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ESTUDIO DE LA VIABILIDAD Y MORFOLOGÍA DE LAS CEPAS *BINGEN, STEINBERG* Y *SAUTERNES* DEL GÉNERO *SACCHAROMYCES CEREVISEAE* EN CONCICIONES DE ESTRÉS

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FINAL PROJECT

THE STUDY OF VIABILITY AND MORPHOLOGY OF YEASTS STRAIN BINGEN, STEINBERG AND SAUTERNES OF SACCHAROMYCES CEREVISIAE IN A STRESSES CONDITIONS

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INTRODUCTION

The *Saccharomyces cerevisiae* yeasts are used for ethanol production by alcohol industries. Apart from obtaining ethanol for alcoholic drinks, they have been used in human and animal feeding. Yeasts contain approximately 40% dry matter of proteins and they can tolerate and produce until 18%-20% (v/v) of ethanol. Nowadays, yeasts are the object of studies of unicells proteins production as well as many works about ethanol tolerance and fermentation.

1. YEAST

Yeasts constitute a highly versatile group of eukaryotic carbon-heterotrophic organisms that have successfully colonized natural habitats. The yeasts are taxonomically diverse and include ascomycetes and basidiomycetes. A third group, the imperfect yeasts, have both ascomycetous and basidiomycetous. Most of these are saprophytes and some are well known as human pathogens. The best-known yeast is *Saccharomyces cerevisiae*, strains of which are widely used in the fermentation of wine, beer, and other alcoholic beverages and in baking. It is a common environmental fungus and transient component of the normal flora of the gastrointestinal tract and skin. They are also found in nature on ripe fruits in winery, on grapes skin or in other fruits. They are anaerobics-phacultatives, they can growth with or without O_2. When oxygen is present in the medium, yeast can make ATP by aerobic respiration. In the absence of oxygen (in anaerobic conditions) the yeast begin anaerobic metabolism, converting the pyruvate from glycolisis to alcohol and CO_2. [1]

1. Growth and Reproduction:

Yeasts are definition like an unicellular fungi, they reproduce asexually by budding. Yeast has a phenomenal growth rate and can duplicate itself every 90 minutes.

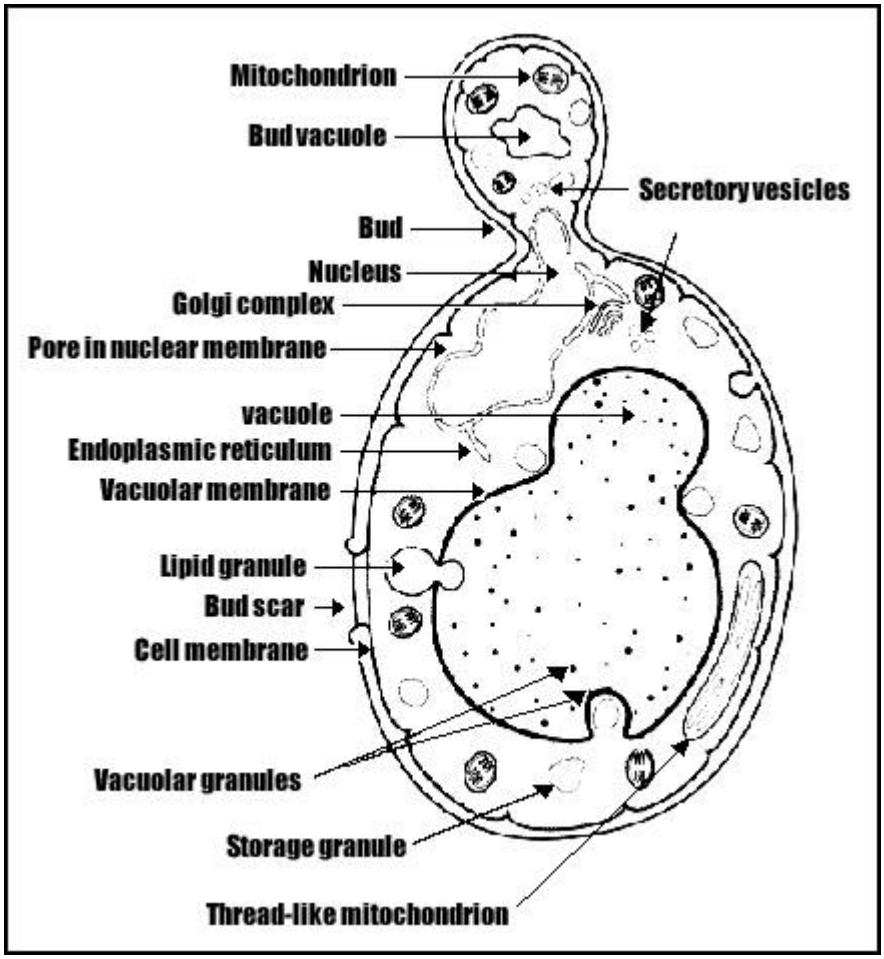
The process of budding begins in a mature yeast cell at predetermined areas of its cell wall. In *Saccharomyces cerevisiae*, for example, budding takes place at the poles of the cell. In these areas when the budding is about to take place, the cell wall is softened and is "blown out" to form the so-called "bud", which will become the new cell. As the bud enlarges, mitosis of the nucleus occurs, with one of the nuclei moving into the newly formed cell. When the cell reaches the approximate size of the original cell, cell wall material is laid down in the passage between the two cells, which will then shortly separate.

To live and grow, yeast needs moisture, warmth, food and nutrients. Commercial yeast is manufactured on an aerated suspension of molasses. Molasses, a form of sugar, provides the food for the yeast so it can reproduce. The molasses is mixed with water and sterilized to kill off unwanted bacteria, clarified by removal of sludge and then held in vats. [2]

1.1. Morphological characteristics:

Yeast cells exhibit great diversity with respect to cell size, shape and colour. Even individual cells from a particular yeast strain of a single species can display morphological and colour heterogeneity. This is mainly due to alterations of physical and chemical conditions in the environment.

Figure 1: Morphological components of cell



The morphological characteristics of yeast can be observed by microscope. The cells can be single, budding or they can grow in pseudomycelium form. The shape of the yeast can be round to oval or elongate, and lemon way form too. Also they are differences with respect to size they measure 1-10 um of wide 2-3 um of length. Mean cell volumes are 29-55 μm^3 for both a haploid or a diploid cell respectively, cell size increases with age. *Saccharomices cerevisiae* cells shape can be spherical or elliptic, and their length and width are between 4-10μm. [2, 3, 4]

1.2. General characteristics:

Temperature:

In general, yeasts are mesophiles, they grow in a range 24-28ºC. Only the 2% of yeasts are psychrophilic, they maximum temperature of growing is below 24ºC, although for the biggest group of psychrophilic yeast the optimal temperature of growing is below 20ºC. The yeast don´t grow around 50ºC, *Pichia polymorpha, Geotrichum capitatum, Saccharomyces cerevisiae* and species of *Candida* and *Debaryomyces*, they can spread around 40ºC. Only few species can live near 0ºC, for example: *Yarrowia lipolytica, Debaryomyces hansenii* and *Pichia membranaefaciens*. [5, 6, 7]

Water Activity:

Majority of yeasts damages foods in a range of water activity below 0.90-0.95. However *Zygosaccharomyces rouxii* can grow around 0.62 water activity in sugar substrate. Around thirty species multiply in the range 0.912- 0.876 of water activity.

In general, yeasts tolerate high concentrate of sugar better than salt. However *Stephanoascus ciferrii, Debaromyces hansenii* and *Lipomyces kononekoae* prefer salt substrate. [5, 6, 7]

pH:

The great majority of yeasts tolerate a pH range 3-10, but they prefer a acid medium between 4,5 and 6,5. However *Issatchenkia orientalis, Pichia membranaefaciens, Dekkera intermedia* and *Saccharomyces exiguous* can grow between 1.3-1.7, if the medium is an inorganic acid. However, *Rhodotorula* and *Crytococcus* yeasts can tolerate alcaline medium, meanwhile *Saccharomycodes, Schizosaccharomyces* and *Dekkera* don´t grow in a medium with pH higher than 8. [5, 6, 7]

Oxygen Conditions:

Yeast in general are aerobic organisms and in such conditions they can do fermentative process. But some genus *Cryptococcus* and *Rhodotorula* don´t have fermentation activity. *Saccharomyces* and a few more genus can make sugar energy fermentative, but they stop their multiplication and grown in the absence of oxygen. [5, 8]

"KILLER" Yeast:

Killer yeasts can synthetic polypeptides, they are toxics for another species of the same genus, as well as inhibit other organisms. Killer yeasts in wine and beers fermentation can produce a contamination and predominate on useful yeast for that fermentation. On the other hand, selected killer yeast can be suppressed to wild undesirable yeast. [5, 9]

2. *SACCHAROMYCES CEREVISIAE*

The classification of *Saccharomyces cerevisiae* is described in Table 1.

Table1: Classification of *Saccharomyces cerevisiae* [10]

DOMAIN	*Eukariota*
KINGDOM	*Fungi*
PHYLUM	*Ascomycota*
SUBPHYLUM	*Saccharomycotina*
CLASS	*Saccharomycetes*
ORDER	*Saccharomycetales*
DIVISION	*Amastigomycota*
SUBDIVISION	*Ascomychtina*
FAMILY	*Saccahromycetacea*
SUBFAMILY	*Saccharomycetoideae*
GENUS	*Saccharomyces*
SPECIES	*Saccharomyces cerevisiae*

Saccharomyces cerevisiae has very high fermentative activity and can fermentate products with high level of sugar, around 20-30%. They can produce ethanol in a range of 10-18% (v/v). The yeasts *Saccharomyces cerevisiae* can fermentate sugar products with C6, these are; glucose, fructose and galactose. The fermentation is regulated by substrate rate and in absence of oxygen.

The transporters have an important role in sugar metabolism. One of transporters of *Sacchraomyces cerevisiae* is the hexose transporter and they transport glucose, fructose and manose. They are two kind of transport, one is facilitated diffusion (a passive precess) and the second is active transport.[11]

2.1. Physiologic characteristics

The alcohol production is until 18 % (v/v), which is important factor; because those yeast are used for fermentation. During fermentation process they produce volatile acids in middle level. They predominant habitat is grape juice and wine.

The yeast are mesophiles, with a maximum temperature of growing around 25º-40ºC, but the optimum temperature is around 20º-30ºC . The normal pH is between 4-4.5. They can´t move by themselves, they move by air, liquids or insects.

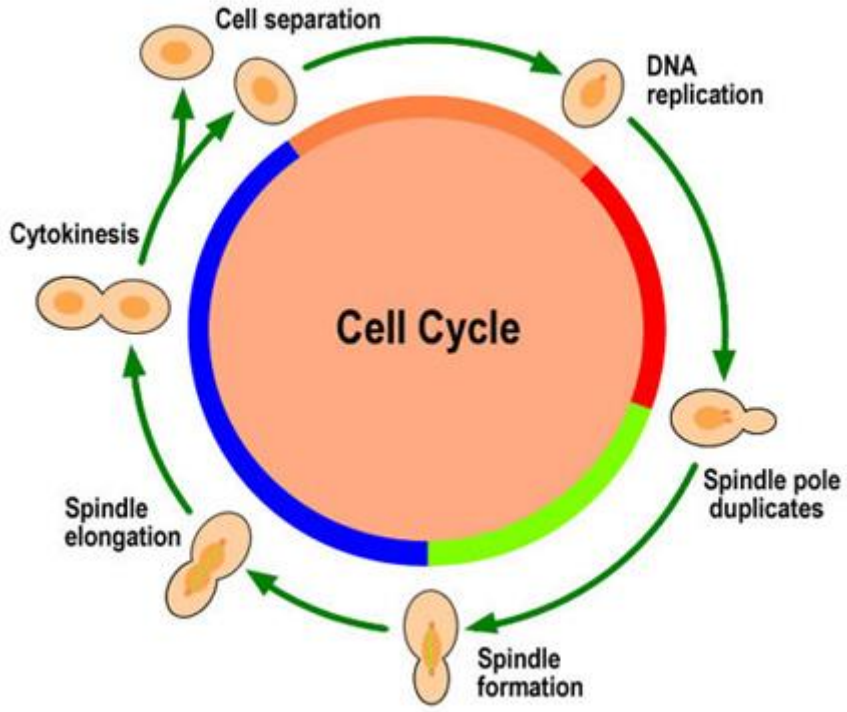
The sugars are the energy source for the yeast. Those sugars are glucose, fructose and galactose.

In a sugar medium, *Saccharomyces cerevisiae*, produce ethanol when pH is 3-4 and they produce glycerol when is higher than 8. [12]

2.2. The Cell

2.2.1. Cell cycle

Figure 2: Cell cycle



As I was mentioned above, yeast vegetative multiplication is via budding. Mitotic cell cycle progression is accomplished through a reproducible sequence of events, DNA replication (S phase) and mitosis (M phase) separated temporally by gaps (G1 and G2 phases).On Figure 3 I show intherphase of cells.

G1, S, and G2 phases are collectively known as interphase. Cdc28 is the catalytic subunit of the cyclin-dependent kinase (CDK) in yeast. At G1 phase Cdc28 associates with G1-cyclins Cln1 to Cln3, while B-type cyclins Clb1 to Clb6 regulate Cdc28 during S, G2, and M phases. Cln3/Cdc28 activity is required for cells to pass through 'Start', the commitment point in G1. When Cln3/Cdc28 accumulates more than a certain threshold, SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) are activated,

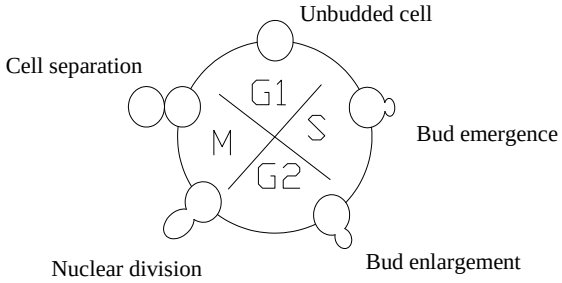
promoting transcription of Cln1, Cln2, and other genes required for S-phase progression. There is size control which operates over 'Start'. Cells can-not pass 'Start' unless a minimum size (which varies according to the growth medium) has been reached. Cln1 and Cln2 interacting with Cdc28 promote activation of B-type cyclin associated CDK, which drives DNA replication and entry into mitosis. Specifically, Cdc28 association with Clb2 and Clb1 promotes entry into mitosis. Cells suffering from DNA damage, spindle misorientation, or spindle assembly defect do not undergo the metaphase-anaphase transition for chromosome segregation and fail to exit from mitosis. The spindle assembly checkpoint activates Mad2, which in turn prevents chromosome segregation by inhibiting degradation of the securin Pds1. Moreover, Pds1 is phosphorylated and stabilized in response to DNA damage in a Chk1-dependent manner. The spindle checkpoint is also involved in the Cdc14 release from the nucleolus. Cdc14 dephosphorylates Swi5, Sic1, and Cdh1, leading to inhibition of Cdc28 and degradation of cyclin required for mitotic exit.

Two fundamentally different patterns of growth occur during different portions of the yeast cell cycle. The emergence of a bud and it is initial growth are finished by polarized growth. Later in the cell cycle approximately early in G2, there is a switch to isotorpic growth, which brings about uniform swelling of the bud.

Finally, during cytokinesis, is the process by which a cell divides its cytoplasm to produce the daughter cells. The separation of one cell in two is accomplished by a structure called the contractile ring. The contractile ring is a structure believed to operate in a way similar to muscle. Myosin is a molecular motor that contracts the actin filaments and form the contractile ring tighter and tighter until the cell is divided in two. The furrow created by this pinching process is also called the cleavage furrow, as it is the site at which cleavage of one cell into two cells occurs. Cytokinesis consists of four steps. The first step is to define the position at which the contractile ring will form. The second step is to assemble the actin filaments that form the contractile ring. The third step is the

actual contraction of the contractile ring. The final step, breakin and refusion of the plasma membrane, occurs once the ring has contracted.[13, 14, 15]

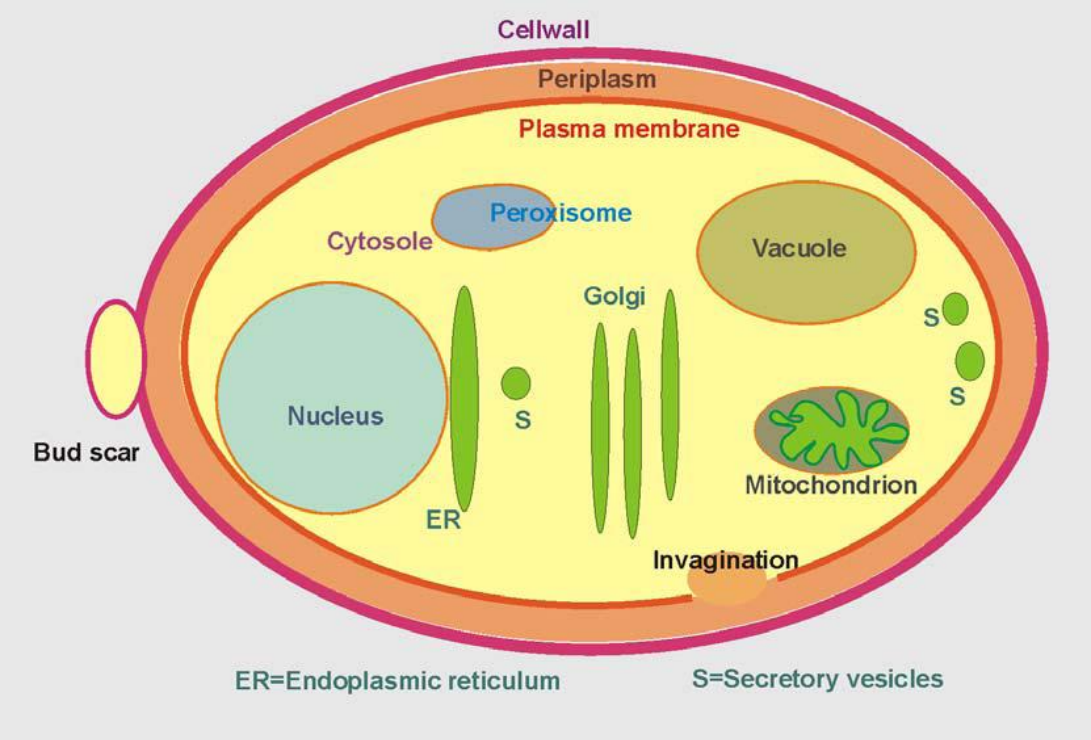
Figure 3: Interphase of cells



2.2.2. Composition cells

Yeast organelles are cell wall, cell membrane, nucleus, ribosomes, mitochondrion, endoplasmic reticulum, Golgi apparatus, and vacuoles. All this cells components are in a cytoplasmic medium.

Figure 4: The organelles cells [1]



Around 75%-80% of the yeast cell is water. The content of proteins is in the range 40%-60% of dry matter. Majority of them are enzymes, and they are locate in the membrane wall. Macromolecular constituents of yeast comprise proteins, glycoproteins, polysaccharides, polyphosphates, lipids, and nucleic acids. In Table 2 I show class of macromolecules in yeast cells.

Table2: Classes of molecules encountered in yeast: [1]

Class of Macromolecule	Categories	Major Components
Proteins	Structural	actin, tubulin (cytoskeleton) histones(H2A, H2B, H3, H4, no H1) ribosomal proteins
	Hormones	α and pheromones
	Enzymes	
Glycoproteins	Cell wall components	mannoproteins
	Enzymes	functional enzymes (invertase)
Polysaccharides	Cell wall components	glucan, mannan, chitin
	Capsular components	
	Storage	glycogen, trehalose
Polyphosphates	Storage	polyphosphate in vacuole
Lipids	Structural	free sterols in membranes
	Storage	lipid particles (sterol esters and triglycerides)
	Functional	phosphoglyceride derivatives, free fatty acids
Nucleic acids	DNA	nucleus (80%); mitochondrial (10-20%)
	RNA	rRNA (80%);mRNA (5% cytosol, ER, mitochondria), tRNA, snRNAs

Main components of *Saccharomyces cerevisiae*;

- 35% of Polysaccharides in the cellular wall
- 7% of minerals
- 5% of phospholipids
- 3% of triglycerides
- 0.5% of DNA, vitamins and fiber

2.2.3. Cell membrane

Plasma membrane forms a relatively impermeable barrier for hydrophilic molecules. Specialized proteins mediate the selective uptake and/or secretion of solutes across this membrane. The membrane separates cell components from the enviromental medium and citoplasmic medium too. [16]

1. *Structure of the plasma membrane*

The plasma membrane forms a lipid bilayer, approximately 7.5 nm wide. It contains a mixture of polar lipids and proteins which, by their interactions, govern the structure of the membrane (Figure 5). The lipids motions on membrane are rotational and transverse (flip-flop). Membrane proteins are often hindered in their lateral motions because of association with other proteins or association with elements of the cytoskeleton or extracellular matrix. [17]

The proteins, in membrane structure are in asymmetric location. There are proteins intrinsic, which span the entire length of the membrane, and others are extrinsic, those are only partially embedded in membrane and protrude on one side of the membrane. The plasma membrane surface proteins are

involved in transport of solutes, signal transduction and synthesis of outer membrane components. [16]. The figure 6 shows classes of membrane proteins found in the *S.cerevisiae* plasma membrane.

Figure 5: Classes of membrane proteins and lipids found in the *S.cerevisiae* plasma membrane

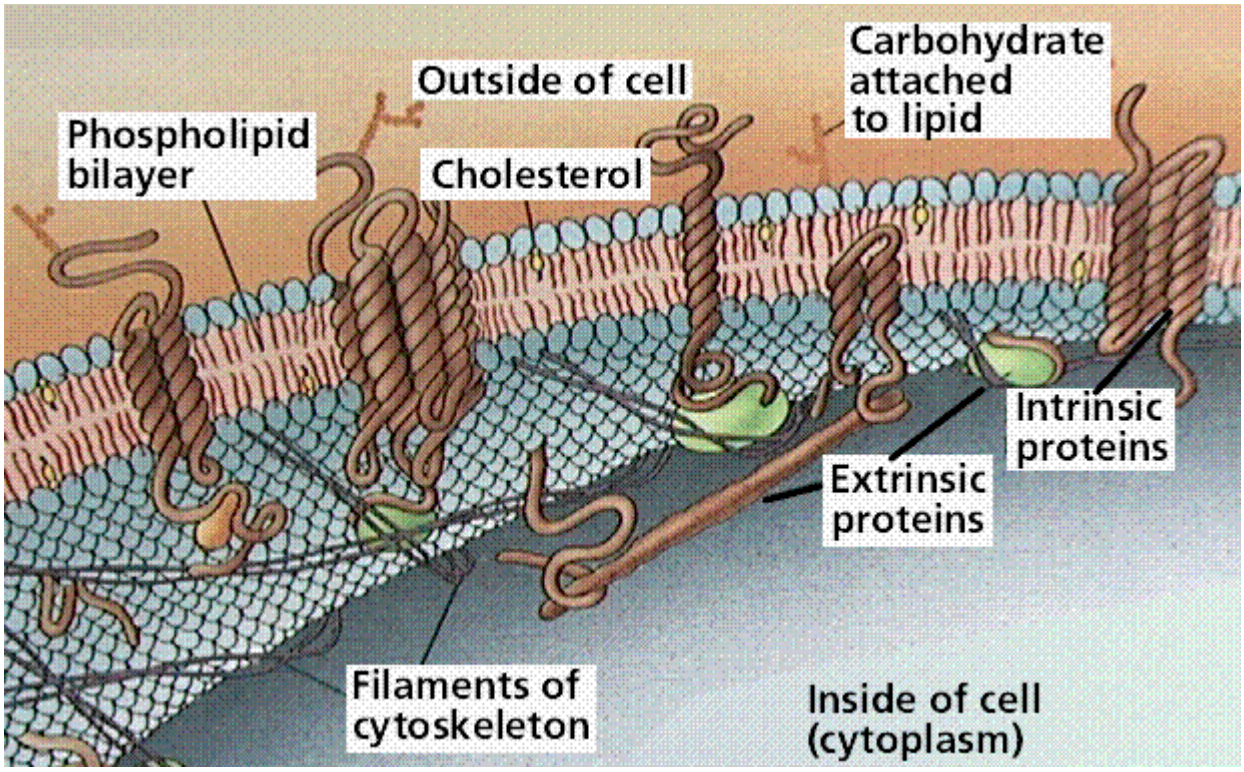
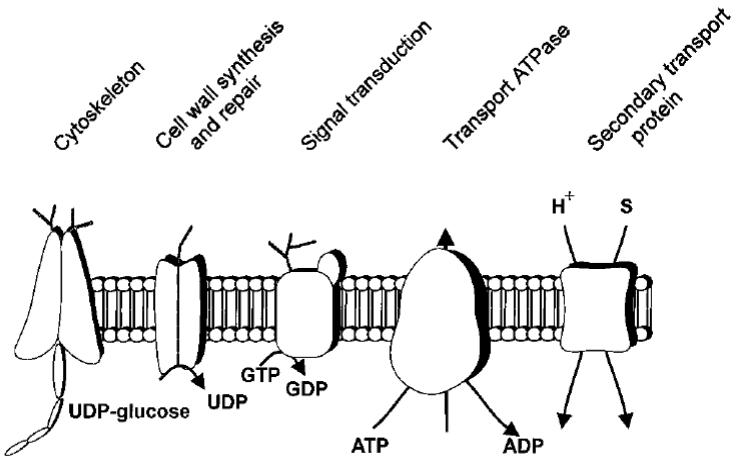


Figure 6: Classes of membrane proteins found in the *S. cerevisiae* plasma membrane



The lipids of the plasma membrane are symmetrically disposed across the bilayer. The plasma membrane lipids are diversities in size and composition. The major lipids classes are glycerophospholipids, sphingolipids, and sterols.

- Glycerophospholipids consist of two fatty acid acyl chains ester-linked to glycerol-3-phosphate. Various substituents such as choline, ethanolamine, serine, myoinosytol, and glycerol can be linked to the phosphoryl group. Diphosphatidyl-glycerol or cardiolipin, is present in yeast cells. [18]

- Sphingolipids have a ceramide backbone which is composed of a long-chain base phytoshingosine that is N acylated with a hydroxyl C26 fatty acid. *S.cerevisiae* contains only three major sphingolipids: inositol-phosphate-ceramide, mannosyl-inositolphosphate-ceramide and mannosyl-diinositolphosphate-ceramide. [18]

- Sterols are compact rigid hydrophobic molecules with a polar hydroxyl group. The yeast plasma membrane contains mainly ergosterol (40%) and minor amounts of zymosterol. [18]

2. *Lipids compositions and principal role of lipids in the plasma membrane*

Phospholipids:

In the following table (Table 3) shows the lipids composition of the plasma membrane of *S.cerevisiae*.

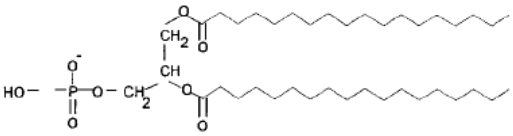
Table 3: Lipid composition of the plasma membrane of *S. cerevisiae* [16, 18, 19]

% Composition according to:		
Lipids	**Patton and Lester**	**Zinser et al.**
PC (phosphatidylcholine)	17,0	16,8
PE (phosphatidylethanolamine)	14,0	20,3
PI (phosphatidylinositol)	27,7	17,7
PS (phosphatidylserine)	3,8	33,6
Sphingolipids	30,7	
Others		6,9

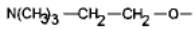
The inner leaflet of the S. cerevisiae plasma membrane is enriched in phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). The differences in PS and PI (table 3) content are most probably caused by differences in the strains, culture conditions, and/or extraction procedures used.

Here showed some of lipids found in *S.cerevisiae.*

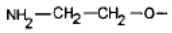
Phosphatidic acid



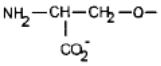
Phosphatidylcholine



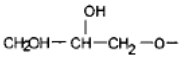
Phosphatidylethanolamine



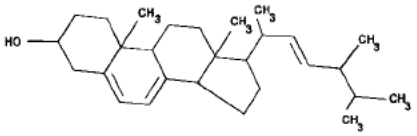
Phosphatidylserine



Phosphatidylglycerol



Ergosterol



Fatty Acyl Chains:

Oleic acid (18:1) and palmitoleic acid (16:1), together with small amounts of palmitic acid (16:0) and stearic acid (18:0), are the principal fatty acyl chains in *Saccharomyces cerevisiae*. The fatty acyl packing of these chains determines to a large extent the membrane fluidity. The fatty acyl packing increases with increasing length of the acyl chains and decreasing extent of unsaturation, which leads to a more ordered structure and decrease in fluidity. Perturbations of the bilayer that decrese the area of a lipid molecule, such as increased hydrostatic pressure, lowering of the temperature, or addition of sterols to phospholipids, also result in a decrease in fluidity. The physiological relevance of fluidity is evident from the adaptations of various yeasts to environmental stress. [20, 21, 22]

Table 4: Fatty acid composition of *S. cerevisiae* [20, 21]

Chain length and saturation	% of total fatty acids
10:0–14:1	7.0
16:0	12.8
16:1	32.3
18:0	8.0
18:1	28.0
18:3	1.4
20–24	8.0

Heads Groups:

The charge of the head groups affects the surface potential of the membrane and also influences in the activity of membrane proteins directly. The size of the head group determines to a large extent the physical state of the membrane. Lipids such as PC (phosphatidylcholine), PS (phosphatidylserine), PI (phosphatidylinositol), and sphingolipids, which have head groups and acyl chains are cylindrical and organize easily in bilayers. Lipids which have smaller head groups than acyl chaisns, such as PE, and sterols are cone shaped and form inverted micelles in solution. High concentration of such lipids in the membrane may locally induce a high membrane curvature and membrane-packing defect, which can create an environment into which proteins can insert without compromising the barrier function of the membrane. [22, 23, 24]

Sphingolipids:

Sphingolipids are ubiquitous constituents of eukaryotic plasma membranes. They are a class of lipids derived from the aliphatic amino alcohol sphingosine. They play important roles in signal transmission and cell recognition. More than 90% of the sphingolipids are located in the plasma membrane and constitute about 30% of the total phospholipids content. [16, 19]

Sterols:

Sterols determine to a large extent the rigidity of the plasma membrane, which, in turn may affect the lateral movement and the activity of membrane proteins. A further role for sterols can be found in cell proliferation, which requires the presence of specific sterols. The yeast plasma membrane contains mainly ergosterol around 40%. The synthetic of sterols is connected with aerobics conditions. [16, 18]

3. *Proteins content of the plasma membrane*

Cell membrane contains a large amount of proteins, they are responsible for various activities. The amount of proteins in a cell membrane is around 50% dry matter. Membrane proteins may be classified as peripheral, integral, and lipid anchored. [16]

Peripheral proteins are on the membrane surface. They are water-soluble, with mostly hydrophilic surfaces. These proteins tend to have only temporary interactions with biological membranes, and once reacted the molecule, dissociates to carry on it is work in the cytoplasm. [24]

Lipids anchor, covalently-bound to single or multiple lipid molecules, hydrophobically inserts into the cell membrane and anchor the protein. The protein itself is not in contact with the membrane. A protein may link to the cytosolic surface of the plasma membrane via a covalently attached fatty acid, for example plamitate or myristate, or it can be an isoprenoid group. [24]

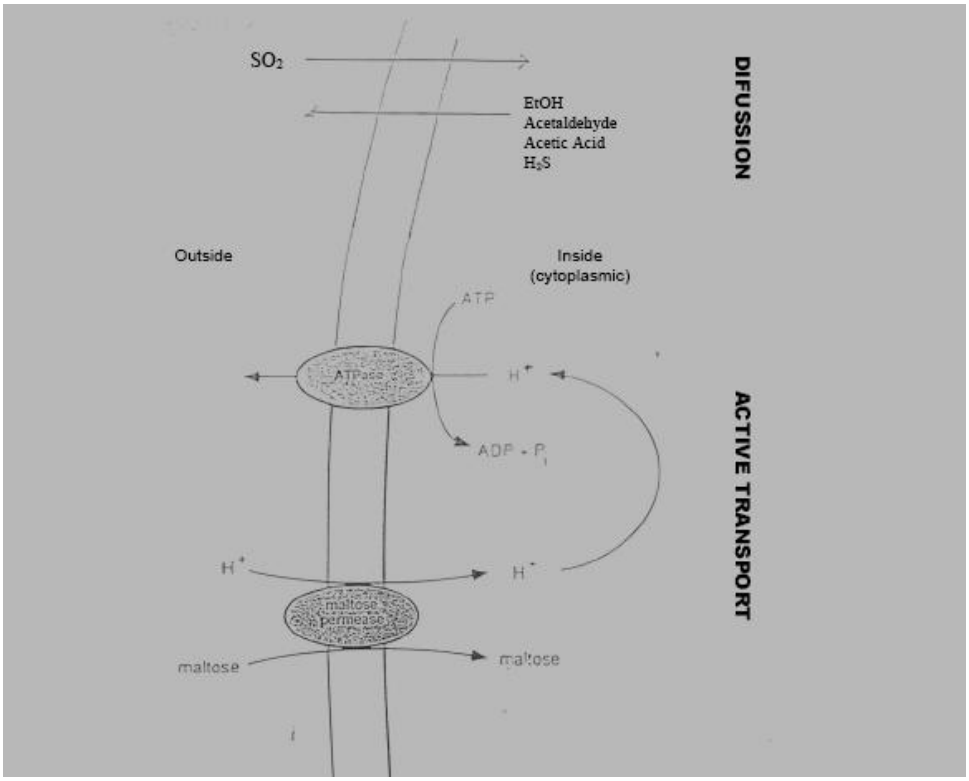
Integral proteins have domains that extend into the hydrocarbon core of the membrane. Often they span the bilayer. Intramembrane domains have largely hydrophobic surfaces that interact with membrane lipids. Generally, integral proteins fit into three categories: marker proteins, transport proteins, and receptor proteins. Marker proteins identify the cell to other cell. Each organism has its own unique marker proteins on its membranes. Transport proteins are responsible for transport and receiving. They move materials in and out of the cell and as well it allow to diffuse substances to diffuse through the membrane. Others act by pulling molecules across the membrane, a process called as active transport. The last one is receptor proteins extend through the cell membrane. They are the communication of the cell; they allow the cell to interact with other cells. The specificity of receptor proteins allow the cell to respond to the outside environment in many different ways.

These three classes of proteins are the real workers of the plasma membrane. They allow the membrane to be a dynamic structure that permits materials to be transported and messages to be communicated to the cell. [24]

4. Membrane Transports

Water, carbon dioxide, and oxygen are among the few simple molecules that can cross the cell membrane by diffusion (or a type of diffusion known as osmosis). Diffusion is one principle method of movement of substances within cells, as well as the method for essential small molecules to cross the cell membrane. As we can see on the figure 7, cells produce ethanol, acetaldehyde, acetic acid an H_2S and they are transported outside and cells get $S0^{2}$ from the medium. This transport is without energy. On the other hand, active transport requires the cells to spend energy usually in the form of ATP. This transport is carry out by enzyms, ATPase and maltose permease. [24]

Figure 7: Membrane transports



2.2.4. Cell wall:

Cell wall is a sturdy structure providing physical protection and osmotic support.. Electron microscopic analysis of the wall using negative staining reveals a layered structure with an *electron-transparent* internal layer of about 70-100 nm thick depending on growth conditions and genetic background, and an *electron-dense* outer layer. In brewing yeast the electron-transparent inner layer may be as thick a 200 nm. The mechanical strength of the wall is mainly due to the inner layer, which consists of β1,3-glucan and chitin, and represents about 50-60% of the wall dry matter. [25]

Table5: Cell wall macromolecules in *S. cerevisiae*:

Macromolecule	Wall dry weight (%)	Site of synthesis
Mannoproteins	35-40	Secretory pathaway
β1,6-Glucan	5-10	*(Plasma membrane)
β1,3-Glucan	50-55	Plasma membrane
Chitin	1-2	Plasma membrane

*The site of synthesis of β1,6-Glucan is uncertain. Those data presented here may vary depending on growth conditions.

The outer layer, which consists of heavily glycosylated mannoproteins emanating from the cell surface, is involved among others in cell-cell recognition events. It also limits the accessibility of the inner part of the wall and the plasma membrane to foreign enzymes. The carbohydrate side chains of the cell surface the proteins contain multiple phosphodiester bridges, resulting in numerous negative charges at the cell surface at physiological pH values. These side chains are responsible for the hydrophilic properties of the wall. The outer protein layer accounts for about a third percent of the wall dry weight.

The cell wall is highly elastic. When yeast cell are transferred to hypertonic solution, they rapidly shrink, and depending on the osmotic stress they may lose mor than 60% of their initial volume. This process is reversible. When transferred back to the original medium, the cells immediately expand to their initial volume. This elasticity of the wall is probably due to the elastic properties of the β1,6-Glucan chains. [25]

3. ETHANOL INFLUENCE ON YEAST

Ethanol is well known as an inhibitor of growth of microorganisms and theirs viability. It has been reported, that ethanol can damage mitochondrial DNA in yeast cells and can cause inactivation of some enzymes. Nevertheless, some strain of the yeast *Saccharomyces cerevisiae* show tolerance and can adapt to high concentrations of ethanol. *Saccharomyces cerevisiae* can tolerate more than 12% of ethanol in culture medium. Many studies have documented the alteration of cellular lipid composition in response to ethanol exposure. It has been found that *S.cerevisiae* cells grown in the presence of ethanol appear to increase the amount of mono-unsaturated fatty acids in cellular lipids. The primary target of ethanol stress is the plasma membrane. The predominant unsaturated fatty acids (UFAs) of *S.cerevisiae* are the mono- UFAs palmitoleic acid and oleic acid. [26]

The ethanol concentration also can inhibit the animoacids transport across membrane. The reasons of that can be different mechanisms, like membrane unpolarizetion or changes on membrane proteins transport.

When the cells are growing and they are exposure to ethanol the cells multiply decrease and if temperature is high also the inhibition effects enhance. The toxicity of ethanol becomes higher with increase of temperature. The ideal temperature for growth of *S.cerevisiae* is around 20ºC, when temperature is the highest; the ethanol effect on cells is the worst. The yeasts which are resistence to the ethanol, they will be tolerate also to the temperature. The ethanol affects the function and the stability of cytoplasmatic enzymes.

The yeasts have acetaldehyde inner the cells, in cytoplasmatic medium. That acetaldehyde is associates to ethanol tolerance. The yeast with low level of cytoplasmatic acetaldehyde content will be more tolerance to ethanol toxic effects. [27]

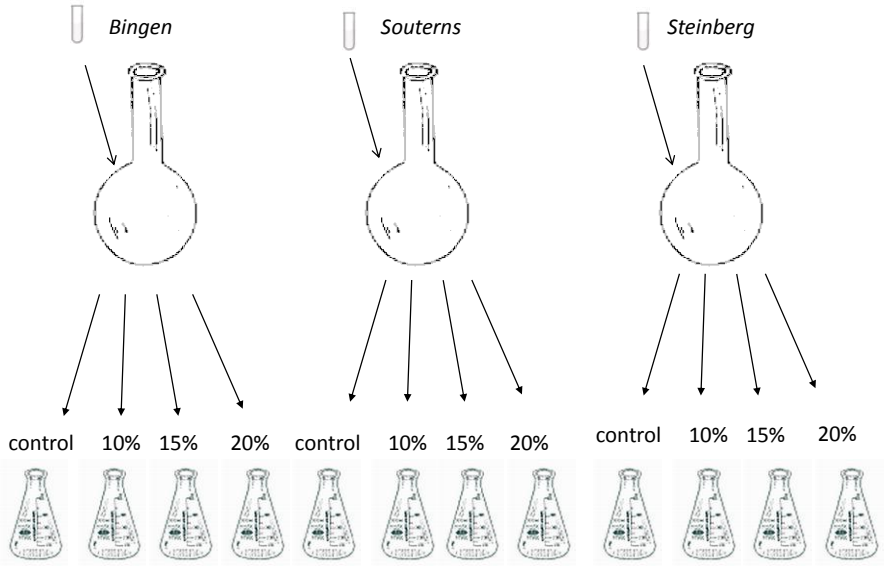
1. Yeast Strain

Saccharomyces cerevisiae strains *Bingen, Steinberg* and *Sauternes* from the culture collection of Department of Food Biotechnology and Microbiology of Warsaw Agricultural University (SGGW) were used for this study.

2. Culture medium

The strains were multiplied in an apple juice medium, containing apparent extract 10ºBrix on the shaker, which name is EDMUND BÜHLER, SH-30CONTROL. This juice was prepared from concentrated apple juice and water in volumetric flask and they were sterilized in autoclave during 15 minutes at 121ºC. After the sterilization, I put inside of each volumetric flask small amount of each strain from test-tube and 80 ml of apple juice. The time of incubation was two days, at 28ºC and at 200 rpm. By shaking, the mixture was oxygenated, for the better multiplication of cells. After two days of incubation, I put inside of each Erlenmeyer flask with ethanol and the control bottle 12 ml of yeast (Figure 8).

Figure 8: Scheme of proofs



3. Ethanol assay

The stress factor in the present work was ethanol. I have studied the effects of different concentrations of ethanol on the yeast. The concentrations were 10% (v/v), 15% (v/v) and 20% (v/v) of ethanol. For the preparation of ethanol I used physiological solution. The physiological solution I prepared in Erlenmeyer flask (Figure 8). Latter they were sterilized in autoclave during 20 minutes at 121ºC. On the table 6 I show the amount of yeast in an apple juice (ml), ethanol (ml) and NaCl (ml). In each Erlenmeyer flask was 60 ml of NaCl. For the preparation of ethanol I took out small amount of NaCl depending on the ethanol concentration of each sample. In control probe there wasn´t any ethanol inside and the volume of solution was 60 ml. However from the probes of 10%, 15% and 20% ethanol I took out 6,8 ml, 10,2 ml, 13,7 ml respectively of NaCl and I put into the Erlenmeyer flask the same amount, 6,8 ml, 10,2 ml, 13,7 ml respectively of ethanol. Altogether in each bottle of

Erlenmeyer was 60ml. Later I put in Erlenmeyer flask, 12ml of yeast in an apple juice. Time of incubation with stress factor was 28 days, at a room temperature.

Table 6: Amount of liquids in each Erlenmeyer flask

Proof	**(0,8%) NaCl (ml)**	**Strain and apple juice (ml)**	**(96%) Ethanol (ml)**
Control	60	12	0
10% (v/v)	53,2	12	6,8
15% (v/v)	49,8	12	10,2
20% (v/v)	46,3	12	13,7

4. Analysis of viability

Saccharomyces cerevisiae strains have been incubated in a different concentrations of ethanol during 28 days. Throughout those days I have studied the viability of cells using methylene blue staining method for identification of dead cells. I observed the cells under microscope using hemocytometer. Dead cells were in blue color and life cells in white. I counted blue cells and white cells from each Erlenmeyer flask, and afterwards I calculated the percentage of life cells. Through the process of incubation, I have noticed the development of life cells below ethanol influence during those days.

5. Analysis of morphology

Morphology has been studied in two intervals. The first one was at the beginning without ethanol stress condition, before incubation. The second one was after 28 days of incubation. In both cases I took photos using photo camera which name is SONY DSC-S7, connected to microscope. For the measure of the cells size I used the computer program LSM Image Browser Rel. 4.2. Then I made the statistical study with SSPS Statistic 17.0 program.

RESULTS

Through five weeks, I studied changes in viability of three strains of *Saccharomyces.cerevisiae* under ethanol stress. Before and after incubation I studied the morphology of the same strains. SPSS Statistics 17.0 program and LSM Image BrowserRel 4.2 I used for analyzed morphology results. Microsoft Office Excel 2007 I used for analyzed viability results. On the following pages, I will show the results of each strain with their own conclusions, tables and graphics.

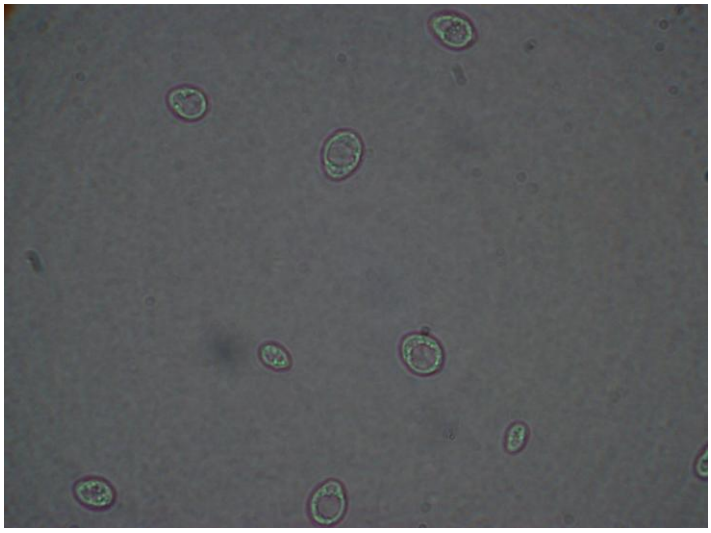
1- MORPHOLOGY

For the morphology study, I measured the width and length of hundred cells before and after incubation in different ethanol concentrations. On figures 9 and 10, I show the cells before incubation and after incubation in 20% of ethanol. I have chosen this concentration because 20% of ethanol is the worst conditions for yeast viability and I have compared with cells before incubation because it is the opposite situation, it is the best situation. With this comparison it can see better the morphology differences.

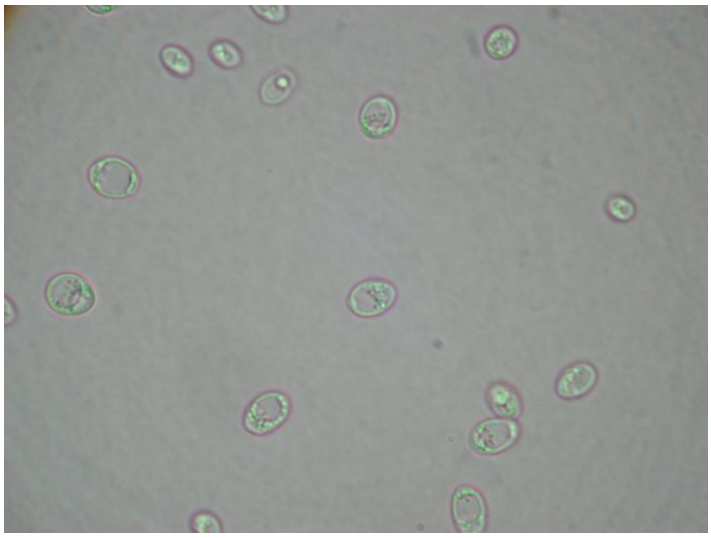
For all the cases, I have count one hundred cells for each sample and I have analyzed descriptive statistics variables. Nevertheless, for the three strains I will concentrate on *mean* (it describes the central location of the data) and *variance* (of a random variable is a measure of the amount of variation within the values of the variable) for results assess. Another useful parameter is the *Outlier*, is an observation that is numerically distant from the rest of the data, but they are often indicative either of measurement error.

Figure 9: The cells before incubation

BINGEN (x 1200)



SAUTERNES (x 1200)



STEINBERG (x1200)

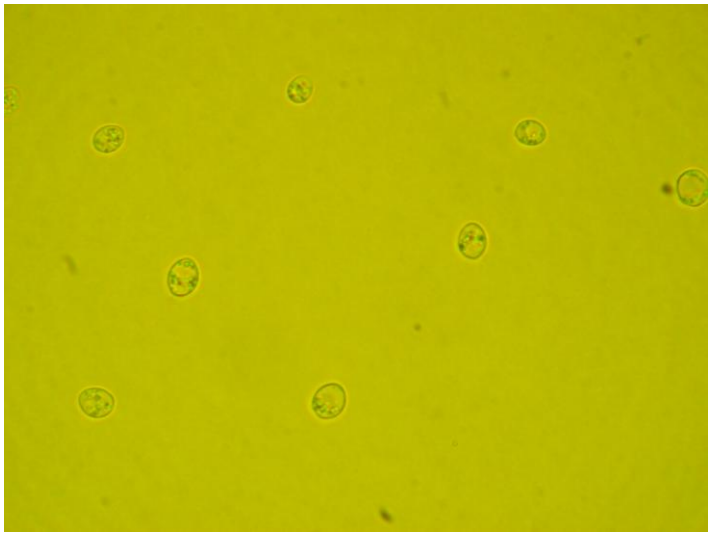
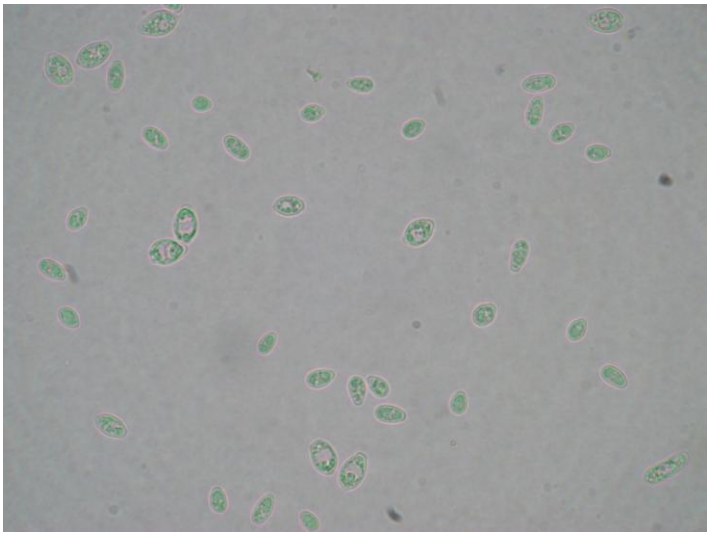
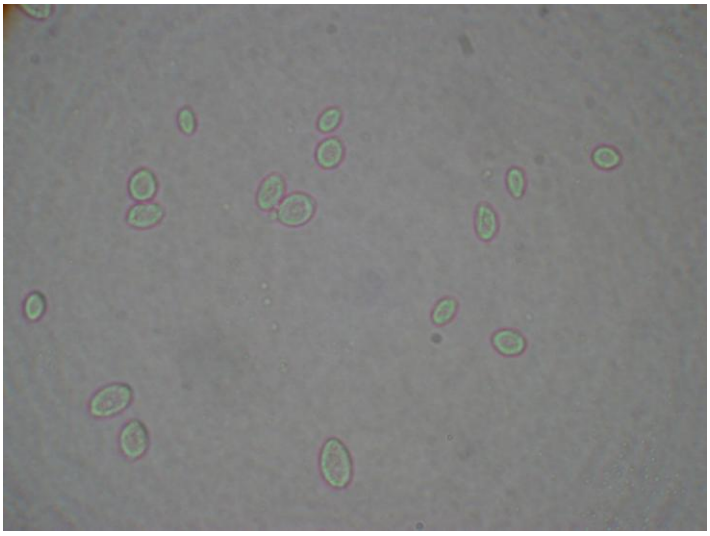


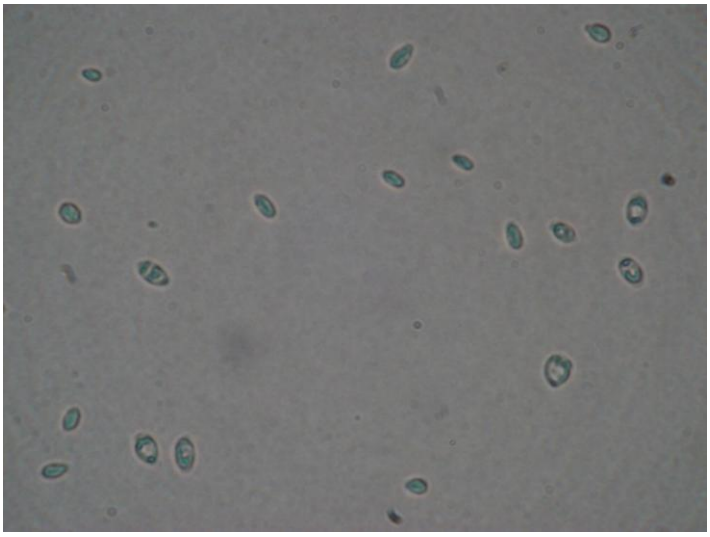
Figure 10: The cells after incubation (20% (v/v) of ethanol):



BINGEN (x 1200)



SAUTERNES (x 1200)



STEINBERG (x 1200)

BINGEN

Table 7: The **width** of *BINGEN* cells (µm).

Sample	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Before Incubation	100	2,4	6,4	4,0	1,06	1,13
Control	100	2,1	6,3	3,8	0,85	0,72
10%	100	2,3	6,4	3,8	0,80	0,64
15%	100	1,8	5,0	3,3	0,70	0,49
20%	100	1,3	3,9	2,6	0,57	0,32

Table 8: The **length** of *BINGEN* cells (µm).

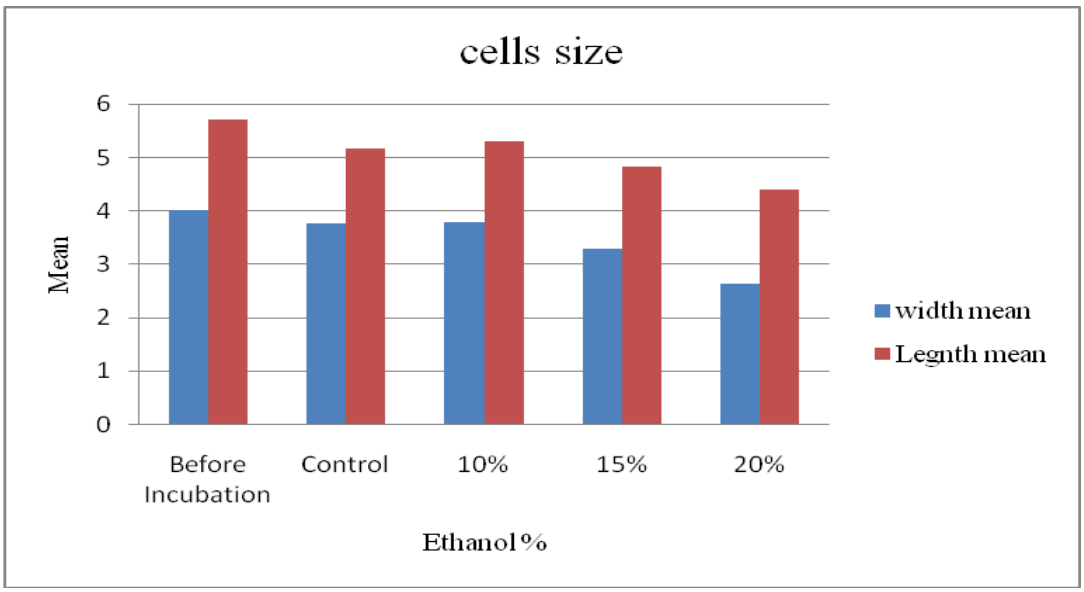
Sample	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Before Incubation	100	3,6	8,6	5,7	1,20	1,43
Control	100	3,0	10,3	5,2	1,27	1,61
10%	100	3,3	8,0	5,3	1,10	1,21
15%	100	3,0	6,8	4,8	0,91	0,83
20%	100	2,8	7,3	4,4	0,89	0,79

The tables 7 and 8 show width and length of *Bingen* cells. Before incubation, the cells were 4.0 µm width and 5.7 µm length. After incubation, the cells in Control and 10% of ethanol samples were similar 3,8 µm width, and 5,2-5,3 µm length. The mean width was almost similar between the control, 10% and before incubation probe, but the mean length decrease after incubation.

The width and length of cells were decreasing, as ethanol concentration was getting higher. The mean length of 15% and 20% of ethanol probes were 4,8 µm and 4,4 µm respectively, and the mean width of cells in 15% and 20% of ethanol probes were 3,3 µm and 2,6 µm. Therefore, that shows, there were cells size differences and the cells size decreased as ethanol concentration rose.

On the table 8, the length of *Bingen*, there is outlier data 10,3 µm, and I suppose is a measurement error.

Graph 1: The mean size of *Bingen* cells



The graph 1 shows the mean width and length of cells in all concentrations of ethanol. The blue color represents width measures and red-length measures. There was a decrease trends in mean width and in mean length from 10% of ethanol probe until 20% of ethanol during the five weeks. In control and 10% of ethanol probes the size cells was similar, but smaller than cells before incubation.

SAUTERNES

Table 9: The **width** of *SAUTERNES* cells (µm).

Sample	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Before Incubation	100	2,0	6,1	4,1	0,90	0,81
Control	100	1,9	8,0	4,3	1,15	1,31
10%	100	2,5	8,2	4,3	1,09	1,18
15%	100	2,3	6,2	3,4	0,61	0,37
20%	100	1,6	5,1	3,3	0,85	0,73

Table 10: The **length** of *SAUTERNES* cells (µm).

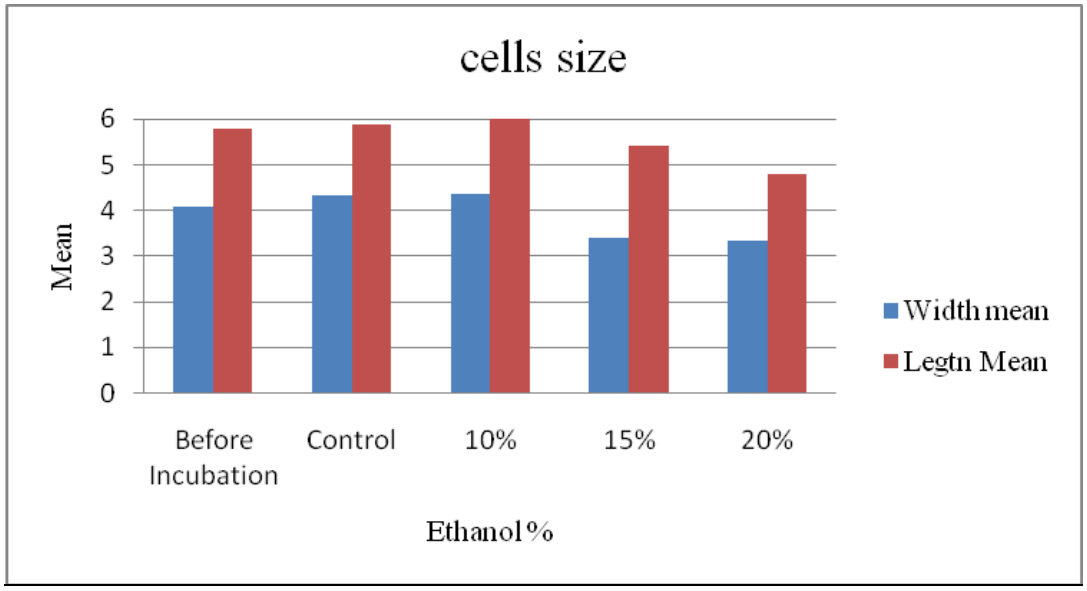
Sample	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Before Incubation	100	3,5	9,8	5,8	1,29	1,66
Control	100	3,1	9,6	5,9	1,24	1,54
10%	100	3,5	11,4	6,2	1,25	1,56
15%	100	2,5	9,9	5,4	1,33	1,77
20%	100	2,5	8,6	4,8	1,29	1,67

The tables 9 and 10 show width and length of *Sauternes* cells respectively.

The mean width of cells was the same in control and 10% of ethanol-4,3 µm. Before incubation, the mean width of cells was slight smaller 4,1 µm, than yeast after incubation in control and 10%ethanol-4,3 µm, but it wasn´t a marked differences. Therefore, the mean width of cells remained similar, before incubation and after incubation in control and in the smallest concentration of ethanol. As the table 9 shows, the width of cells in 15% and 20% of ethanol samples were similar-3,4 µm and 3,3 µm respectively and there is significant different respect to the control and 10% of ethanol samples. The mean width of cells decreases gradually, from 10% of ethanol until 20% of ethanol.

The table 10 shows the length of *Sauternes* cells. The cells before incubation and control sample, remained similar, 5,8 μm and 5,9 μm respectively. However, the yeast after incubation, in 10% of ethanol sample showed, a slight rise of the mean length of cells until 6,2 μm. Then, the cells in 15% and 20% of ethanol samples were 5,4 μm and 4,8 μm long respectively and they decreased respect to control, 10% of ethanol and before incubation samples. The increase of ethanol makes effects in *Sauternes* cells decreasing the mean width and length of cells.

Graph 2: The mean size of *Sauterns* cells



The graph 2 shows the changes of mean width and length of *Sauternes* cells in different ethanol concentration. As we can see the length trend, at the beginning, the cells before incubation were 5,8 µm long. Then yeast in control and 10% of ethanol raised slightly until 5,9 µm and 6,2 µm respectively. The cells before incubation should be bigger than cells after incubation in 10% of ethanol, but the differences are not too high.

The mean width of yeast remained steady in control and 10% of ethanol-4,3 µm. The mean width of cells before incubation-4,1 µm, was a slight smaller than control and 10% of ethanol. However, from 10% of ethanol until 15% of ethanol the trends of width yeast decreased from 4,3 µm to 3,4 µm respectively. At the end, after incubation in 20% of ethanol, the mean width of cells was 3,3 µm. It was almost similar to 15% of ethanol.

STEINBERG

Table 11: The **width** of *STEINBERG* cells (μm).

Sample	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Before Incubation	100	2,1	5,8	3,8	0,9	0,9
Control	100	1,7	5,0	3,0	0,7	0,5
10%	100	1,7	7,3	3,6	1,2	1,4
15%	100	1,7	5,4	3,5	1,0	0,9
20%	100	1,3	4,4	2,7	0,7	0,5

Table 12: The **length** of *STEINBERG* cells (μm).

Sample	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Before Incubation	100	2,4	8,1	5,2	0,8	0,6
Control	100	2,5	6,9	4,5	0,8	0,7
10%	100	2,7	8,2	5,2	1,4	1,9
15%	100	2,8	7,0	5,1	0,8	0,6
20%	100	2,3	5,7	4,1	0,7	0,5

The tables 11 and 12 show width and length of *Steinberg* cells respectively.

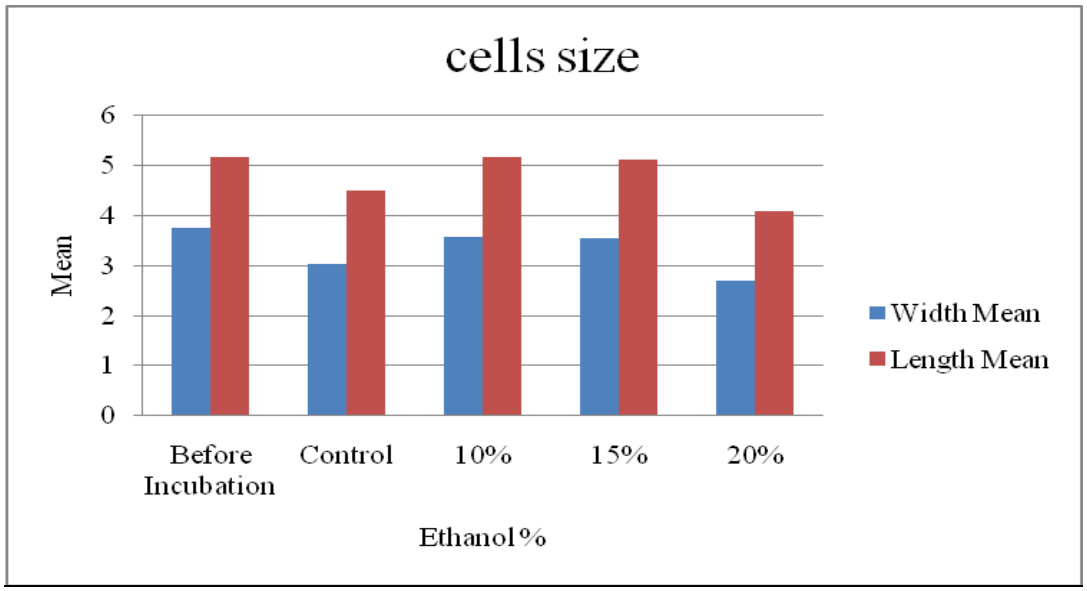
Both tables show, that the mean size of cells in control sample, was smaller than after incubation in 10% of ethanol and before incubation.

The table 11 shows the width of *Steinberg* cells. As I said before, the mean width of yeast from control sample was 3,0 μm, that was smaller in comparison with cells in 10% of ethanol and before incubation, 3,6 μm and 3,8 μm respectively. Then, from 10% ethanol until 20% ethanol the mean width decrease gradually.

The length of *Steinberg* cells (table 12) was in the same situation like in the table 11. The cells in control probe, 4,5 μm long, were smaller than cells after incubation in 10% ethanol-5,2 μm, and before incubation, 5,2 μm, samples. However, the long of *Steinberg* in 15% and 20% of ethanol

probes decrease slightly. Therefore, in both tables, the control sample can be a measurement error and it is not representative.

Graph 3: The mean size of *Steinberg* cells



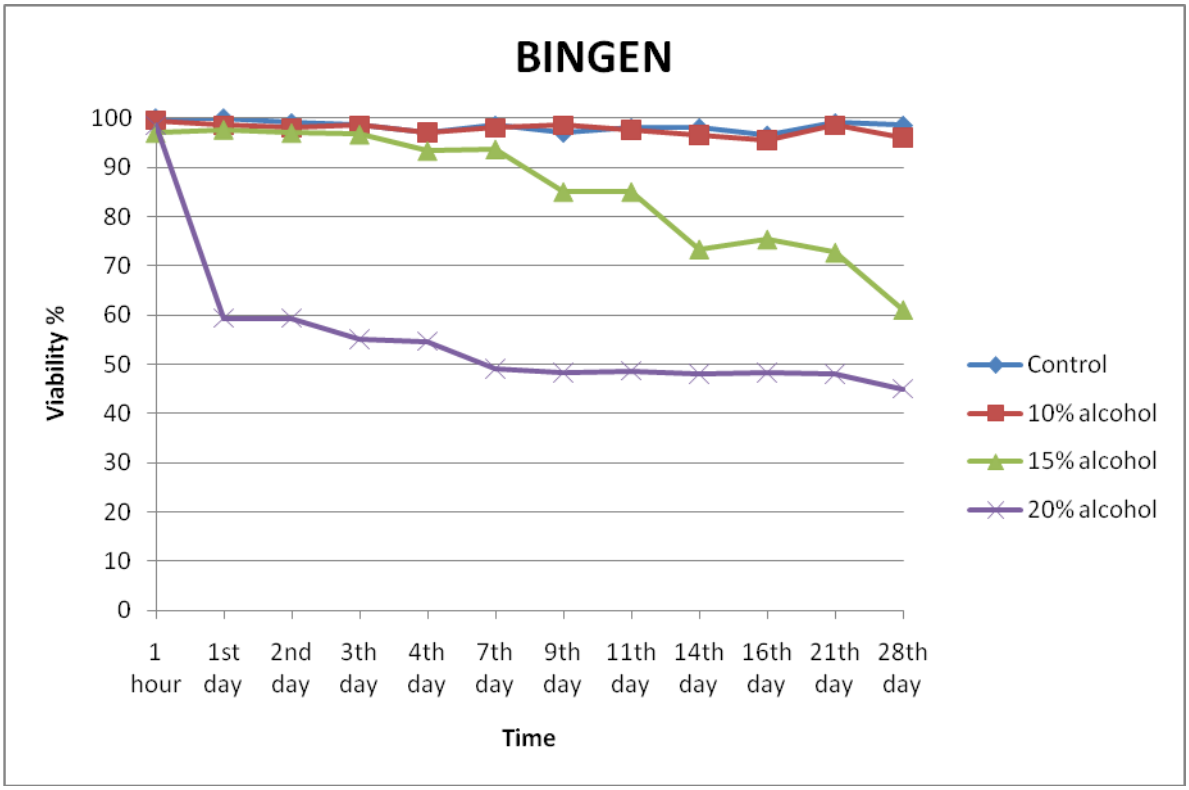
The graph 3 shows the mean width and length in connection with different ethanol concentration. The trend shows erratic way in both cases. The mean width and length of control yeast was smaller than cells after incubation in 10% of ethanol and before incubation. Nevertheless, from 10% of ethanol until 20% of ethanol the trend is decreasing gradually. Therefore, the control sample is not representative, but the rest of probes show the development of the size of *Steinberg* cells.

2- VIABILITY

For the viability I used Methylene Blue staining method for identify dead cells under microscope. In the following pages, I will show the results of each strain.

BINGEN

Graph 4: The viability of *Bingen* cells

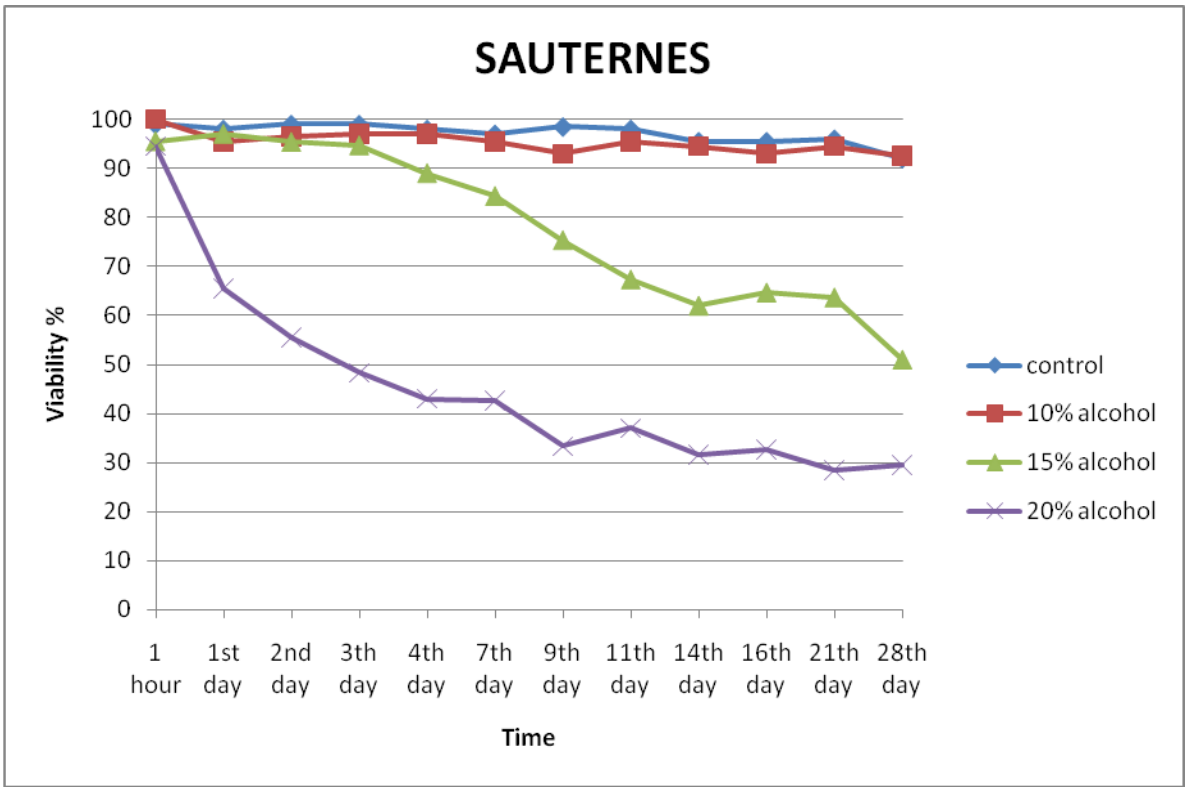


The graph 4 shows *Bingen* yeast changes of viability during 28 day of incubation in ethanol concentrations. After the first hour of incubation all the curves were at the same point. As the graph number 4 shows, the most characteristic was the decrease of viability in 20% ethanol after the first day of experiment. The percentage of viable cells was this time 60%. Then, the percentage of alive cells decreased slightly until 7th day, 50% of viability, and then remained on the similar level to the end of incubation, until 45% of viability.

The rest of samples, control, 10% and 15% of ethanol had similar trend until 7th day. Then, the 15%of ethanol curve decreased until the last day, but control and 10% of ethanol remained steady until 28th day.

SAUTERNES

Graph 5: The viability of *Sauternes* cells



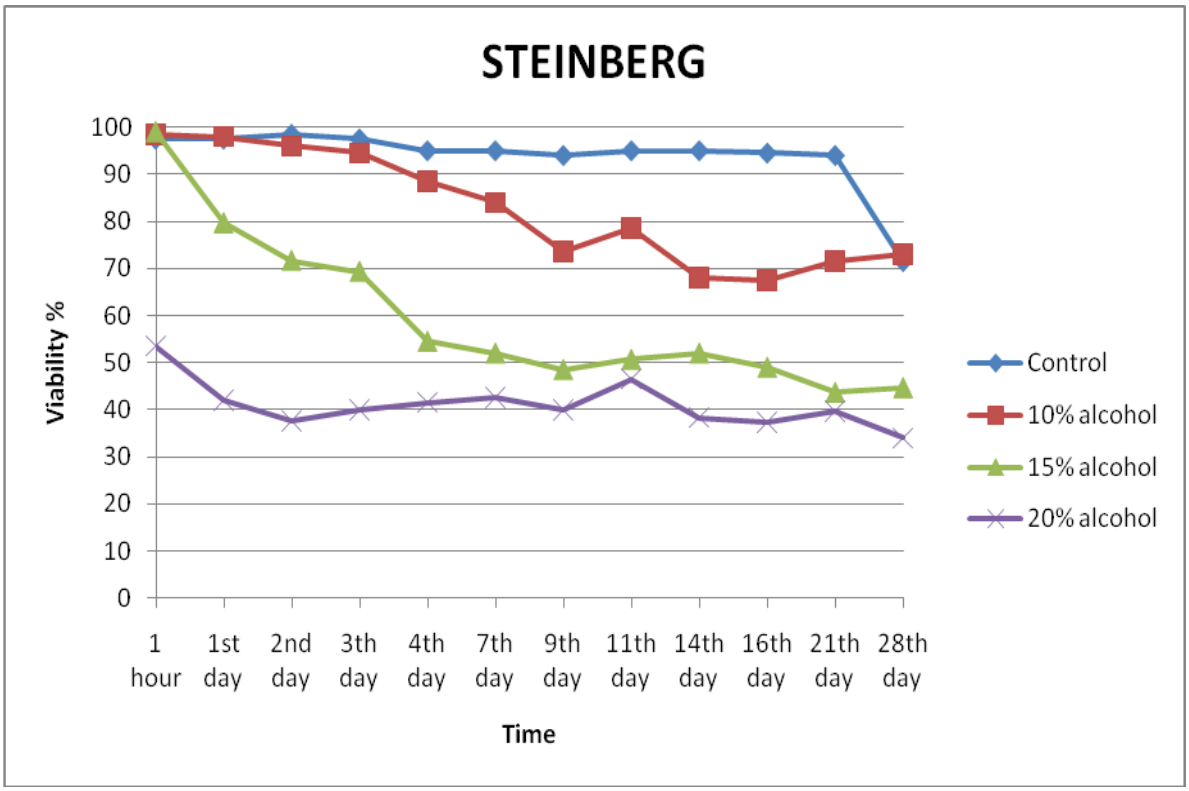
The graph 5 shows the influence of ethanol on the viability of *Sauternes* yeast during 28 days of incubation in ethanol. The most significant feature in this graph is the curve of viability in 20% ethanol. This curve was decreasing from the first day, with almost 100% of viability, until 9th day with 35% of viability. Then it showed slight erratic trend until last day.

The viability in 15% of ethanol curve remained steady from the first hour until 3th (94,7%) day, but then fell sharply until 14th day (60%). The end of this curve showed a strange trend, the 16th day showed a slight rise until 21th day but then declined until last day with 50% of viability.

The curves of viability in control and 10% of ethanol remained almost at similar level from the beginning until last day.

STEINBERG

Graph 6: The viability of *Steinberg* cells



The graph 6 shows the changes of *Steinberg* yeast viability during 28 day of incubation in different ethanol concentrations. The most significant feature in this graph is the curve of yeast viability in 20% ethanol. In the first hour of experiment, the viability in 20% of ethanol was at 55%. This curve showed erratic trend until the last day.

The curve of yeast viability in 15% of ethanol declined rapidly until 9th day (40%), and then, the trend remained steady until 28th day.

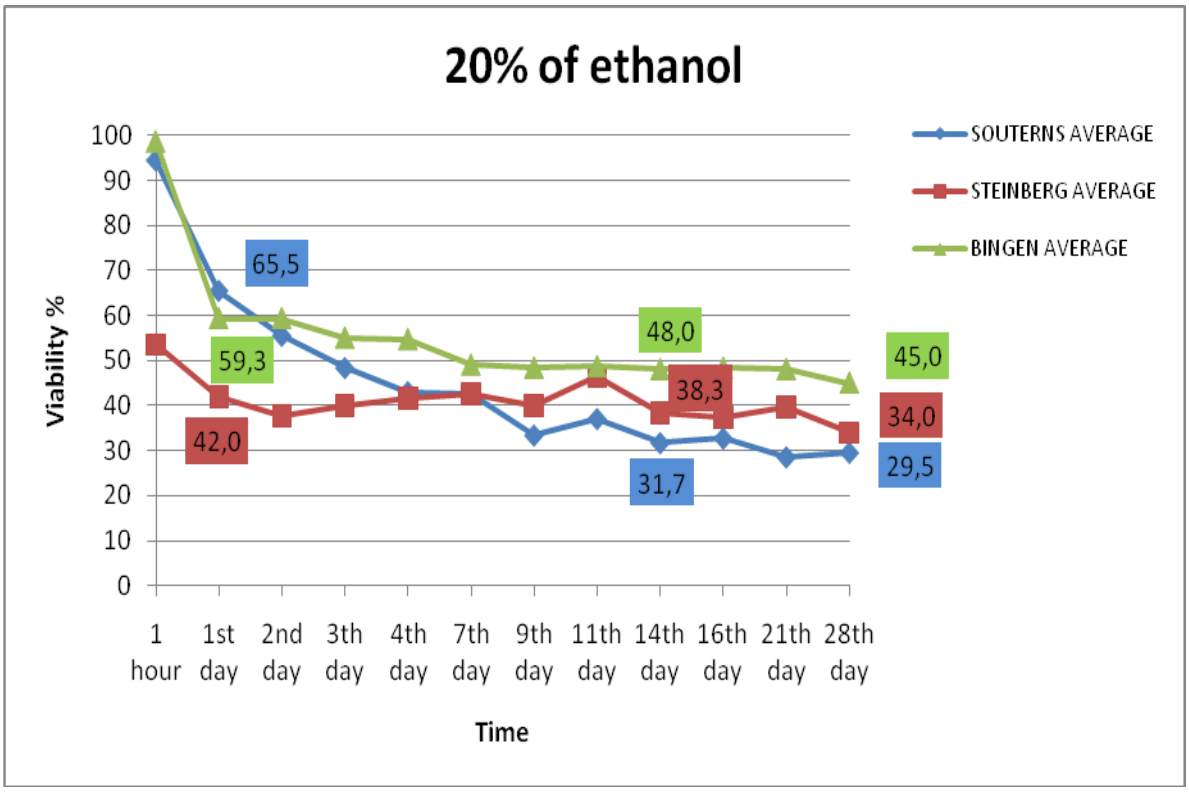
Another feature in this graph comparing with the graphs before, are the control and 10% of ethanol curves. On the graphs 4 and 5, those curves were in similar level during 28 days of incubation, but no in graph 6 (the *Steinberg* strain).

The curve of viability in 10% of ethanol showed a decreasing trend until 9th day, after that rose slightly but then remained in similar level until last day of experiment, when the percentage of viable cells was 73%.

On the other hand, the curve of viability in control probe graph 6 remained steady until 21th day, and the trend declined sharply through last week.

20% ETHANOL

Graph 7: The viability of each strain in 20% of etanol



The graph 7 shows the viability of each strain in 20% of ethanol during 28 days. As I said in previous pages, 20% of ethanol is the highest-level stress condition in this study. The biggest differences were at the beginning, after first hour of the proofs, *Steinberg* viability decreased until over 50% and the rest, *Sauternes* and *Bingen* had around 100% viability. Then, between the first hour and the first day the viability showed a sharp fell in *Sauternes* and *Bingen* cases. The viability of *Sauternes* and *Bingen* strains, after the first day fell down from 100% until 65,5 % and 59,3% respectively. *Steinberg* strain had a slight dip between first hour and first day.

Therefore, from the first day until the last day, the viability trend in all the strains remained steady, with almost the same values between 14th and 28th days.

Table 13: The viability of each strain in marked days in 20% of ethanol

20% OF ETHANOL			
STRAINS	1st DAY	14th DAY	28th DAY
Bingen	59 %	48 %	45 %
Steinberg	42 %	38 %	34 %
Sauternes	66 %	32 %	30 %

The table shows the intermediate days and results of yeast viability. In this way at first sight, I can show the changes of viability in 20% of ethanol.

Bingen was the most resistance strain to ethanol influence. At the last day the viability of *Bingen* was 45%. Nevertheless, at the beginning, *Steinberg* viability was 42% and it was lower than *Sauternes* 66%. However, after 14 day of incubation, the viability of *Steinberg*, 38% was higher than viability of *Sauternes*, 32%. From 14th day until the last day, the percentages of viability remain steady in all the strains.

CONCLUSIONS

BINGEN

In general, I can say that 10% and 15% of ethanol don´t have too much effect in this strain in a matter of cells size. However, 20% of ethanol has influence in cells size. After 28 days, *Bingen* was the most resistance strain of incubation in 20% of ethanol, the viability still just over 50%.

SAUTERNES

All concentration of ethanol don´t has too much influence on cells size on this strain. The 10% and 15% of ethanol don´t have too much influence on cells size. Nevertheless, the viability of this strain after 28 days of incubation in 20% of ethanol is rather low if we compare with *Bingen*. The viability of *Sauternes* is around 30%.

STEINBER

The influence of ethanol on morphology of this strain is significant. Therefore, the control sample is not representative, it can be a measurement error. The rest of probes show properly the changes of *Steinberg* cells size. In general, the cells were smaller after incubation in ethanol influence than before incubation.

In viability, the curve of 10% of ethanol was much lower than the rest of the strain. After 28 days of incubation in 20% of ethanol, the viability of *Steinberg* is around 35%, a little higher than *Sauternes* yeast viability.

Therefore, in general conclusions about morphologies results, *Bingen* and *Sauternes* after incubation in 20% of ethanol were smaller than before incubation. However, *Steinberg* morphology results it can be a measurement error in control sample, but shows properly the development of size cells below ethanol influence.

On the other hand, the viability of *Bingen* strain gets the best result, that means that is the most resistant and it can alive in those bad condition more than other strains studied. Nevertheless, *Sauternes* and *Steinberg* have approximately the same results in the worst conditions, but as well, they have significant differences in the curves of control and 10% of ethanol.

Then, *Bingen* strain if I compare with *Sauternes* and *Steinberg* strains can bear high level of ethanol, so in fermentation process with ethanol addition *Bingen* can be still alive better than *Sauternes* and *Steinberg* strains.

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IMAGENS:

Figure 1: http://www.bakeinfo.co.nz/school/school_info/types_of_bread.php

Figure 2: http://www.ethelqueraltlaboratory.com/home.php

Figure3: CARMEN DELEUZE JIMENEZ. By Autocad 2005 program.

Figure 4: http://biochemie.web.med.uni-muenchen.de/Yeast_Biol/02%20Yeast%20Cell%20Architecture%20and%20Function.pdf

Figure 5: Image from Purves et al., Life: The Science of Biology, 4th Edition, by Sinauer Associates (www.sinauer.com) and WH Freeman (www.whfreeman.com), used with permission.

Figure 6: http://mmbr.asm.org/cgi/reprint/59/2/304.pdf

Figure 7: http://www.utu.edu.uy/Escuelas/departamentos/canelones/vitivinicultura/Bioquimica%20Enologica/Teoricos/Tolerancia%20al%20etanol.pdf

ANNEXES I:

ANALYSIS OF MORPHOLOGY

TABLE I: BINGEN´S DATAS

Nº CELLS	BEFORE INCUVATION WIDTH	BEFORE INCUVATION LENGTH	CONTROL WIDTH	CONTROL LENGTH	10% (v/v) WIDTH	10% (v/v) LENGTH	15% (v/v) WIDTH	15% (v/v) LENGTH	20% (v/v) WIDTH	20% (v/v) LENGTH
1	4,79	6,21	3,92	5,44	4,32	4,87	3,05	6,12	3,65	5,65
2	2,81	4,57	4,14	5,65	4,05	6,25	2,6	3,57	3,49	5,01
3	5,99	6,84	3,17	5,07	4,69	5,77	2,29	3,73	3	4,24
4	3,23	5,85	3,99	5,74	3,9	6,23	3,78	5,39	3,29	4,86
5	6,09	7,62	4,36	5,7	3,75	5,29	3,12	4,76	3,05	3,97
6	3,16	5,62	3,32	4,43	3,03	4,16	3,6	5,3	2,2	3,1
7	4,14	6,28	4,69	7,41	3,77	4,92	3	3,28	3,66	4,87
8	5,55	7,08	5,12	5,83	3,59	4,71	2,65	4,91	2,05	2,79
9	4,21	6,13	3,1	6,89	5,34	7,73	2,97	4,19	2,18	3,92
10	5,34	7	4,92	8,45	3,33	5,75	4,15	6,79	2,08	3,67
11	2,89	4,82	3,81	4,79	4,86	7,25	3,71	5,3	1,95	4,48
12	5,6	7,5	3,62	4,31	3,12	5,44	4,09	5,59	2,4	3,92
13	3,17	4,95	3,24	4,62	4,03	5,12	2,6	3,79	2,39	3,7
14	5,44	7,72	4,53	4,95	4,25	4,72	3,4	5,86	1,94	3,86
15	4,82	6,48	3,37	4,68	3,48	4,25	3,55	5,6	3,08	4,73
16	5,16	7,08	6,31	7,19	3,25	4,7	3,01	4,23	2,87	6,01
17	5,29	6,12	4,92	5,82	4,92	6,58	3,44	4,55	3,44	5,8
18	5,12	5,77	4,96	10,3	3,4	4,97	4,03	5,66	2,51	4,1
19	5,01	6,51	4,99	6,47	3,48	6,32	3,94	5,77	2,63	3,94
20	3,07	3,7	4,14	5,14	4,14	6,1	3,81	5,8	2,65	4,17
21	2,68	6,08	3,48	3,7	3,23	5,94	2,78	3,82	3,28	5,07
22	5,87	5,87	3,56	4,23	4,78	7,28	2,93	3,9	2,9	5,68
23	3,99	4,83	4,39	4,71	5,66	8,01	3,71	4,8	2,7	3,7
24	3,11	4,1	5,44	6,31	3,46	5,19	4,17	5,26	3,05	5,62
25	2,96	4,48	4,17	5,86	4,87	5,8	2,92	4,72	2,18	3,49
26	3,86	5,82	3,55	5	4,58	6,56	4,03	4,97	3,47	5,63
27	3,16	4,48	3,73	4,5	3,51	4,29	2,7	4,23	3,31	4,97
28	3,1	4,59	3,16	5,01	4,66	6,92	4	5,38	3,49	4,3
29	5,64	7,61	5,88	7,12	6,43	7,09	3,71	6,34	3,47	5,63
30	3,37	5,01	5,55	5,99	6,01	6,45	3,48	5,31	3,59	3,81
31	6,38	7,4	4,03	5,01	3,32	4,68	3,39	4,85	3,16	6,78
32	3,27	4,44	3,7	4,9	3,9	4,79	3,7	5,66	3,53	4,5
33	3,7	4,79	4,58	6,15	5,33	7,73	3,86	5,22	2,47	6,05
34	5,7	6,04	2,97	4,35	4,41	5,58	4,63	5,79	3,55	4,77
35	6,25	6,25	3,41	4,41	3,71	4,68	3,86	5,17	1,96	4,03
36	6,13	7,53	5,41	6,31	3,55	4,27	3,34	4,55	2,85	4,05
37	4,25	5,31	4,58	6,1	3,53	3,85	4,08	6,78	3,93	5,37

38	4,05	5,16	3,77	5,8	5,02	6,02	3,53	5,1	3,13	4,82
39	3,7	5,1	3,1	4,1	3,28	3,85	2,59	4,65	3,21	5,62
40	2,69	4,78	4,14	5,77	4,81	7,13	3,86	5,2	2,69	4,41
Nº CELLS	**BEFORE INCUVATION WIDTH**	**BEFORE INCUVATION LENGTH**	**CONTROL WIDTH**	**CONTROL LENGTH**	**10% (v/v) WIDTH**	**10% (v/v) LENGTH**	**15% (v/v) WIDTH**	**15% (v/v) LENGTH**	**20% (v/v) WIDTH**	**20% (v/v) LENGTH**
41	3,77	6,45	3,59	5,34	3,7	4,39	3,72	5,2	3,64	4,97
42	6,25	7,14	3,85	4,56	3,33	5,26	4,11	5,03	2,36	4,19
43	5,66	6,86	4,14	5,11	4,94	6,39	4,39	5,78	2,31	3,7
44	5,23	5,99	3,57	4,15	3,85	5,77	3,93	6,5	2,51	4,03
45	3	5,14	4,31	7,53	4,73	6,64	3,72	5,8	2,18	3,59
46	4,94	6,3	5,88	6,64	4,23	6,23	2,73	4,82	2,27	2,92
47	3,19	4,17	3,66	5,02	4,47	7,74	3,7	5,62	3,05	6,1
48	3,55	6	5,27	7,85	4,47	5,62	4,69	5,67	3,1	6
49	4,9	6,53	4,59	6,96	3,35	4,62	4,35	5,23	2,4	5,75
50	2,97	5,1	3,48	3,27	4,49	6,23	3,62	5,81	2,38	4,01
51	2,48	4,39	3,37	4,14	2,27	5,2	4,1	4,97	2,88	4,44
52	4,32	6,6	3,32	4	4,16	5,02	3,16	5,99	2,03	4,55
53	4,39	5,19	3,75	4,82	3,31	4,55	3,49	5,72	2,62	3,81
54	3,12	6,78	2,73	4,15	2,75	5,19	4,25	4,68	3,02	4,19
55	3,48	3,6	2,97	4,21	3,27	4,03	3,08	4,87	1,46	3,22
56	3,24	5,15	3,05	3,48	2,94	4,35	2,75	4,79	2,42	4,53
57	3,6	5,52	3,01	3,51	3,32	5,98	2,51	3,81	2,11	3,32
58	4,23	7,24	3,05	3,72	2,72	5,12	3,55	4,68	2,45	3,86
59	5	7,24	3,21	3,94	3,1	4,85	2,75	4,51	1,32	4,05
60	4,17	8,58	3,46	4,04	2,33	4,92	2,01	3,31	2,46	5,93
61	3,07	4,12	4,17	6,49	2,93	6,63	2,7	4,14	2,03	3,33
62	2,5	4,5	2,69	3	3,42	7,08	3,92	5,66	2,79	3,9
63	4,46	5,86	3,27	4,25	3,66	5,8	3,54	4,99	2,96	4,62
64	3,92	5,93	4,25	5,34	4,24	4,63	4,96	6,52	1,93	3,12
65	2,9	4,12	3,27	5,79	3,8	5,2	3,39	4,4	2,81	5,31
66	3,94	7,21	2,69	3,87	3,72	4,8	2,38	4,4	1,96	3,8
67	3,42	4,99	2,83	3,6	2,6	4,09	2,79	4,16	3,53	5,02
68	3,28	4,89	3,59	5,34	4,26	6,14	3,78	5,29	1,96	3,59
69	3,96	5,13	4,59	6,06	3,02	5,23	2,93	3,75	2,2	3,16
70	2,93	4,03	2,96	4,05	2,55	3,57	2,38	3,41	2,72	4,89
71	5,85	7,21	3,99	6,25	3,83	5,79	4,03	5,55	2,39	4,65
72	4,82	6,04	2,05	4,82	3,93	5,41	2,09	3,13	2,38	3,83
73	4,57	6,98	3,12	4,73	3,72	5,25	3	4,15	2,87	4,1
74	2,78	5,07	2,57	3,87	2,72	3,96	4	5,86	2,01	3,93
75	4,38	6,82	2,29	3,46	3,49	5,39	3,44	5,41	2,79	3,9
76	3,78	6,12	2,79	3,72	3,42	4,73	2,45	3,72	1,46	3,71
77	2,7	5	3,03	4,55	3,22	3,86	2,93	4,13	2,81	4,64
78	3,02	5,34	4,2	6,74	3,35	4,02	2,1	4,31	2,7	3,93

79	3,24	3,63	4,2	5,94	4,21	3,26	3,29	5,28	2,14	3,63
80	3,72	5,84	4,59	5,76	5,25	4,05	2,3	3,27	2,24	4,87
81	3,32	4,89	3,16	4,08	3,27	5,66	3,92	4,68	3,31	5,85
82	3,62	4,15	2,73	3,8	3,48	5,23	3,81	4,96	3,51	4,89
№ CELLS	**BEFORE INCUVATION WIDTH**	**BEFORE INCUVATION LENGTH**	**CONTROL WIDTH**	**CONTROL LENGTH**	**10% (v/v) WIDTH**	**10% (v/v) LENGTH**	**15% (v/v) WIDTH**	**15% (v/v) LENGTH**	**20% (v/v) WIDTH**	**20% (v/v) LENGTH**
83	3,17	4,15	3,02	4,69	2,93	3,55	3,62	5,53	2,78	4,77
84	2,72	4,62	3,73	5,26	3,82	5,96	2,05	3,28	2,23	7,28
85	3,57	5,38	2,83	4,7	3,59	6,64	3,16	4,71	2,19	4,37
86	4,23	7,38	3,05	5,23	3,94	5,23	2,29	3,17	2,29	3,42
87	2,93	4,61	2,84	4,2	3,93	4	3,81	5,44	2,29	4,26
88	4,8	7,24	3,04	3,6	3,31	4,49	2,61	3,05	2,31	3,31
89	4,65	6,89	3,91	7,09	3,65	4,98	3,05	5,54	2,48	3,96
90	3,09	4,26	3,16	4,81	4,86	7,42	1,79	3,31	1,76	3,57
91	2,68	4,24	2,76	4,01	3,52	3,93	2,7	4,62	2,47	4,02
92	3,86	7,84	4,33	6,82	3,02	3,89	3,27	3,81	2,39	3,27
93	2,75	4,23	4,04	5,07	3,86	4,17	2,4	3,88	2,62	3,88
94	3,66	4,02	3,11	3,77	3,12	4,87	3,78	5,65	2,18	4,49
95	4,4	7,4	4,34	6,13	3,9	4,77	2,14	4,1	3,39	4,86
96	4,69	6,75	2,94	3,92	2,72	4,42	2,31	3,02	2,3	3,7
97	4,36	7,2	3,32	3,66	3,16	4,17	4,12	5,17	2,01	3,77
98	2,4	4,03	3,16	6,1	2,99	4,23	3,76	5,36	2,33	4,12
99	2,93	4,73	4,87	6,47	2,72	4,57	2,14	3,23	1,96	3,23
100	4,63	5,38	3,77	5,7	3,09	3,96	2,31	4,17	2,67	4,76

TABLE II: SAUTERNSE´S DATAS

Nº CELLS	BEFORE INCUVATION WIDTH	BEFORE INCUVATION LENGTH	CONTROL WIDTH	CONTROL LENGTH	10% (v/v) WIDTH	10% (v/v) LENGTH	15% (v/v) WIDTH	15% (v/v) LENGTH	20% (v/v) WIDTH	20% (v/v) LENGTH
1	4,53	7,2	5,81	7,33	5,26	8,21	2,94	6,79	3,93	4,08
2	4,42	5,73	3,68	5,84	5,16	6,61	3,27	5,16	3,7	3,81
3	4,2	6,25	6,28	7,84	4,77	6,05	3,35	5,8	3,59	3,6
4	2,88	3,9	5,67	7,55	4,31	5,16	3,8	4,97	4,08	4,85
5	5,07	7,13	3,39	4,81	5,12	6,1	3,44	6,59	3,66	4,95
6	5,1	7,39	7,01	6,17	6,17	6,72	3,48	4,38	3,38	4,93
7	5,55	7,58	3,76	5,76	5,8	7,25	3,7	4,82	2,33	3,03
8	5,44	7,37	4,45	5,91	4,03	4,9	2,85	4,7	3,14	4,55
9	3,16	5	4,88	6,72	5,86	5,86	3,16	3,59	1,8	3,99
10	3,46	4,17	3,96	5,96	5,36	6,75	3,58	5,75	2,54	4,09
11	3,77	6,24	5,33	7,21	4,87	5,9	4,69	5,79	2,03	3,42
12	5,01	6,61	6,89	5,38	5,39	6,89	2,78	4,42	4,2	5,07
13	3,96	4,86	5,09	6,63	3,99	8,06	3,49	7,29	2,33	2,72
14	3,37	4,57	5,09	4,98	6,07	7,73	4,01	5,86	2,27	3,51
15	5,27	7,19	5,01	7,17	3,51	4,59	3,7	3,81	3,35	4,83
16	5,24	8,16	5,62	6,5	4,1	5,26	2,84	6,35	1,63	2,53
17	4,14	5,15	5	6,04	5,62	7,73	3,85	6,04	2,24	4,23
18	5,89	8,13	3,92	5,88	4,35	4,59	3,59	5,55	2,76	3,2
19	4,53	6,07	4,39	6,94	5,47	7,24	3,38	5,41	2,51	3,92
20	4,2	4,95	5,29	5,62	5,57	6,98	2,73	4,76	3,51	4,43
21	4,25	4,36	5,08	7,25	5,28	8,87	2,93	4,16	3,35	4,78
22	4,92	6,47	5,09	5,97	5,16	7,04	3,38	5,33	3,37	4,57
23	3,33	4,23	5,64	7,16	5,14	5,78	2,73	4,9	1,9	2,45
24	5,36	5,48	5,32	7,07	4,83	5,93	3,16	4,7	4,53	5,32
25	5,03	7,61	5,09	5,82	5,17	7,4	2,99	3,99	4,14	4,77
26	5,41	7,08	4,98	7,12	4,06	6,71	3,2	6,64	1,72	3,83
27	3,66	6,54	6,44	7,92	5,93	6,64	3,51	4,44	3,1	5,3
28	3,18	4,93	3,77	4,77	6,45	7,26	3,54	4,24	2,48	2,75
29	4,83	7,47	5,42	6,19	4,64	7,1	3,07	3,96	4,01	5,44
30	3,57	4,71	6,09	8,02	8,17	11,42	3,91	4,62	3,92	4,03
31	5,55	6,42	3,98	4,19	6,1	8,53	4,15	5,12	3,51	4,63
32	3,62	5,11	7,19	7,19	5,23	6,1	3	3,77	2,16	2,62
33	3,27	5,33	4,3	5,24	3,65	5,72	2,31	3,88	3,85	4,71
34	3,03	4,99	5,89	7,08	4,76	6,92	3,23	5,14	3,59	3,81
35	5,39	7,31	4,51	5,53	4,29	4,73	2,96	4,5	4,25	4,87
36	4,9	5,12	3,92	5,04	4,99	6,41	2,72	5,02	3,99	5,32
37	3,41	4,73	5,35	8,02	3,68	5,34	2,47	2,54	4,51	3,79
38	4,17	5,86	4,3	5,95	3,38	5,14	3,38	4,71	2,47	3,16
39	5,12	5,13	4,21	5,05	7,4	7,4	3,6	5,76	3,57	4,74

40	6,14	6,91	3,93	6,78	4,63	6,98	3,2	5,2	2,43	4,1
Nº CELLS	BEFORE INCUVATION WIDTH	BEFORE INCUVATION LENGTH	CONTROL WIDTH	CONTROL LENGTH	10% (v/v) WIDTH	10% (v/v) LENGTH	15% (v/v) WIDTH	15% (v/v) LENGTH	20% (v/v) WIDTH	20% (v/v) LENGTH
41	2,55	4,09	3,39	5,84	6,04	7,9	3,46	6,12	2,87	4,39
42	3,57	4,38	3,64	4,85	6,01	9,4	3,38	6,75	2,4	3,22
43	4,52	6,94	5,32	7,17	3,7	5,05	3,82	6,21	4,2	6,09
44	4,79	6,99	4,06	5,2	5,83	6,87	3,32	6,35	4,39	4,41
45	5,36	6	3,78	4,57	6,32	7,42	3,05	3,92	2,62	3,1
46	5,66	7,16	4,33	5,06	3,81	4,9	3,02	5,73	2,73	4,63
47	5,23	5,01	5,77	7,81	4,76	7,55	2,87	5,44	2	3,47
48	3,7	5,23	7,97	9,61	3,55	6,22	2,87	4,53	2,2	3,55
49	3,55	4,97	5,31	5,31	3,05	5,22	3,54	4,16	3,27	6,2
50	3,7	6,21	3,87	5,6	3,23	5,12	2,48	4,93	4,62	6,04
51	2,83	5,16	4,04	6,09	2,54	4,31	4,15	7,45	4	8,17
52	5,78	4,31	4,44	6,45	4,14	4,93	3,49	4,14	3,03	4
53	3,49	5,77	4,38	6,18	4,7	6,31	3,12	4,54	2,61	5,44
54	3,89	6,48	2,69	3,14	4,27	5,84	4,68	5,44	2,29	4,14
55	3,2	4,69	2,94	4,46	3,62	5,3	3,42	7,93	3,92	6,1
56	3,48	4,39	2,5	4,19	3,07	5,16	4,25	5,12	4,47	7,16
57	3,51	5,45	3,42	4,63	4,25	6,39	3,82	4,14	3,75	4,89
58	1,95	5,86	4,1	7,92	3,31	5,12	3,59	4,73	2,53	4,33
59	3,27	3,48	5,23	5,12	3,22	5,28	3,29	7,91	4,08	5,26
60	4,35	5,16	5,28	6,47	3,59	4,46	3,92	4,79	2,78	4,82
61	3,93	5,26	3,46	5,66	2,83	4,39	3,93	7,89	3,85	5,93
62	3,27	3,65	3,6	7,41	4,55	6,29	2,61	4,25	4,86	5,86
63	2,59	5,31	3,14	4,44	2,99	4,89	3,44	7,28	4,03	4,79
64	2,63	4,53	3,12	3,93	4,23	6,62	2,33	4,63	3,54	4,81
65	3,65	5,67	4,26	7,47	3,44	5,03	3,85	8,25	3,27	4,46
66	4,24	6,31	4,44	6,72	2,55	6,01	3,7	6,21	3,72	4,17
67	3,7	6,08	1,85	5,23	4,99	6,23	4,14	6,01	4,18	5,66
68	4,14	3,6	4,15	6,19	4,31	5,1	3,75	8,8	2,2	3,41
69	3,32	4,57	4,63	6,43	3,47	7,55	2,31	4,1	4,24	6,39
70	4,39	5,41	3,87	5,89	3,39	4,95	2,97	7,12	4,08	5,78
71	3,37	6,01	2,4	3,93	4,25	6,32	3,83	8,24	2,58	4,23
72	3,16	7,72	2,78	3,7	3,85	6,39	3,7	4,3	2,19	3,18
73	2,84	7,68	3,17	5,78	3,99	5,62	3,05	4,4	3,05	4,29
74	4,56	9,78	3,72	4,35	3,87	6,37	2,31	4,01	3,07	5,41
75	4,55	6,54	4,71	6,04	3,75	6,35	3,34	5,1	2,48	4,14
76	3,75	4,57	4,26	5,8	3,81	6,56	4,34	5,75	2,2	4,18
77	5,47	6,11	2,69	4,15	4,15	5,18	3,84	7,14	2,97	4,86
78	3,86	4,35	2,78	3,49	3,57	5,42	2,27	3,78	2,85	3,77
79	4,03	6,78	2,07	4,42	3,38	6,43	3,16	6,32	4,1	7,79
80	4,84	4,94	4,32	5,77	3,14	5,51	4,36	4,68	3,32	5,55

81	4,03	6,16	3,27	4,1	3,68	7,4	3,79	7,36	2,36	4,29
82	4,28	7,97	4,25	6,11	5,12	7,16	3,22	4,19	3,16	3,85
Nº CELLS	**BEFORE INCUVATION WIDTH**	**BEFORE INCUVATION LENGTH**	**CONTROL WIDTH**	**CONTROL LENGTH**	**10% (v/v) WIDTH**	**10% (v/v) LENGTH**	**15% (v/v) WIDTH**	**15% (v/v) LENGTH**	**20% (v/v) WIDTH**	**20% (v/v) LENGTH**
83	3,94	4,02	4,31	5,87	2,96	6,8	3,34	5,1	2,95	4,33
84	4,8	6,46	3,79	5,31	4	5,79	4,57	4,37	3,57	7,12
85	4,03	6,79	3,55	4,89	3,77	4,88	3,54	9,85	4,17	7,99
86	2,66	7,01	4,36	4,9	3,56	5,8	6,21	6,21	4,99	7,64
87	3,9	6,67	2,81	4,43	3,57	4	3,81	4,35	3,32	5,75
88	4,25	5,24	4,9	6,86	3,85	6,1	4,03	4,25	4,34	5,94
89	3,33	5,65	2,62	5,73	3,41	5,34	2,73	4,76	3,29	5,12
90	3,81	8,06	2,83	4,58	4,52	7,13	3,16	7,07	4,53	6,04
91	3,16	3,96	4,15	7,45	4,28	6,32	3,79	6,19	2,62	4,9
92	4,73	4,69	5,02	7,52	2,87	5,75	2,99	3,12	2,78	5,56
93	4,34	4,78	2,73	4,68	3,82	6,05	3,47	6,93	4,59	8,64
94	2,89	6,66	3,99	7,16	4,14	5,32	4,06	6,01	4,31	6,77
95	3,61	3,59	4,25	5,34	4,07	7,93	2,73	4,28	4,68	6,38
96	2,73	5,62	3,8	4,57	3,29	4,2	3,32	6,16	4,33	6,53
97	4,05	4,48	4,58	7,85	2,69	4,53	3,94	5,41	3,99	6
98	2,99	8,58	2,92	3,9	4	6,43	3,16	6,53	3,22	4,89
99	4,23	4,93	4,12	5,89	3,07	6,39	2,93	4,82	5,07	6,77
100	3,05	5,51	3,72	5,84	2,79	3,46	3,84	5,36	2,9	3,66

TABLE II: STEINBERG´S DATAS

Nº CELLS	BEFORE INCUVATION WIDTH	BEFORE INCUVATION LENGTH	CONTROL WIDTH	CONTROL LENGTH	10% (v/v) WIDTH	10% (v/v) LENGTH	15% (v/v) WIDTH	15% (v/v) LENGTH	20% (v/v) WIDTH	20% (v/v) LENGTH
1	3,55	4,92	3,51	5,28	3,5	4,83	2,62	2,83	2,84	4,05
2	5,01	5,99	3,51	3,74	6,63	6,66	5,41	6,68	3,16	3,9
3	3,7	5,22	5,01	5,78	5,56	6,79	2,83	4,14	2,62	4,27
4	3,16	4,42	2,7	3,55	4,7	6,77	4,24	5,47	2,78	3,86
5	3,16	4,89	4,17	5,65	5,36	6,28	3,03	4,83	3,27	3,77
6	2,83	4,9	3,47	3,47	5,89	6,45	3,07	5,44	3,47	3,85
7	3,16	5,45	2,4	2,5	5,16	6,13	3,46	4,81	2,63	3,2
8	3,17	4,92	3,61	4,85	2,55	6,15	2,79	4,53	4,23	5,51
9	2,72	4,88	1,95	4,17	5,07	7,34	4,24	5,31	3,55	3,58
10	3,81	5,34	2,54	4,57	3,85	6,6	4,53	5,14	1,77	4,2
11	2,75	4,31	2,24	3,93	5,65	7,52	4,51	6,89	3,55	4,95
12	2,08	4,39	2,34	3,32	4,54	7,21	3,02	4,39	2,51	5,56
13	2,78	3,6	2,75	4,48	4,9	6,64	3,63	4,08	2,29	3,49
14	2,36	2,39	2,83	5,23	4,31	6,55	4,03	4,92	3,62	4,57
15	2,4	4,46	3,68	4,8	3,51	4,84	4,25	4,42	2,45	4,48
16	2,57	4,53	3,26	4,75	3,54	6,48	4,14	5,79	3,08	3,7
17	2,39	4,97	3,54	4,56	4,02	6,91	3,49	4,83	3,12	4,41
18	3,81	4,35	4,68	6,42	5,16	6,75	2,29	3,28	2,72	4,79
19	3,74	4,92	4,84	3,38	4,29	6,45	3,7	5,12	3,07	5,36
20	4,1	5,51	4,25	4,9	4,77	7,01	4,97	6,11	2,83	4,9
21	4,41	5,77	2,72	4,35	6,03	7,61	2,18	3,4	1,96	4,46
22	2,29	3,92	2,65	3,89	2,83	5,67	4,68	4,9	3,03	4,48
23	3,85	5,93	4,03	5,59	5,96	6,29	4,53	5,28	3,08	5,16
24	2,94	5,13	2,51	4,79	7,25	8,16	3,17	4,41	3,53	3,96
25	5,27	5,44	2,93	4,62	6	8,12	4,54	5,51	3,85	5,56
26	3,46	4,45	2,69	3,96	4,51	5,77	4,68	4,68	2,94	5,24
27	4,36	5,34	3,02	4,97	2,96	5,37	4,25	5,34	3,07	4,07
28	3,68	5,24	3,37	4,82	4,65	5,76	3,34	4,74	3,46	3,46
29	2,87	4,89	2,84	3,99	4,7	6	3,2	4,74	4,38	4,38
30	3,49	5,23	2,86	5,73	4,49	6,47	2,93	3,93	2,61	2,5
31	4,87	5,49	2,78	4,4	3,47	6,58	5,23	5,23	3,75	4,69
32	2,4	4,25	2,51	3,72	4,69	6,9	2,5	4,79	3,85	5,24
33	3,7	4,14	2,87	4,15	2,57	5,85	2,93	4,45	1,95	2,88
34	4,56	5,77	2,86	3,96	3,66	5,46	4,1	5,23	3,17	5,66
35	2,45	5,7	4,28	5,47	3,68	7,45	3,8	5,79	2,53	3,78
36	4,79	5,03	3,18	4,99	4,25	5,16	4,68	4,57	2,27	5,02
37	3,7	4,58	3,99	6,43	3,16	4,8	3,87	5,32	2,85	4,62
38	4,03	5,23	3,5	6,44	3,63	6,31	4,14	4,9	2,83	4,79
39	4,43	5,47	4,86	6,89	3,96	6,25	4,46	5,55	2,93	4,02

40	4,21	5,28	3,48	5,57	2,82	5,3	2,19	3,53	2,72	5,12
Nº CELLS	BEFORE INCUVATION WIDTH	BEFORE INCUVATION LENGTH	CONTROL WIDTH	CONTROL LENGTH	10% (v/v) WIDTH	10% (v/v) LENGTH	15% (v/v) WIDTH	15% (v/v) LENGTH	20% (v/v) WIDTH	20% (v/v) LENGTH
41	4,05	5,75	3,16	4,94	5,23	6,36	2,86	4,46	2,18	3,73
42	3,87	4,97	2,72	4,9	3,73	5,8	3,21	3,8	2,51	4,17
43	3,47	4,29	4,33	6,21	4,4	6,47	3,4	3,91	3,52	4,68
44	3,12	4,92	2,49	4,97	4,32	5,88	3,85	4,93	2,43	4,99
45	3,59	4,23	3,5	4,51	4,35	7,08	2,94	4,4	3,07	4,69
46	2,54	4,08	3,25	4,29	3,42	6,6	1,74	3,2	3,36	5,14
47	4,84	5,71	3,78	5,55	4,87	5,03	2,18	4,31	2,39	5,22
48	2,69	3,82	3,62	4,92	3,26	6,58	1,85	3,92	4,09	4,3
49	4,25	5,33	3,81	6,23	3,05	4,79	4,79	5,66	4,16	4,39
50	4,79	5,23	2,29	3,92	2,35	6,55	4,14	4,98	2,42	4,25
51	2,5	4,4	3,28	4,73	2,92	3,72	3,16	5,44	1,32	2,8
52	4,79	6,17	2,51	4,92	3,27	3,9	3,81	6,35	2,11	3,79
53	2,85	5,23	2,07	5,23	3,05	4,25	3,05	5,47	2,35	3,82
54	3,49	5,68	1,96	3,84	2,07	3,59	2,42	5,04	2,68	4,87
55	3,59	5,03	2,83	2,94	2,55	3,24	2,24	4,97	3,31	3,9
56	4,32	5,68	2,85	4,62	1,85	3,71	2,95	5,14	2,07	3,76
57	4,38	5,52	3,25	4,97	2,16	3,72	2,51	5,86	3,31	3,9
58	3,34	4,75	1,7	3,89	2,14	3,61	1,66	5,38	2,93	4,78
59	3,39	5,47	3,55	4,86	2,53	4,73	2,55	5,6	2,99	5,23
60	3,77	4,93	2,93	3,86	1,75	3,81	4,52	5,77	1,42	3,49
61	4,46	6,11	3,39	5,01	2,54	4,2	4,79	6,43	3,7	4,09
62	3,72	4,9	2,08	3,62	3,72	3,82	3,04	5,76	2,9	3,27
63	3,25	5,07	3,16	4,27	3,56	4,29	5,12	5,44	3,41	5,38
64	3,38	4,89	3,16	4,82	2,53	4,73	2,33	5,31	3,62	4,01
65	4,42	5,53	3,38	4,46	4,2	4,7	3,05	5,67	3,58	4,82
66	4,03	5,44	3,27	3,8	2,48	3,85	2,93	5,07	2,53	3,89
67	4,97	8,1	3,55	4,78	4,94	5,62	4,35	5,13	1,88	3,65
68	4,17	6,4	3,28	4,34	2,35	4,15	3,7	6,98	2,72	5,01
69	5,76	5,89	3,16	4,17	2,79	3,08	2,45	5,01	2,18	3,05
70	2,69	4,09	3,16	5,07	1,74	3,76	2,31	4,39	2,88	3,8
71	5,73	6,16	3,22	4,97	2,39	3,48	2,6	5,47	1,76	3,35
72	2,61	5,23	2,55	4,12	2,17	4,13	2,87	5,32	3,51	4,28
73	3	5,39	2,18	3,38	2,53	4,77	4,07	5,03	2,69	4,48
74	3,89	5,03	2,83	5,24	3,39	4,39	2,86	5,68	2,48	3,11
75	2,5	5,44	4,35	3,7	3,27	3,31	4,2	4,56	1,54	2,33
76	3,17	4,69	2,75	4,73	2,72	3,72	4,39	6,39	2,09	3,78
77	5,22	5,75	3,12	4,74	2,65	5,17	4,7	6,5	1,66	3,08
78	2,93	4,65	1,96	3,85	2,4	4,19	3,49	6,97	1,85	3,65
79	4,62	6,26	2,33	4,01	2,14	3,31	2,18	5,01	2,4	3,88
80	3,28	4,76	2,66	4,12	2,75	4,61	2,87	4,99	2,65	3,46

81	2,51	5,14	3,27	4,03	2,78	4,12	2,5	5,48	2,4	3,65
82	3,07	4,58	2,43	4,06	2,49	3,39	3,51	5,42	1,66	3,08
Nº CELLS	**BEFORE INCUVATION WIDTH**	**BEFORE INCUVATION LENGTH**	**CONTROL WIDTH**	**CONTROL LENGTH**	**10% (v/v) WIDTH**	**10% (v/v) LENGTH**	**15% (v/v) WIDTH**	**15% (v/v) LENGTH**	**20% (v/v) WIDTH**	**20% (v/v) LENGTH**
83	3,7	5,99	2,61	4,14	3,22	5,32	4,35	5,02	1,52	3,07
84	4,53	5,3	2,24	3,24	2,83	4,25	2,77	5,86	1,96	4,01
85	2,72	4,36	2,2	3,99	3,02	4,25	4,87	5,52	2,35	3,82
86	3,21	4,75	3,17	5,12	3,71	4,51	3,12	5,01	2,62	3,76
87	5,16	6,43	2,33	4,53	2,47	3,16	4,7	4,85	1,96	3,8
88	4,69	6,31	2,87	3,9	2,94	3,05	4,36	5,77	2,31	3,6
89	5,68	6,02	2,97	3,17	2,72	2,83	4,39	5,78	2,62	3,51
90	5,41	5,7	2,79	4,33	2,78	3,8	2,61	4,69	1,59	3,63
91	4,8	6,21	2,66	3,59	3,36	4,15	1,87	4,56	1,74	4,01
92	5,16	5,92	2,39	4,16	2,94	4,91	4,91	5,44	1,82	4,16
93	4,7	6,57	1,96	3,94	2,3	4,25	4,75	6,25	2,18	4,06
94	5,36	5,96	3,47	4,81	2,51	4,55	4,57	4,57	1,93	3,09
95	4,03	4,81	2,72	4,25	2,42	4,53	4,44	4,98	1,85	3,7
96	4,34	5,92	2,49	4,15	3,44	4,29	3,12	5,51	3,05	3,48
97	3	3,7	1,93	3,17	4,72	2,69	3,37	5,33	2,07	3,59
98	3,31	4,38	3,36	5,6	2,4	4,25	2,18	5,72	2,62	3,55
99	4,57	4,46	2,35	2,97	2,42	4,02	5,23	5,01	1,52	2,96
100	5,12	5,72	2,62	4,38	2,11	3,67	4,2	6,19	1,96	3,78

ANNEXES II:

ANALYSIS OF VIABILITY

TABLE I: SAUTERNES PROOFS RESULTS

	SAUTERNES											
TIME	Control			10%			15%			20%		
	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof
1 hour	100	98		100	100		97	94		98	91	
1st day	100	96		100	91		100	94	97	52		79
2nd day	99	99		96	97		97	92	97	36		75
3th day	99	99		96	98		94	96	94	30	33	82
4th day	99	97		97	97		89	86	92		28	58
7th day	97	97		98	93		92	78	83	44	36	48
9th day	99	98		88	98		78	63	85	25	23	52
11th day	97	99		96	95		66	60	76	30	22	59
14th day	96	95		91	98		48	62	76	23	22	50
16th day	94	97		89	97		44	76	74	19	30	49
21th day	98	94		93	96		52	76	63	26		31
28th day	88	96		88	97		28	71	54	24		35

NOTE: Boxes in blue means that I didn´t take datas for each days. Because it was necessary.

TABLE II: SAUTERNES AVERAGE

	SAUTERNES			
TIME	Control	10%	15%	20%
	AVERAGE	AVERAGE	AVERAGE	AVERAGE
1 hour	99	100	95,5	94,5
1st day	98	95,5	97	65,5
2nd day	99	96,5	95,3	55,5
3th day	99	97	94,7	48,3
4th day	98	97	89	43,0
7th day	97	95,5	84,3	42,7
9th day	98,5	93	75,3	33,3
11th day	98	95,5	67,3	37,0
14th day	95,5	94,5	62,0	31,7
16th day	95,5	93	64,7	32,7
21th day	96	94,5	63,7	28,5
28th day	92	92,5	51	29,5

TABLE III: SAUTERNES AVERAGE AND ALL PROOFS

	SAUTERNES															
TIME	Control				10%				15%				20%			
	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE
1 hour	100	98		99	100	100		100	97	94		95,5	98	91		94,5
1st day	100	96		98	100	91		95,5	100	94	97	97	52		79	65,5
2nd day	99	99		99	96	97		96,5	97	92	97	95,3	36		75	55,5
3th day	99	99		99	96	98		97	94	96	94	94,7	30	33	82	48,3
4th day	99	97		98	97	97		97	89	86	92	89		28	58	43,0
7th day	97	97		97	98	93		95,5	92	78	83	84,3	44	36	48	42,7
9th day	99	98		98,5	88	98		93	78	63	85	75,3	25	23	52	33,3
11th day	97	99		98	96	95		95,5	66	60	76	67,3	30	22	59	37,0
14th day	96	95		95,5	91	98		94,5	48	62	76	62,0	23	22	50	31,7
16th day	94	97		95,5	89	97		93	44	76	74	64,7	19	30	49	32,7
21th day	98	94		96	93	96		94,5	52	76	63	63,7	26		31	28,5
28th day	88	96		92	88	97		92,5	28	71	54	51	24		35	29,5

NOTE: Boxes in blue means that I didn´t take datas for each days. Because it was necessary.

TABLE IV: STEINBERG PROOFS RESULTS

	STEINBERFG											
TIME	Control			10%			15%			20%		
	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof
1 hour	99	96		99	98		98	100		47	60	
1st day	99	96		100	96		85	84	70	21		63
2nd day	100	97		94	98		68	81	66	28	32	53
3th day	97	98		92	97		63	61	84	28	32	60
4th day	95	95		81	96			43	66		20	63
7th day	93	97		71	97		48	49	59		35	50
9th day	92	96		53	94			48	49		38	42
11th day	93	97		65	92		28	50	74		41	52
14th day	93	97		52	84			55	49	23	47	45
16th day	93	96		51	84		43	60	44	24	37	51
21th day	98	90		54	89		24	60	47	37	47	35
28th day	52	91		54	92		29	60	45	24		44

NOTE: Boxes in blue means that I didn´t take datas for each days. Because it was necessary.

TABLE V: STEINBERG AVERAGE

	STEINBERFG			
TIME	Control	10%	15%	20%
	AVERAGE	AVERAGE	AVERAGE	AVERAGE
1 hour	97,5	98,5	99	53,5
1st day	97,5	98	79,7	42,0
2nd day	98,5	96	71,7	37,7
3th day	97,5	94,5	69,3	40,0
4th day	95	88,5	54,5	41,5
7th day	95	84	52,0	42,5
9th day	94	73,5	48,5	40,0
11th day	95	78,5	50,7	46,5
14th day	95	68	52,0	38,3
16th day	94,5	67,5	49,0	37,3
21th day	94	71,5	43,7	39,7
28th day	71,5	73	44,7	34,0

TABLE VI: STEINBERG AVERAGE AND ALL PROOFS

	STEINBERFG															
TIME	Control				10%				15%				20%			
	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE
1 hour	99	96		97,5	99	98		98,5	98	100		99,0	47	60		53,5
1st day	99	96		97,5	100	96		98	85	84	70	79,7	21		63	42,0
2nd day	100	97		98,5	94	98		96	68	81	66	71,7	28	32	53	37,7
3th day	97	98		97,5	92	97		94,5	63	61	84	69,3	28	32	60	40,0
4th day	95	95		95	81	96		88,5		43	66	54,5		20	63	41,5
7th day	93	97		95	71	97		84	48	49	59	52,0		35	50	42,5
9th day	92	96		94	53	94		73,5		48	49	48,5		38	42	40,0
11th day	93	97		95	65	92		78,5	28	50	74	50,7		41	52	46,5
14th day	93	97		95	52	84		68		55	49	52,0	23	47	45	38,3
16th day	93	96		94,5	51	84		67,5	43	60	44	49,0	24	37	51	37,3
21th day	98	90		94	54	89		71,5	24	60	47	43,7	37	47	35	39,7
28th day	52	91		71,5	54	92		73	29	60	45	44,7	24		44	34,0

TABLE VII: BINGEN PROOFS RESULTS

	BINGEN											
TIME	Control			10%			15%			20%		
	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof	1ºProof	2ºProof	3ºProof
1 hour	100	100		100	99		98	96		99	98	
1st day	100	100		100	97		99	96	98	39	47	92
2nd day	99	99		98	98		97	95	99	65	45	68
3th day	98	99		98	99		98	96	96	44	41	80
4th day	97	97		96	98		92	92	96	39	56	69
7th day	98	99		99	97		91	96	94	39	53	55
9th day	95	99		100	97		77	84	94	37	42	66
11th day	98	98		98	97		79	87	89	41	44	61
14th day	99	97		95	98		56	74	90	40	48	56
16th day	96	97		93	98		59	84	83	41	56	48
21th day	100	98		100	97		54	89	75	37	57	50
28th day	98	99		94	98		39	75	69	38	49	48

NOTE: Boxes in blue means that I didn´t take datas for each days. Because it was necessary.

TABLE VIII: BINGEN AVERAGE

	BINGEN			
TIME	Control	10%	15%	20%
	AVERAGE	AVERAGE	AVERAGE	AVERAGE
1 hour	100	99,5	97	98,5
1st day	100	98,5	97,7	59,3
2nd day	99	98	97	59,3
3th day	98,5	98,5	96,7	55,0
4th day	97	97	93,3	54,7
7th day	98,5	98	93,7	49,0
9th day	97	98,5	85	48,3
11th day	98	97,5	85	48,7
14th day	98	96,5	73,3	48,0
16th day	96,5	95,5	75,3	48,3
21th day	99	98,5	72,7	48,0
28th day	98,5	96	61	45,0

TABLE IX: BINGEN AVERAGE AND ALL PROOFS

	BINGEN															
TIME	Control				10%				15%				20%			
	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE	1ºProof	2ºProof	3ºProof	AVERAGE
1 hour	100	100		100	100	99		98,5	98	96		97,0	99	98		98,5
1st day	100	100		100	100	97		43	99	96	98	97,7	39	47	92	59,3
2nd day	99	99		99	98	98		55	97	95	99	97,0	65	45	68	59,3
3th day	98	99		98,5	98	99		42,5	98	96	96	96,7	44	41	80	55,0
4th day	97	97		97	96	98		47,5	92	92	96	93,3	39	56	69	54,7
7th day	98	99		98,5	99	97		46	91	96	94	93,7	39	53	55	49,0
9th day	95	99		97	100	97		39,5	77	84	94	85,0	37	42	66	48,3
11th day	98	98		98	98	97		42,5	79	87	89	85,0	41	44	61	48,7
14th day	99	97		98	95	98		44	56	74	90	73,3	40	48	56	48,0
16th day	96	97		96,5	93	98		48,5	59	84	83	75,3	41	56	48	48,3
21th day	100	98		99	100	97		47	54	89	75	72,7	37	57	50	48,0
28th day	98	99		98,5	94	98		43,5	39	75	69	61,0	38	49	48	45,0

TABLE X: 20% OF ETHANOL RESULTS

	20% of ethanol		
TIME	SOUTERNS	STEINBERG	BINGEN
	AVERAGE	AVERAGE	AVERAGE
1 hour	94,5	53,5	98,5
1st day	65,5	42,0	59,3
2nd day	55,5	37,7	59,3
3th day	48,3	40,0	55,0
4th day	43,0	41,5	54,7
7th day	42,7	42,5	49,0
9th day	33,3	40,0	48,3
11th day	37,0	46,5	48,7
14th day	31,7	38,3	48,0
16th day	32,7	37,3	48,3
21th day	28,5	39,7	48,0
28th day	29,5	34,0	45,0