 - The structure of the endoplasmic reticulum

1.6 Vacuoles

Vacuole is formed from the endoplasmic reticulum, when the channels thicken and join forming large cavities. They are filled with the cell sap. They also contain nutrients and reserve substances: glycogen, trehalose and fats.

When yeast cells are introduced into a fresh nutrient medium, the vacuoles disappear, and they appear again with aging of yeast cells.

Vacuoles are important in the regulation of osmotic pressure in mature or adult yeast cells, while in young cells the Golgi apparatus and endoplasmic reticulum regulate it [1-4].

1.7 Golgi apparatus

A part of the smooth membranes that are separated from the endoplasmic reticulum and are organized into structures is called the Golgi apparatus.

They take part in the construction of the cell membrane and the formation of vacuoles and lysosomes, as well as transportation of secreted substances inside the cell [4].

1.8 Ribosomes

Ribosomes are granules in the form of balls consisting of protein and RNA. Proteins and enzymes are synthesized in ribosomes [4].

1.9 Lysosome

Lysosome is an organoid surrounded by a cell membrane, and contains a variety of hydrolytic enzymes [4].

2 Multiplication of yeast

Yeast, like all fungi, can have asexual and sexual reproduction. The most common method of yeast multiplication is asexual reproduction by budding (Fig. 2.1).

A small daughter cell is formed on the maternal cell in the form of a bud. The nucleus of the maternal cell divides into a daughter nucleus and passes into the daughter cell. The daughter cell continues to grow until its size reaches the size of the maternal cell, the daughter cell separates from it and a new cell is formed.

In the process of yeast multiplication, the cell wall is important. Two buds are never formed at the same place on the cell wall. Consequently, each time the bud separates from the cell wall it leaves a new bud scar on the wall of the maternal cell.

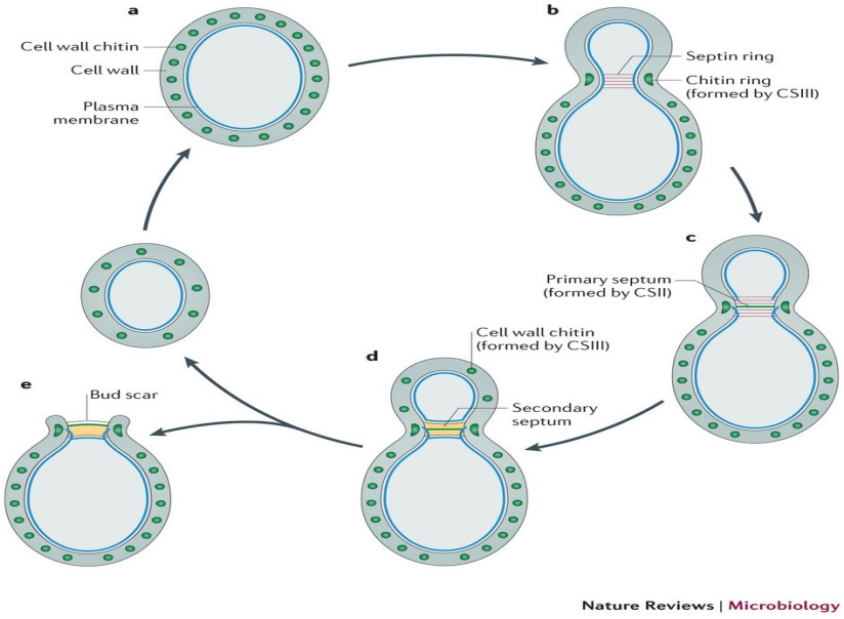

Figure 2.1 - Multiplication of yeast

During yeast multiplication a chitin septum is formed at the site of bud separation; with the increasing number of buds, the number of the forming chitinous

septa on the cell wall increases. These septa do not allow the transfer of nutrients from the environment into the cell and it leads to decrease in yeast viability [1-4].

Cell cycle

A cell cycle is a sequence of stages that occur in cells and lead to cell division and replication (duplication).

The cell cycle is divided into two phases, interphase and mitotic phase (Fig. 2.2). During interphase, the cell prepares, grows and accumulates nutrients for mitosis and also duplicates the DNA. In the mitotic phase, the cell splits itself into two distinct cells known as 'daughter cells'; and the final phase is cytokinesis, where the newly formed cells are completely divided.

In the cell cycle there are four phases: phase G_1, phase S (synthesis), phase G_2 and phase M. Phases G_1, S and G_2 together are called interphase.

G_1 is the presynthetic phase, where the enzymes required by yeast for budding and replication of the DNA are synthesized. This phase ends when the bud is formed on the cell wall.

S is synthetic phase. In this phase, DNA replication occurs and ends when the size of the bud is 1/3 of the size of the maternal cell.

G_2 is the phase, where nuclear material is distributed between the daughter and the maternal cell.

The M phase or mitotic phase has two processes: first, the cell chromosomes are divided between two cells and then the cell cytoplasm divides into half, forming two different cells (cytokinesis) [1, 3, 4].

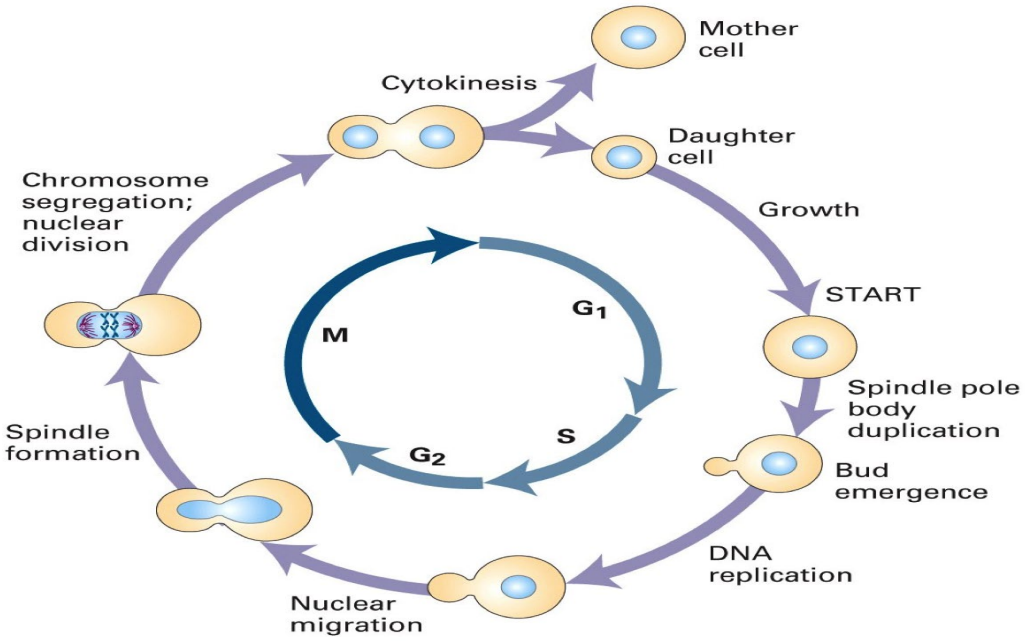

Figure 2.2 – Life cycle of a cell

3 Factors determining the biosynthetic activity of yeast

3.1 Yeast nutrition

Physiologically active yeast can be obtained only in the absence of nutrient deficiency. Deficiency of nutrients increases with the use of undermodified malt, grain unmalted materials, maltose syrup and sugar. As a result, the intensity of yeast multiplication and fermentation rate decrease, duration of fermentation increases, and the final degree of wort fermentation is reduced. This, in turn, leads to a change in the beer taste profile, complexity of seed yeast removal and a decrease in their physiological activity.

In some cases, to prevent the decrease in the intensity of multiplication and fermentation activity of yeast, it is necessary to add the missing nutrients (amino acids

or ammonium salts, mineral salts) and vitamins. To select preparation conditions that include nutrients for yeast, and dosage, considered should be yeast growth factors, mineral components and their effect on the fermentation process.

Nutrients are required for multiplication and yeast growth. Nutrients are also used for the synthesis of secondary metabolites, which are crucial in the flavor and aroma formation in brewing.

The nutrient intake depends on cultivation condition, such as temperature and pH, and also on yeast seeding rate. Yeast nutrients are usually divided in two groups: basic such as carbons, oxygen, hydrogen and nitrogenous substances and nutrients required in small quantities such as trace elements, macronutrients and vitamins that are vital in the process of yeast metabolism. Food yeasts are divided into exogenously and endogenously nurtured yeasts. With exogenous nutrition, nutrients enter the cell from the environment and exogenous yeast uses reserve substances such as glycogen, trehalose and lipids [1, 3, 4, 8-11].

Carbon nutrition

Yeasts use carbon from organic compounds, such as monosaccharides like glucose, fructose and mannose, disaccharides, such as sucrose and maltose, and trisaccharides such as maltotriose, while lactose (milk sugar) is not fermented by yeast. Yeast consumes maltose only in the absence of fructose and glucose in the medium. Maltose is fermented almost completely during the stationary phase of yeast growth. Cleaved by the enzyme, sucrose is inverted into glucose and fructose and they enter the cell.

Maltotriose enters the cell with a permease enzyme where it splits into glucose. Depending on yeast strains, the trisaccharide raffinose is fermented either completely (strains of beer bottom fermentation yeast) or by 1/3 (baker's yeast and brewer's

yeast). Organic acids serve as a source of carbon and their choice depends on the type of yeast, acid concentration and the length of the carbon chain. Organic acids with a carbon chain length from C2 to C4 (for example acetic or lactic) are good sources of carbon. Fatty acids with an average length of the carbon chain from C6 to C10 and at very low concentrations in the medium (0.02-0.05 %) are least consumed by yeast, while higher concentrations suppress yeast growth and multiplication [1, 4, 8, 9, 11].

Nitrogen nutrition

Nitrogen ranges from 50 to 60 % of the total dry matter, mainly proteins and free amino acids. Yeasts use amino acids contained in wort as a source of nitrogen for the cell protein synthesis. They also effectively use nitrogen sulfate and ammonium phosphate, urea, ammonium salts. It should be noted that yeasts do not utilize nitrates and nitrites. For normal metabolism, the wort must contain at least 140 mg of nitrogen per 1 liter of wort. To consume organic nitrogen (amino acids, amides), many yeast strains require vitamins (biotin, pantothenic acid, thiamine, etc.) [1, 3, 4, 8, 12].

3.2 Growth Factors

Yeasts differ in relation to growth factors, i.e. to those substances that are part of the cells, but at the same time cannot be synthesized. Growth factors for all yeast strains are biotin (vitamin B_7), pantothenic acid (vitamin B_3) and mesoinosite (vitamin B_8). Some strains of bottom-fermented yeast also need pyridoxine (vitamin B_6). In addition to these vitamins, thiamine (vitamin B_1) is an activator of fermentation. The vitamins in cells and their role in the metabolism of substances in yeast are given in Table 3.1 [1, 4].

Table 3.1 - Growth factors (vitamins) in brewer's yeast.

Vitamin	Vitamin content, mg / 100 g DM content	Role in physiological state regulation of yeast
Thiamine	8-15	Stimulates alcoholic fermentation, participates in biomass synthesis
Pantothenic acid	2-20	Participates in the synthesis of unsaturated fatty acids, steroids
Biotin	0.1-1.0	Regulates carbohydrate, nitrogen and fat metabolism of yeast
Inositol	200-500	Regulates the synthesis of lipid membranes, growth and multiplication of cells

Biotin

Biotin is one of the important factors of yeast growth. All yeast strains are auxotrophs for biotin, i.e. unable to grow and multiply in its absence. Biotin is part of the enzyme compost, participates in the transfer of the carboxyl group, and is important for yeast metabolism. In the synthesis of oxaloacetate, biotin affects the synthesis of fatty acids and pyruvate carboxylase activity, which ensures the intensity of the tricarboxylic acid cycle. The Acetyl-S-CoA-carboxylase composition is a key enzyme for fatty acids synthesis.

The growth of biotin-seeking microorganisms in media containing suboptimal vitamin concentrations has been studied to get an idea of biotin part in the metabolism of living cells. It is shown that microorganisms grown under these conditions lose the ability to perform certain metabolic reactions, mainly those that are associated with

carboxylation.

Since the process of CO_2 transfer is of fundamental importance in the metabolism of living cells, it should be expected that any deterioration of this process will lead to a serious disruption of the metabolism of cells. In the absence of biotin in the nutrient medium, the rate of oxaloacetate formation decreases, the work of the cycle slows down and adversely affects the formation of fatty acids. Hence, the yield of biomass and the economic coefficient decrease and ethanol synthesis increases.

The importance of biotin in the synthesis of fatty acids

Biotin is important in the synthesis of fatty acids in the cell membrane. Its deficiency in a natural medium causes disturbance of the metabolic process in the cell.

The source of the compound for the synthesis of fatty acids in the cytosol cell is cytoplasmic Acetyl-CoA, which enters the cytosol from the mitochondria.

Acetyl-CoA is formed in the mitochondria either from pyruvate or from the oxidation of fatty acids containing from 4 to 10 carbon atoms in the carbon chain. These low-molecular fatty acids freely pass through the mitochondrial membrane.

Biotin is a part of pyruvate carboxylase, the main enzyme in the process of formation of oxaloacetate from pyruvate with ATP, Zn and CO_2 in the mitochondria. Biotin also belongs to the Acetyl-S-CoA carboxylase (as a coenzyme), a key enzyme in the formation of high-molecular fatty acids from Acetyl-CoA. Acetyl-CoA is converted into high-molecular fatty acids in the presence of biotin, Mn, ATP and CO_2 and this process occurs in the cytoplasm.

Usually, to compensate for the lack of biotin in complex nutrient media, for example, molasses or malt wort, it is added at the rate of 0.25 mg per gain of 1 kg of yeast with a mass fraction of solids of 25 %. It should be noted that the role of biotin in the process of fatty acids formation is tightly bound to oxygen, when its deficiency in the nutrient medium leads to an increase in the concentration of Acetyl-CoA in the cell, and hence the accumulation of keto-compounds [1, 4, 8].

Thiamin (B1)

In yeast metabolism, vitamins are cofactors in a variety of enzyme complexes. Thiamine (vitamin B1) is found in a variety of cells in their base forms (thiamine), thiamine monophosphate (TMP), and thiamine diphosphate (TDP). Under the normal fermentation conditions, the yeasts utilize the glycolytic pathway to convert glucose to pyruvate to generate energy in the form of ATP for the cell. During this process, when glyceraldehyde-3-phosphate is oxidized to 1,3-bisphosphoglycerate, NAD+ is reduced to NADH. The process of NADH formation leads to imbalance in the redox cell state and prevents yeast growth due to the inability to generate energy. Yeast undergoes fermentation, where pyruvate is decarboxylated to acetaldehyde, which is subsequently reduced to ethanol by alcohol dehydrogenase with the concurrent oxidation of NADH to NAD+. This is due to the fact that thiamine is a coenzyme of pyruvate-decarboxylase; an enzyme is required to convert pyruvate to acetaldehyde. This requirement for thiamine was noted by several authors; without this vitamin the production of ethanol can be difficult, and fermentation may be incomplete [1, 4].

3.3 Mineral nutrition of yeast

Macronutrients.

The main mineral components necessary for the growth and reproduction of yeast include nitrogen, phosphorus, potassium, sulfur and magnesium, which constitute the bulk of the ash (Table 3.2). The majority of cell components are substances like proteins, free amino acids, and nucleic acids (up to 60 % of dry matter (DM). For their synthesis, yeast preferably uses amino acids from the wort. They can also assimilate inorganic nitrogen (NH^{4+}), which is converted by cells into amino

acids. For normal exchange in wort, at least 140 mg of amine nitrogen per liter of wort should be contained. Note that yeast does not utilize nitrates, nitrites, amino acids, proteins, and peptides.

Table 3.2 - Component content in yeast (% of DM)

Component	Amount
Nitrogen	4.8- 10.0
Phosphorus (calculated as P_2O_5)	1.9-5.5
Potassium (calculated as K_2O)	1.4-4.3
Magnesium (calculated as MgO)	0.1-0.7
Sulfur (calculated as SO_3)	0.01-0.05

The nitrogen exchange is closely related to the exchange of phosphorus, potassium and magnesium. Phosphorus is part of nucleic acids, ATP, phospholipids, cell wall polymers, and it can accumulate in the cell as polyphosphates. For the physiological needs of yeast, about 10-13 mg of phosphorus is consumed for the growth of 10 billion cells.

Potassium is found in yeast in significant quantities: up to 4.3 % of DM. This is comparable only with the content of nitrogen (up to 10 % of DM) and phosphorus (up to 5.5 % of DM), which indicates its importance for yeast metabolism. In addition, it participates in the regulation of ion transportation through the cell wall and through the mitochondrial membrane. Potassium activates about 40 different enzymes, stimulates the digestion of maltose and maltotriose. It is closely related to the growth of yeast and the speed of fermentation.

Magnesium is important in the energy metabolism of yeast associated with the

growth and multiplication of cells. The economic coefficient per ions of consumed magnesium varies from 300 to 900 g of dry biomass per 1 g of magnesium, for yeast this value is usually 540 g / g of magnesium.

For normal yeast multiplication, sulfur is necessary, as it is involved in the synthesis of amino acids such as cysteine and methionine. A small amount of sulfur is required for the formation of sulfogroup with some coenzymes such as biotin, coenzyme A, lipoic acid, thiamine and pyridoxin. It was found that limitation of sulfur content in the medium leads to a decrease in respiratory activity of yeast cells, the same as with limitation of iron in yeast. The economic coefficient per ion $S0_4^{-2}$ for yeast is 100 g of dry biomass per 1 g of sulfur [1, 3, 4, 11].

Microelements.

The trace elements that are necessary for yeast growth are: Ca, Mn, Fe, Co, Cu, and Zn (Table 3.3). Elements required for yeast growth in smaller quantities are B, Na, Al, Si, Cl, V, Cr, Ni, As, Se, Mo, Sn, and I.

Table 3.3 - The role of certain microelements in the life of yeast

Microelement	Role
Cu	necessary for the synthesis of several respiratory enzymes. Increases yeast fermentation activity. In small amounts stimulates cell budding
Fe	necessary for the synthesis of a number of respiratory enzymes. In a small amount (up to 0.2 mg / l) stimulates cell budding, highly toxic for yeast

Table 3.3 continuation

Microelement	Role
Mn	activates numerous enzymes, such as decarboxylases, dehydrogenases, kinases, oxidases, peroxidases, peptidases, etc., increases fermentation activity
Zn	activates numerous yeast cell enzymes. Stimulates the multiplication of cells, fermentation activity. Facilitates sedimentation of yeast

The average values of the economic coefficient, i.e. the amount of biomass grown (g) per 1 g of consumed trace element, are given in Table 3.4.

Table 3.4 -The economic coefficient of biomass yield (kg) per 1 g of element

Chemical element						
Ca	Fe	Mn	Zn	Cu	Co	Mo
0.54	10.8	54.0	5.4	135.0	100	100

The need for microelements may increase several times when the culture is stressed, for example, by increasing the temperature above the optimum.

Reproduction of yeast in brewer's wort is limited due to a lack of assimilable nitrogen, zinc salts, iron and pantothenic acid (Table 3.5). Iron deficiency can be compensated by magnesium ions, the concentration of which is several times higher than the yeast needs, while the limit of zinc ions, pantothenic acid and amine nitrogen can be replenished by adding only these components to brewer's wort. Thus, brewing process requires enriching the medium with growth substances and microelements, various preparations and "dressing" for yeasts (Table 3.6).

Table 3.5 - Concentration of mineral, nutrient and growth substances in wort (per 100 kg of fermentable carbohydrates)

Nutrients and growth substances	The required number for the output of 8.1% in the calculation of the ADB	The content in 1280 litres of wort with 12 % DM	Excess or deficit in the wort
Fermented carbohydrates	100 kg	100 kg	
Assimilated nitrogen	750 g	256 g	-
Phosphate P_2O_5	300 g	1100 g	+
Ions			
Potassium	150 g	704 g	+
Sulfur	82 g	115 g	+
Magnesium	15 g	128 g	+
Calcium	15 g	44.8 g	+
Sodium	7.5 g	38.4 g	+
Zinc	1.5 g	0.192 g	-
Iron	0.75 g	0.128 g	-
Manganese	0.15 g	0.166 g	+
Copper	0.06 g	0.128 g	+
Growth substances			
Biotin	3 mg	8.32 mg	+
Pantothenic acid	1215 mg	320 - 640 mg	-
Inositol	16.2 g	51.2 g	+

Table 3.6 - Products for intensification of yeasts growth and multiplication and increase in their fermentation activity

Product name	Company	Composition
Yeast food «Yeast Food GF»	Quest International	Inorganic substances (salts of ammonium, potassium, zinc, manganese). Organic substances (soy flour). Growth factor
Alcotek	Murphy and son LTD	A mix of B vitamins and amino acids
Yeast feeding «Rhodia Zumesite»	Rhodia LTD companies	A mix of inorganic substances (salts of ammonium, manganese, zinc)
HY-VIT	Hydralco Hydracolloide GmbH	Inorganic substances (salts of calcium, zinc, ammonium). Amino acid. B vitamins. Peptone
Yeastex	I. E. Siebel Sons Company	Mixture of inorganic substances (salts of ammonium, manganese, zinc)

The product improves the physiological state of pure culture and seed yeast, increases the coefficient of their growth, intensifies the main fermentation process, and improves the organoleptic properties of beer, by increasing the yeast's resistance to autolysis and a high degree of fermentation.

The effect of applying «dressing» depends on the duration and storage conditions of yeast seeds (their physiological state), the composition of the wort (especially the content of amine nitrogen), the number of generations of seed yeast, the main fermentation method, and beer type (light, dense, dark).

With an increase in the number of generations, the physiological state of the yeast deteriorates due to yeast aging and the adsorption of protein-tanning complexes, hop-like bitter substances and beer infection bacteria on their surface. In this case, broth consumption is increased to the recommended maximum. Yeast washing with orthophosphoric acid (pH 3-3.5) gives a positive effect before applying dressing.

With significant contamination of yeast by extraneous microorganisms, it is not recommended to use broth that stimulates yeast multiplication, since intensified multiplication and fermentation of wort leads to multiplication of extraneous microorganisms [1, 3, 4, 8, 13].

3.4 The role of aeration

Aeration of the nutrient medium is used in the beginning of fermentation or while obtaining a pure yeast culture during brewing. The air oxidation is important for energy metabolism and synthesis of unsaturated fatty acids and ergosterol in yeasts.

With a single-shot oxygen saturation of 12 % of the wort supplied for fermentation to an average of 8 mg O_2 / l, an increase in yeast biomass up to $40 \cdot 10^6$ cells / ml is observed. However, this value can change due to nutrient medium disbalance in macro and microelements, which are not determined in brewing.

When diluting a pure yeast culture in order to obtain a guaranteed result, it is recommended to use about 120 mg O_2 / g ADB, as the wort has only a limited ADB potential, reducing the specific consumption of oxygen nutrients.

The yeast cell requires not less than 30-35 mg O_2 / g ADB for synthesis of the

vital level of fatty acids under the brewing conditions. The concentration of fermented carbohydrates determines the specific rate of oxygen digestion. In wort, depending on the temperature of fermentation and the physiological state and viability of introduced yeast (viability of yeast preserved under water is lower than that in yeast after fermentation for 24 hours), the specific rate of oxygen consumption ranges from 2 to 33 mg O_2 / (g ADB · h).

The maximum specific oxygen consumption rate at a fermentation temperature of 15 ºC equal to 13 mg O_2 / (g ADB · h) is used as the average value for carrying out model calculations.

With glucose concentrations exceeding 0.1 g / l, oxygen serves not as a hydrogen acceptor in the tricarboxylic acid cycle, but as a necessary element for the synthesis of unsaturated fatty acids and sterols, which are extremely important for yeast multiplication.

Sterols are synthesized from squalene. First, it is converted into squalene-2,3-epoxide, and then cyclized into ergosterol. The fact that squalene is the precursor of sterols becomes clear also because there is a significant quantitative and qualitative difference between aerobic and anaerobic fermented yeast, since anaerobic yeast reveals a significant content of squalene and low content of sterols and vice versa.

Sterols in most cases are presented in the form of esters. The greatest amount of cymosterol found in aerobically grown yeast cells is 5 mg per gram of dry matter. Sterol synthesis, which begins in half an hour after yeast inoculation, is closely related to yeast growth. If the yeast is aerated, then at the same time the transformation of squalene begins. Therefore, one mg of the resulting sterol then corresponds to the production of 1000 mg of yeast dry matter. It is obvious that the sterol synthesis is limited by the intracellular concentration, i.e. it stops when the concentration reaches a certain value.

There is also a connection between the synthesis of sterols and the storage of glycogen in yeast cell. To accumulate glycogen, yeasts consume about 0.25 % of the extract during fermentation. There is a stoichiometric relationship between the consumed glycogen at the onset of fermentation and the resulting sterol. For every gram of glycogen, 69 mg of sterol is formed. If there is little glycogen in a mature yeast, then further fermentation runs slower [1, 3, 4, 10].

4 Production-related signs associated with the physiological state of yeast

The physiological state of yeast determines:

- speed and degree of wort fermentation (fermentation activity),
- synthesis of fermentation by-products, forming a sensory profile of beer,
- yeast's ability to flocculate.

4.1 Flocculation ability

Flocculation is a reversible aggregation or agglutination of yeast cells. Such indicators as the degree of wort fermentation, organoleptic properties of beer, as well as its biological and colloidal stability are related to this yeast property.

Flocculation is defined by strain characteristics of yeasts, as well as their physiological activity. These characteristics determine the z-potential of cells and the intensity of the aggregation process.

The yeast that has settled as a result of flocculation is metabolically inactive and is unable to use the remaining sugar in the wort. As a lot of metabolic processes take place on the cell surface, the main fermentation cycle is shortened in yeast with a high degree of flocculation which results in a beer with high residual extract. Such

beers have low biological stability and are characterized as under-fermented. With slow yeast settling, the beers are poorly clarified, and the excessive content of yeast cells during pre-fermentation leads to the appearance of autolysis yeast taste and the smell of hydrogen sulfide.

The intensity of flocculation is influenced by several environmental factors:

-wort composition: the early flocculation of yeast is facilitated by the unbalanced composition of the wort, namely the lack of amine nitrogen, growth factors and low ratio of fermentable carbohydrates to unfermented carbohydrates.

- fermentation temperature: when the temperature is raised to 20 ºC and higher, the yeast deflocculation occurs, which continues with a decrease in temperature to 2 – 5 ºC.

- the medium pH value. The intensity of yeast flocculation increases with pH decreasing from 5.6 to 4.3-4.0. Yeast deflocculate when the pH value increases (more than 6) or when it decreases (less than 3).

- quantity and quality of seed yeast. The process of flocculation is accelerated with an increase in the rate of seed yeast and a decrease in their biosynthetic activity [1, 3, 4, 9, 14].

4.2 Fermentation activity

The yeast fermentation activity determines the duration of the main fermentation, the physicochemical properties of beer, its biological and colloidal stability, as well as the sensory profile and its stability during storage. Fermentation activity is assessed by:

- the rate of consumption of fermentable carbohydrates,
- the amount of carbon dioxide released during fermentation;
- the final degree of wort fermentation.

The rate and degree of beer wort extract fermentation depends on the composition of the nutrient medium and the ratio between the fermented sugars (glucose, maltose, maltotriose) in particular. With an increase in glucose concentration in the medium, the activity of permeases that transport maltose and maltotriose in the cells decreases, while there is a decrease in the rate of wort fermentation. This phenomenon does not always occur, as there are yeast strains with glucose repression.

Yeast fermentation activity is interrelated with the rate of their reproduction, which is important for rapid wort fermentation. The rate of growth and multiplication of cells depends on the balance of the wort composition (α-amino nitrogen, growth factors and microelements contained), and the presence of the dissolved oxygen (more than 8 mg / dm^3).

The physiological state of yeasts and their concentration in the wort is important. With the increase in seeding, the rate of sugars fermentation in wort increases, and as a result, main fermentation time decreases. Long-used yeast as well as yeast that has been stored incorrectly have low fermentation activity [1, 3, 4].

4.3 Stress

Osmotic stress

The technology of dense brewing is widely used at present. In these conditions, a high concentration of wort causes osmotic stress in yeast cells.

The osmotic pressure arises from the tendency of water to penetrate through the semipermeable membrane towards a more concentrated of the two solutions separated by the membrane. This pressure is directly proportional to the concentration of molecules that cannot pass through the membrane. The cytoplasmic membrane of

yeast cells is characterized by semi-permeability to water and hydrophilic compounds with high molecular weight. Consequently, when the nutrient concentration of solids is low, yeast cells experience hypo-osmotic stress and water content in the cells increases. In a more concentrated environment (for example, in a wort) a hyperosmotic stress occurs in cells, which leads to water leakage from the cells. In this case, yeasts, regardless of their strain morphological features, acquire a rounded shape and their surface becomes wrinkled.

The response of cells to osmotic stress depends on the wort density and its carbohydrate composition, yeast physiological conditions and the growth stage of the cells. Breeding cells (log phase of growth) are more sensitive to stress than cells in stationary growth phase. This is due to different chemical compositions of yeast, in particular the content of reserve carbohydrates, glycogen, and trehalose.

The hyperosmotic stress experienced by cells at the time of their introduction into the nutrient medium requires some time to adapt to the given conditions before the yeast multiplication process starts. This cell development stage is called the lag phase. During this period, the synthesis of glycerol-3-phosphate dehydrogenase increases substantially, which leads to an increase in intracellular concentration of glycerol. However, it may take several hours before the registration of a significant increase in the intracellular glycerol content. Normally, glycerol can penetrate through the cytoplasmic membrane, but under hyperosmotic stress the pores through which it leaves are closed and glycerol is not released into the medium during the lag phase. Stress is removed during the process of fermentation, and the intracellular concentration of glycerol decreases [1, 4, 15-18].

Ethanol stress

Alcohol is formed during fermentation, and its effect on yeast is defined as

ethanol stress.

The toxic properties of ethanol result in the increased permeability and porosity of the cell membrane, which leads to problems with transportation of nutrients. In addition, there is a deficiency of water available to the cytoplasm. The response of cells to ethanol stress under laboratory conditions is manifested in the increase of undersaturation of fatty acids present in the plasma membrane, increase of ergosterol content, the synthesis of trehalose and the production of specific thermal shock proteins. When the ethanol content in the medium is higher than 1.2 %, the specific yeast growth rate decreases. The concentration of alcohol in a medium of 2.0 % or more leads to a decrease in biomass. Yeast growth is fully inhibited at 8.0-9.5 % ethanol.

Ethanol affects the duration of the yeast cell generation time. The concentration of ethanol from 0 to 1.0 % increases the generation time from about 2.3 to 3.5 hours, and with ethanol concentration of 3.8 % up to 6.9 hours.

Industrial yeasts in dense brewing are exposed to high concentrations of ethanol. With 23 % extractiveness of the initial wort, the volume fraction of alcohol is more than 9.0 %. The resulting alcohol inhibits both yeast reproduction speed and the fermentation process [4, 15, 16, 18, 19].

Stress caused by carbon dioxide

At concentrations equivalent to gas pressure above 0.2 atm, carbon dioxide stimulates cell growth. At a pressure of about 0.5 atm, the cycle of tricarboxylic acids is inhibited, but alcoholic fermentation continues up to a pressure of 4.0 atm. Cell division ceases at a pressure of about 2.5-3.0 atm. In this case, the cells pass through the S-phase (the DNA synthesis phase), but they do not form buds. Therefore, they are characterized by a double DNA composition and a larger than usual size. At

concentrations of carbon dioxide that allow cells to grow, there is a clear influence on the formation of sensory characteristics of beer.

Researchers have not yet come to a consensus on the bio-chemical mechanism of influence of carbon dioxide [1, 4, 15, 16].

Oxidative stress

Numerous studies have been conducted on the ability of yeast to resist oxidative stress. Cells have protective mechanisms at the substrate level, for example, glutathione, polyamines, and metal ions. These protective mechanisms can also be obtained by enzymes - catalase, superoxiddismutase, glutathione peroxidase, thioredoxine, reductase and DNA restore enzymes. During beer fermentation, the value of oxygen stress is not so great, as the cells are exposed to oxygen for a short period of time only at the beginning of fermentation. Thus, during reactions in mitochondria, cells receive a very small amount of active oxygen, which can negatively affect yeast.

As a substrate, oxygen is especially important for biosynthesis of unsaturated fatty acids and ergosterol required for cell growth [1, 4, 15, 16, 20-22].

Temperature stress

Temperature has a significant effect on energy and functional exchange of cells and, therefore, affects the specific growth rate of yeast and their generation time.

Under certain operating conditions, cells may experience temperature stress (shock). This effect manifests itself if the yeast is exposed to relatively high but non-lethal temperature for a short period of time. Yeast resistance to negative external influences is associated with trehalose, the content of which is determined by the

strain characteristics of yeast and cultivation conditions.

It is established that cells that have survived the effect of high temperatures acquire not only thermal stability, but also alcohol and osmotic resistance [1, 4, 15, 16].

Other types of stress

The vital activity of yeast cells is adversely affected by:

- sharp fluctuations in pH;

- hydrostatic stress;

-mechanical stress due to the action of large tangential stresses (pumps, agitators, and control valves).

The pH value affects the transport system of nutrients, the degree of dissociation of the medium components, dispersity, spatial organization and activity of enzyme proteins, and yeast flocculation.

The optimal pH for the propagation of brewer's yeast cells is 4.8, since at this pH level maltose permease, an enzyme that transports maltose to the cell, has the maximum activity. At lower pH values, consumption of amine nitrogen is accelerated.

As the medium becomes acidified, the cell charge decreases, and the mutual repulsion of cells and the increased flocculation are observed. In general, yeast live and multiply in a wide range of pH from 2 to 6. However, sharp fluctuations of this parameter can also affect enzyme activity, disruption of biosynthetic activity of yeast and an increase in the number of dead cells.

Hydrostatic stress is observed with fermentation of wort in high fermentation apparatus (cylindro-conical tanks - CCT), with the height 17-22 m. This changes the permeability of cell membranes and enzymatic activity of cells.

Mechanical stress occurs as a result of large shearing stresses during yeast

mixing, pumping them from one container to another. These mechanical operations can "peel" the surface layer of the yeast cell membrane, which reduces the flocculation properties of cells. This fact leads to disturbances in the process of fermentation and flocculation and hinders natural clarification of beer [1, 4, 15, 16, 23].

5 The assessment of the physiological state of yeast

The metabolic activity of yeast is assessed:

- by viability, i.e. number of living cells;
- by physiological activity ("life force").

The viability of yeast is the ability of cells to grow and multiply. Thus, the viability indicator measures the number of living cells. In many cases, it is sufficient to determine the number of viable cells in the population to assess the quality of yeast, but it should be considered that viable cells can have different degrees of resilience, affecting viability.

5.1 Determination of cell viability

The viability of cells is determined either by methods using cultivation of microorganisms, or by staining methods.

5.1.1 Methods of intravital staining

Methods of intravital staining are based either on the use of microscopy in visible light or on fluorescence microscopy. The most widely used dye for light-field microscopy is methylene blue (“methylene stain”), which is reduced by living yeast

cells to colorless compounds under the action of enzymes. The effectiveness of this method depends not only on the state of the cell membrane, but also on the activity of certain oxidoreductases in the cell, and thus it reflects both the viability and vitality of the yeast (dead cells are dyed blue). It was found that in cases where viability is below 90 %, this method slightly overestimates the viability.

Despite its relative simplicity, this method is quite subjective and poorly reproducible, because cells can be viable even with partially damaged cell membranes and reduced enzymatic activity.

Methylene blue (pH 4.6) can be replaced by methylene violet (pH 10.6). In an alkaline environment, a more reliable and less subjective result is achieved with better correlation with cell multiplication and oxidative capacity.

Since there are many limitations to optical brighteners (dyes used in light-field microscopy), fluorescent dyes were proposed instead. The action of these dyes is based on:

- integrity of the membrane. Dyes Mg-ANS - magnesium salt of 1-anilino-8-naphthalene sulfonic acid, propidium iodide, *Sytox* orange; *Berberinej TZK, FUN-1*;

- membrane potential (*Oxonol and Rhodamine* 123);

- application of esterase-containing substrates (fluorescein diacetate, carboxyfluorescein diacetate and calcein acetic methyl ester).

To assess the viability of brewer's yeast, Mg-ANS, *Oxonol, Sytox* Orange and *Berberinej TZK* are used. Some brewer’s enterprises use Mg-ANS to yield good results in the study of yeasts with low viability, sometimes observed with the increased wort concentration. The advantage of fluorescent dyes lies in their application with flow cytometry. This increases the sensitivity and objectivity of the method and reduces its labor content [1, 4, 24].

5.1.2 Methods based on multiplication of cells

There are two main methods, based on multiplication of cells: plate cultivation and slide cultivation. The method of plate cultivation is based on the incubation of cells on the surface of an agar nutrient medium poured into a Petri dish. Viability is assessed by the amount of CFU (colony forming units) after incubation for 72 hours at a temperature of 27 ºC. The ratio of CFU to the total number of cells in the solution is expressed in %. With this method, coefficient of variation (relative standard deviation) is 20 %. This method can be used to directly determine the ability of the cell to multiply, but it takes a long time (three days) to obtain the results. In addition, there are difficulties if cells have high flocculating ability.

The method of slide cultivation is based on the microscopic observation of micro colonies grown on a film of nutrient agar within 18 hours of cultivation. This method determines the ability of an individual cell to multiply oneself. Thus, both methods directly determine the ability of the cell to multiply, but it takes considerable time to obtain results [4].

5.2 Determination of physiological state (vitality) of yeast

Vitality of yeasts is their activity or the ability to recover from physiological stress. The physiological state of brewer's yeast is evaluated by measuring the concentration of intracellular components (stored glycogen, the amount of sterol or ATP), or by evaluating metabolic activity. The latter is evaluated in various ways, including measuring oxidative ability, magnesium ions production, average cell age, intracellular pH value, rate of sugar utilization, ethanol formation, CO_2 release, oxygen consumption and enzyme activity [4, 25, 26].

5.2.1 The content of sterols and unsaturated fatty acids

Sterols and unsaturated fatty acids are important components of yeast cell membrane, especially when growing in anaerobic conditions.

Oxygen molecules are necessary for biosynthesis of ergosterol and unsaturated fatty acids. Sterols and unsaturated fatty acids are the limiting factors of yeast growth in the production of beer and their content determines the physiological state of yeast cells.

Consequently, the initial content of sterols and unsaturated fatty acids in the cell is an important indicator of yeast ability to grow and ferment. On the other hand, the use of yeast with a low sterol content in the cell before fermentation can lead to a normal fermentation, if yeast in the early stages of fermentation is provided with sufficient oxygen. Although sterols and unsaturated fatty acids are important factors in determining the course of fermentation, it is difficult to use their initial content as an indicator of the vitality of yeast [4].

5.2.2 Content of ATP

The identification of ATP is a popular method for assessing the viability of yeast, as "dead" cells do not contain ATP. In that case, if the cells are in the same physiological state, the method measures the number of living cells. However, since the ATP rate in the cell varies with the temperature and the level of nutrients, this method does not always give a true yeast vitality level.

Therefore, this method has its limitations, but it can be useful to determine the physiological state of yeast in combination with other methods [4].

5.2.3 Measurement of CO_2 and oxygen consumption rate

The amount of released CO_2 measured with a Warburg manometer is a standard microbiological method adapted for evaluation of brewing yeast in wort. The formation of CO_2 is determined by the pressure rise in closed vessels.

This method gives reproducible results that directly correlate with yeast fermentation activity, but provides little information about yeast growth.

On the other hand, there is a correlation between the course of fermentation and the consumption of oxygen in yeast with a viability of less than 90 %. Reduction in oxygen consumption rate goes in parallel with a decrease in the content of lipids (glycogen) in yeast and the value of the acidification strength, and changes in viability and fermentation process. Although the physiological significance of the oxygen consumption rate is unclear, these two methods complement each other and are useful for determination of yeast vitality variations [4, 25].

5.2.4 Acidification strength test

This method was developed as an indicator of yeast metabolic activity. It was adapted to predict the progress of fermentation, conducted by brewer's yeast, to establish the relationship between the value of the acidification strength test and the fermentation process.

The acidification strength test is a method that measures the decrease in value of extracellular pH of yeast suspension before (spontaneous reduction) and after glucose addition. The level of spontaneous acidification is an indicator of glycogen content, and the glucose-induced level of acidification is an indicator of glycolytic pathway rate. This method is useful, fast, and convenient for determining the viability of yeast cells.

Variation of yeast activity in brewing conditions is very insignificant, especially in yeast with high vitality. This method is recommended when the yeast state of different samples is significantly different from each other [4].

5.2.5 Method for measuring intracellular pH

The method of measuring intracellular pH is more sensitive to brewer's yeast. The value of intracellular pH as well as extracellular pH depends on the proton pump and the effect of ATP in membranes, the activity of which is interrelated with the intensity of cell multiplication. There is a definite dependence between the acidification strength (Y) and the value of intracellular pH (X) [4]:

$$Y = 3.40 – 6.68X + 2.13X^2 – 0.17X^3 \quad (1)$$

5.2.6 Measurement of enzymatic activity

The drawback of the methods that measure the rate of yeast multiplication, the rate of fermentation, the drop in pH, the number of certain by-products of fermentation is that the results become known only after the fermentation of test samples is completed. The process takes usually up to three days and by this time, yeast is already used in production. It should be considered that yeasts with weak fermentation activity may cause slow fermentation, weak reduction of diacetyl, and decrease in the quality of beer.

In the process of biomass accumulation during preparation of a pure yeast culture at different temperatures, the following enzymatic activities are usually studied: PDH (pyruvate dehydrogenase), PDC (pyruvate carboxylase), ADH (alcohol dehydrogenase), MAL (maltase) and PFK (pyruvate phosphokinase) [1, 4].

6 Laboratory work

6.1 Acidification strength test

The state (vitality and viability) of the inoculated yeast is a particularly important factor for the correct fermentation process, which ensures the production of beer of the required quality. The state of yeast is defined by terms of viability and vitality. Viability is measured by the number of "dead or living" cells; viability and vitality are a measure of the metabolic rate or physiological state of living cells.

The "acidification strength" test is used when there are significant differences in physiological state of the compared yeast samples.

The method includes measuring the extracellular pH of the yeast suspension before (within 10 min) and after the addition of glucose (for 20 min). The decrease in extracellular pH value, caused by the release of yeast ions H^+, determines the physiological state of the yeast (Fig. 6.1). The greater the difference between the initial and final pH, the higher the yeast activity [4].

Objectives:

- study the influence of beer color on the physiological state of yeast;
- study the influence of the number of generation of seed yeast on the physiological activity of yeast;
- study the influence of temperature stress on the physiological state of yeast;
- study the influence of osmotic stress on the physiological state of yeast.

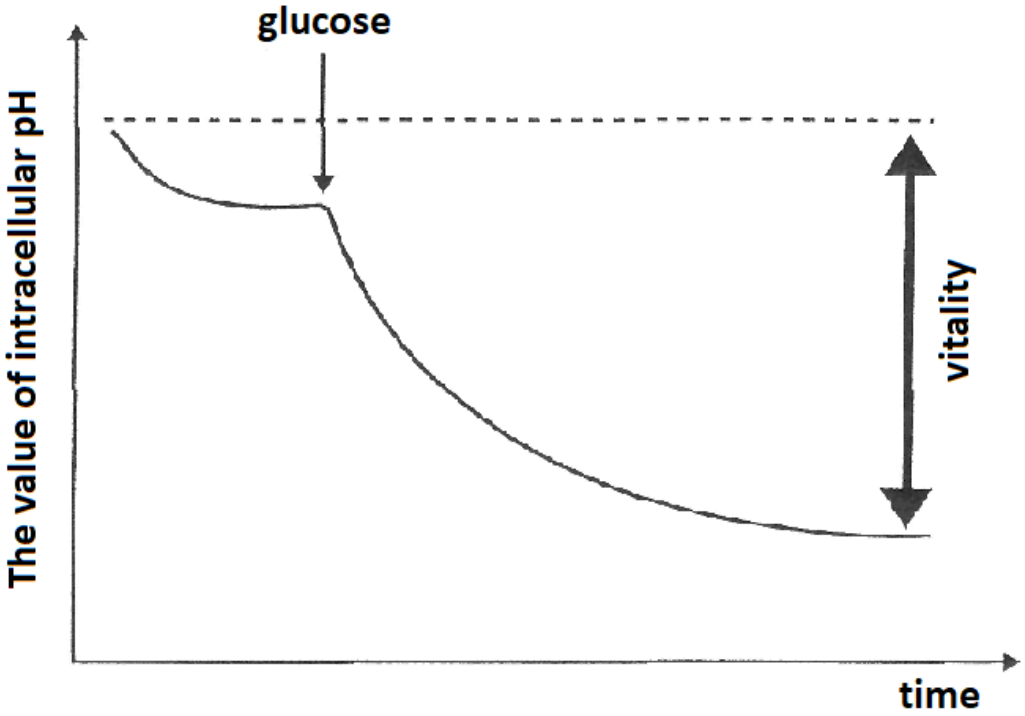

Figure 6.1 - Acidification strength measurement

Instruments and labware:

- laboratory ionometer I-160 M (pH-meter);
- table with a magnetic stirrer;
- analytical balance;
- centrifuge;
- glass beaker with a capacity of 200 ml;
- pipette with a capacity of 10 ml;
- glass rod;
- measuring cylinder with a capacity 100 ml.

Reagents:

- water,
- 20 % glucose solution.

Materials:

- yeast.

Method.

Weigh a yeast sample of 4 grams on an analytical balance with an accuracy of up to 0.1 g. Add tap water (100 ml). The suspension is centrifuged 10 minutes at N = 6000 rpm, then thoroughly mixed into 100 ml of water. Then, the initial pH value of the suspension is determined. Further, to establish the reserve of glycogen, which is consumed during the lag phase of yeast growth, pH values are measured after 5 and 10 minutes. Values should be recorded in Table. 6.1. Next, according to Formula (2), the rate of fall of pH - V_1 is determined. The greater the rate of pH decrease, the higher the content of reserve carbohydrates in the cells, the more intensive the metabolism and the shorter the growth phase of the yeast.

$$V = \ln [pH1 / pH2] / [t_2 - t_1] \quad (2)$$

Table 6.1 - The pH values of the yeast suspension prior to the introduction of the carbohydrate substratum

№ of experiment	Time, min			V_1
	0	5	10	
1				
2				
3				
average value				

After taking the readings (after 10 minutes), add 5 ml of 20 % glucose solution and mix for another 10 minutes. During incubation for 20 minutes, the pH values of

the suspension are measured every minute. The actions should be repeated 3 times, the results should be recorded in Table. 6.2.

Table 6.2 - The pH values of the yeast suspension prior to the introduction of the carbohydrate substratum

№ of experiment	Time, min					Vitality of yeast
	11	12	13	……	30	
1						
2						
3						
average values						

According to the average values of the data given in Tables 6.1 and 6.2, plot a graph of change in pH against the duration of yeast incubation (Fig. 6.2) and determine yeast viability by the difference between the initial pH value and the pH value after 30 min of cell incubation ($pH_o - pH_{30}$) [4].

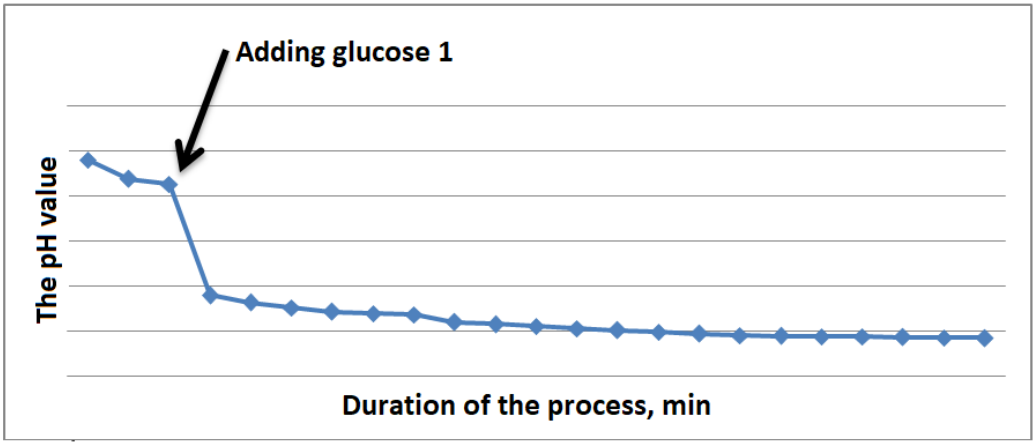

Figure 6.2 - Dynamics of pH change of yeast suspension on a medium with glucose.

6.2 Methods based on staining of cell preparations

Methods of staining are based either on microscopy in visible (ordinary) light, or fluorescence microscopy. The magnesium blue and Lugol's solution are used for light microscopy. Fluorescent dyes used are the magnesium salt of 1-anilino-8-naphthalenesulfonic acid (Mg-ANS), dihydrorhodamine (Table 6.3) [4, 24].

Table 6.3 - Classification of dyes

Dyes for light microscope	Fluorescent dyes
Methylene blue	Rhodamine
Methylene blue + safranin	Mg-ANS
Lugol	
Iodonitrotetrazolium chloride (INT)	

6.2.1 Staining of yeast with methylene blue

The most widely used method to detect dead cells is staining with methylene blue.

After contact with the cell's cytoplasm this dye is restored to colorless compounds in live yeast cells due to the action of reductase enzymes. Dead cells are stained blue. The effectiveness of this method depends not only on the state of the cell membrane, but also on the activity of oxidoreductase in the cell.

Instruments and labware:

- a microscope;
- slides and coverslips;
- a thin glass rod.

Reagents:

- methylene blue.

Materials:

- yeast suspension with a concentration of about 10^7 cells / ml.

Method of staining.

To determine the number of dead cells on a slide, one drop of unfiltered yeast suspension and methylene blue solution (0.01 %) is applied. The drop is covered with a cover slip, excess fluid is collected by a sheet of filter paper and after 2 minutes the colored preparation is observed using a microscope. The total number of yeast cells is counted in the field of view of the microscope, then only the blue ones, after which the preparation is moved and the count is taken in a new field of view. Thus, the total number of cells (at least 500) in five fields of view is counted. After counting, calculate the number of dead cells in percent [4].

6.2.2 Cell staining with methylene blue and safranin

Staining of fixed cells with methylene blue, tannin and safranin gives more complete information about the physiological state of the yeast. Safranin is used to identify cell nuclei that are colored red. If the cells are alive and contain oxidoreductases that cleave the methylene blue, the colored preparation acquires a reddish rather than a purple hue.

Instruments and labware:

- a microscope;
- slides and coverslips;
- a thin glass rod.

Reagents:

- 0.01 % solution of methylene blue;

- 1 % solution of safranin in water;

- 5 % solution of tannin in water;

- physiological solution (0.9 % NaCl solution).

Materials:

a yeast suspension with a concentration of about 10^7 cells / ml.

Method of staining.

Yeast should be centrifuged for 5 minutes at 4000 rpm. Drain the supernatant, rinse with saline and centrifuge again. Prepare the yeast suspension.

Apply a drop of yeast suspension to a glass slide degreased with soap. Leave to dry in air at room temperature. After the droplet dries, fix the preparation (run the slide through the flame of the alcohol lamp10 times). Do not warm hot, leave it to cool down. Flood the slide with a solution of methylene blue and let stand 4 minutes at room temperature. Rinse the dye with warm water. Flood the slide with a freshly prepared tannin solution for 2 minutes. Rinse the dye under running water. Flood the slide with a solution of safronin for 16 minutes. Rinse off the dye. Microscopy is performed with a non-fluorescent oil at 400x magnification [4, 24].

6.2.3 Staining cells with Lugol's solution

The physiological state can also be determined by the composition of the cell, for example, by reserve carbohydrates, one of which is glycogen.

The amount of glycogen in yeast cells varies depending on their age and cultivation conditions. The Lugol’s solution is used for evaluation. The Lugol’s solution stains the cells yellow, and glycogen brown.

The amount of glycogen in yeast varies depending on their age and cultivation conditions. In mature cells, glycogen occupies from 1/3 to 2/3 of the cells and more. In cells with low physiological activity, the colored glycogen occupies less than 1/4 of

the cell. In young cells, glycogen is absent; and the cells when stained with iodine solution acquire a pale yellow color.

Instruments and labware:

- microscope;
- degreased slip and cover glasses;
- thin glass rod.

Reagents:

- dye - Lugol's solution.

Materials:

- yeast suspension with a concentration of about 10^7 cells / ml;
- filter paper.

Preparation of Lugol's solution.

2 g of potassium iodide is dissolved in 5 ml of distilled water;

1 g of crystalline iodine is added to the solution. After dissolution of iodine, the resulting solution is brought to a volume of 300 ml with distilled water.

Method of staining.

For a qualitative assessment of the glycogen level in yeast cells, a small amount of yeast suspension and 1-2 drops of Lugol's solution are applied to the slide glass. The drop is covered with coverslip, the extra liquid is removed by a filter paper and then the glass is microscopized. After 2-3 min, yeast cells are colored yellow, and glycogen is colored brown [4, 24].

7 Practical part

7.1 Yeast nutrition

The nutrient medium has to provide yeast with the following substances:

1. basic elements: C, H, O, N;
2. macronutrients: P, K, S, Mg;
3. microelements: Zn, Mn, Co, Ca, Fe, Cu and others;
4. vitamins.

In some cases, the culture medium for the cultivation of microorganisms does not contain all the necessary elements, micro and macro elements and vitamins, or the culture medium for the cultivation of microorganisms contains them in insufficient quantities. Therefore, the skills of calculating the composition of nutrient media are very important for cultivation processes [1, 4].

Table 7.1 - Examples of nutritional deficiencies [4]

Vitamin	The amount of vitamin in 100 g of absolutely dry yeast biomass, mg	The amount of vitamin in 5 g of absolutely dry yeast biomass, mg	The content of vitamin in 1 liter of malt wort (DS 12 %), mg
Biotin	0.1 – 1.0	0.005 – 0.05	0.0065
Pantothenic acid	2 - 20	0.1 – 1.0	0.45 – 0.65
Inositol	200 - 500	10 - 25	55

We can calculate biotin deficiency using the data from table 7.1:

$$Biotin\ deficiency = 0.05 - 0.0065 = 0.0435\ mg \quad (3)$$

Here, 0.0435 mg of biotin must be added to the wort to obtain 5g of absolutely dry yeast biomass.

Absolutely dry biomass (ADB) is calculated by

$$\mathrm{ADB} = \mathrm{m(yeast)} \cdot \frac{(100 - \mathrm{moisture})}{100}\ , \quad (4)$$

where moisture (humidity) is an indicator of water content (usually in %).

The biomass increment is calculated as

$$\Delta ADB = ADB2 - ADB1\ , \quad (5)$$

where ADB1 is ADB at the beginning of the cultivation process, and

ADB2 is ADB at the end of the cultivation process.

7.2 Tasks

Task 1. According to Annemuller [1], 1 kg ADB of yeast contains 0.185 g of Zn; 1l malt wort (DS 12 %) contains 0.15 mg Zn. We plan to get a 5g ADB yeast increment from 1l of malt wort. How much $ZnCl_2$ must be added to the wort?

Step-by-step explanation of Task 1 is presented in Table 7.2.

Table 7.2 - Task 1 explanation

Step №	Decision
1	1000 g ADB of yeast - 0.185 g zinc ions 5 g ADB of yeast - X g X = 0.000925 g = 0.925 mg
2	How much zinc ions must be added to the wort? 0.925 – 0.15 = 0.775 mg
3	How much $ZnCl_2$ must be added to the wort? M ($ZnCl_2$) = 65.38 + 35.5·2 = 136.38 g/mole 136.38 g $ZnCl_2$ - 65.38 g Zn^{+2} X mg $ZnCl_2$ - 0.775 mg X=1.61 mg $ZnCl_2$ must be added to the wort
Total	1.61 mg $ZnCl_2$ must be added to the wort

Task 2. According to Annemuller [1], an increment of 100 g ADB of yeast requires 0.25 mg of biotin; 1l of malt wort (DS 12 %) contains 0.0065 mg of biotin. How much biotin must be added to the wort: 1) for obtaining a pure culture? 2) for fermentation? The increment of yeast biomass: 5 g ADB in aerobic conditions; 1.7 g ADB in anaerobic conditions.

Step-by-step explanation of Task 2 is presented in Table 7.3.

Table 7.3 - Task 2 explanation

Step №	Decision
1	How much biotin is needed for the increment of yeast biomass 5 g ADB? 100 g ADB - 0.25 mg biotin 5 g ADB - X g X = 0.0125 mg
2	How much biotin must be added to the wort for obtaining a pure culture? X = 0.0125 – 0.0065 = 0.006 mg
3	How much biotin is needed for the increment of yeast biomass 1.7 g ADB? 100 g ADB - 0.25 mg biotin 1.7 g ADB - X g X = 0.00425 mg
4	How much biotin must be added to the wort for fermentation? X = 0.00425 mg < 0.0065 mg
Total	The wort contains a sufficient amount of biotin for fermentation

Task 3. According to Meledina et. al. [4], «crude protein» of yeast is 37-50 % from ADB. We plan to get a 5g ADB increment from 1l of synthetic medium. How much $(NH_4)_2SO_4$ should we use?

In this task we use the concept of "crude protein".

"Crude protein" is calculated by the formula:

$$\text{"crude protein"} = N \cdot 6.25. \quad (6)$$

The «crude protein» is calculated by multiplying 6.25 by the total nitrogen in the cells, determined by the Kjeldahl method. This value is always higher than the content of the true protein, since the total nitrogen in the cell is represented by the nitrogen of the protein, amino acids, nucleic acids, ammonia nitrogen.

The protein content in the cells depends on many factors: the conditions of cultivation, the composition of the nutrient medium, the amount of seeding. The average «crude protein» of the yeast cell is 30-50 %.

In the calculations below, the content of «crude protein» is taken as 50 %.

Step-by-step explanation of Task 3 is presented in Table 7.4.

Table 7.4 - Task 3 explanation

Step №	Decision
1	50% · ADB = N · 6.25 *N* = 50% · ADB / 6.25 = 8% · ADB We want to get ADB = 5 g So: *N* = 8% · 5 g / 100% = 0.4 g
2	M ($(NH_4)_2SO_4$) = 14·2 + 1·8 + 32 + 16·4 = 132 g/mole 132 g $(NH_4)_2SO_4$ - 28 g N^{-3} X g $(NH_4)_2SO_4$ - 0.4 g X=1.89 g $(NH_4)_2SO_4$
Total	1.89 g $(NH_4)_2SO_4$ must be added to the medium

Task 4. According to Meledina et. al. [4], the amount of P_2O_5 in the yeast ash is 1.9 to 5.5 % from yeast ADB; K_2O in the yeast ash is 1.4 – 4.3 % from yeast ADB. We plan to get a 5g ADB increment from 1l of synthetic medium. How much K_3PO_4, KCl should we use?

Step-by-step explanation of Task 4 is presented in Table 7.5.

Table 7.5 - Task 4 explanation

Step №	Decision
1	How much P_2O_5 is contained in 5 g of ADB? 5 ·5.5 /100 = 0.275 g
2	How much P^{+5} is contained in 0.275 g of P_2O_5? M (P_2O_5) = 31·2 + 16·5 = 142 g/mole 142 g P_2O_5 - 62 g P^{+5} 0.275 g P_2O_5 - X g P^{+5} X=0.12 g P^{+5}
3	How much K_3PO_4 should we use? M (K_3PO_4) = 39·3 + 31 + 16·4 = 212 g/mole 212 g K_3PO_4 - 31 g P^{+5} X g K_3PO_4 - 0.12 g P^{+5} X= 0.82 g K_3PO_4 we should use
4	How much K_2O is contained in 5 g of ADB? 5·4.3 /100 = 0.22 g
5	How much K^+ is contained in 0.22 g of K_2O? M (K_2O) = 39·2 + 16 = 94 g/mole 94 g K_2O - 78 g K^+ 0.22 g K_2O - X g K^+ X = 0.18 g K^+

Table 7.5 continuation

Step №	Decision
6	How much K^+ is contained in 0.22 g of K_3PO_4? M (K_3PO_4) = 39·3 + 31 + 16·4 = 212 g/mole 212 g K_3PO_4 - 117 g K^+ 0.82 g K_3PO_4 - X g K^+ X = 0.45 g K^+ we added with K_3PO_4
7	How much KCl must we use? 0.45 g K^+ we added with K_3PO_4 0.18 g K^+ need to get a 5g ADB increment 0.45 > 0.18
Total	We must use 0.82 g K_3PO_4. We do not need to use KCl

Task 5. According to Meledina et. al. [4], the amount of the ash in yeast is 2 – 10 % from yeast ADB. MgO is 3 – 8.1 % from the yeast ash. We plan to get a 5g ADB increment from 1l of synthetic medium. How much $MgSO_4 \cdot 7H_2O$ should we use?

Step-by-step explanation of Task 5 is presented in Table 7.6.

Table 7.6 - Task 5 explanation

Step №	Decision
1	How much ash can we get from 5 g of ADB? 5 · 10/100 = 0.5 g
2	How much MgO is contained in 5 g of ADB? 0.5 g · 8.1 /100 = 0.04 g

Table 7.6 continuation

Step №	Decision
3	How much $Mg^{2+}$is contained in 0.04 g of MgO? M (MgO) = 24 + 16 = 40 g/mole 40 g MgO - 24 g Mg^{2+} 0.04 g MgO - X g Mg^{2+} X = 0.024 g Mg^{2+}
4	How much $MgSO_4 \cdot 7H_2O$ should we use? M ($MgSO_4 \cdot 7H_2O$) = 24 + 32 + 16·4 + 18·7= 246 g/mole 246 $MgSO_4 \cdot 7H_2O$ g - 24 g Mg^{2+} X g $MgSO_4 \cdot 7H_2O$ - 0.024 g Mg^{2+} X = 0.246 g
Total	We should use 0.246 g $MgSO_4 \cdot 7H_2O$

Task 6. Calculate the amount of salt based on the following data: P_2O_5 in the yeast ash is 4 % from yeast ADB; K_2O in the yeast ash is 2.5 % from yeast ADB. We plan to get a 3 g ADB increment from 1l of synthetic medium. How much $(NH_4)_2SO_4$, KH_2PO_4, KCl should we use?

Step-by-step explanation of Task 6 is presented in Table 7.7.

Table 7.7 - Task 6 explanation

Step №	Decision
1.1	Using formula 4, let the content of «crude protein» be 50% 50 % · ADB = N · 6.25 N = 50 % · ADB / 6.25 = 8 % · ADB We want to get ADB = 3 g So: N = 8 % · 3 g / 100 % = 0.24 g
1.2	M ($(NH_4)_2SO_4$) = 14·2 + 1·8 + 32 + 16·4 = 132 g/mole 132 g $(NH_4)_2SO_4$ - 28 g N^{-3} X g $(NH_4)_2SO_4$ - 0.24 g X= 1.13 g $(NH_4)_2SO_4$
2.1	How much P_2O_5 is contained in 3 g of ADB? 3 ·4 /100 = 0.12 g
2.2	How much P^{+5} is contained in 0.12 g of P_2O_5? M (P_2O_5) = 31·2 + 16·5 = 142 g/mole 142 g P_2O_5 - 62 g P^{+5} 0.12 g P_2O_5 - X g P^{+5} X = 0.05 g P^{+5}
2.3	How much KH_2PO_4 should we use? M (KH_2PO_4) = 39 + 1·2+ 31 + 16·4 = 136 g/mole 136 g KH_2PO_4 - 31 g P^{+5} X g KH_2PO_4 - 0.05 g P^{+5} X = 0.22 g KH_2PO_4
3.1	How much K_2O is contained in 3 g of ADB? 3 ·2.5 /100 = 0.075 g

Table 7.7 continuation

3.2	How much K^+ is contained in 0.075 g of K_2O? M (K_2O) = 39·2 + 16 = 94 g/mole 94 g K_2O - 78 g K^+ 0.075 g K_2O - X g K^+ X = 0.062 g K^+
3.3	How much K^+ is contained in 0.22 g of KH_2PO_4? M (KH_2PO_4) = 136 g/mole 136 g KH_2PO_4 - 39 g K^+ 0.22 g KH_2PO_4 - X g K^+ X = 0.063 g K^+ we added with KH_2PO_4
Total	We should add to the medium: 1.13 g $(NH_4)_2SO_4$, 0.22 g KH_2PO_4. We do not need to use KCl

Task for individual work. We plan to get X g ADB increment from 1l of synthetic medium. Calculate the amounts of salts based on the data in your variant. How much $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ should we use? What amount of S^{+6} is added to the medium (calculate on SO_3)? Is this enough for our increment of yeast (For calculations to accept that SO_3 - 0.05 % from yeast ADB)?

Variants of task for individual work are presented in Table 7.8.

Table 7.8 – Variants of task for individual work

Variant №	ADB increment, g	The content of MgO from yeast ADB, %
1	5.5	0.7
2	3.5	0.5
3	4.0	0.3
4	6.0	0.4
5	6.5	0.1
6	3.2	0.6
7	4.5	0.2
8	7.1	0.3
9	5.8	0.5
10	6.7	0.4

Your reply should contain:

1. m ($(NH_4)_2SO_4$), g;
2. m ($MgSO_4 \cdot 7H_2O$), g;
3. m (SO_3), i.e. the amount of SO_3 added by these salts ($(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$),g;
4. Is this sufficient for a given increment of ADB (yes - sufficient / no – not sufficient)?

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YEAST. MORPHOLOGY AND PHYSIOLOGY

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