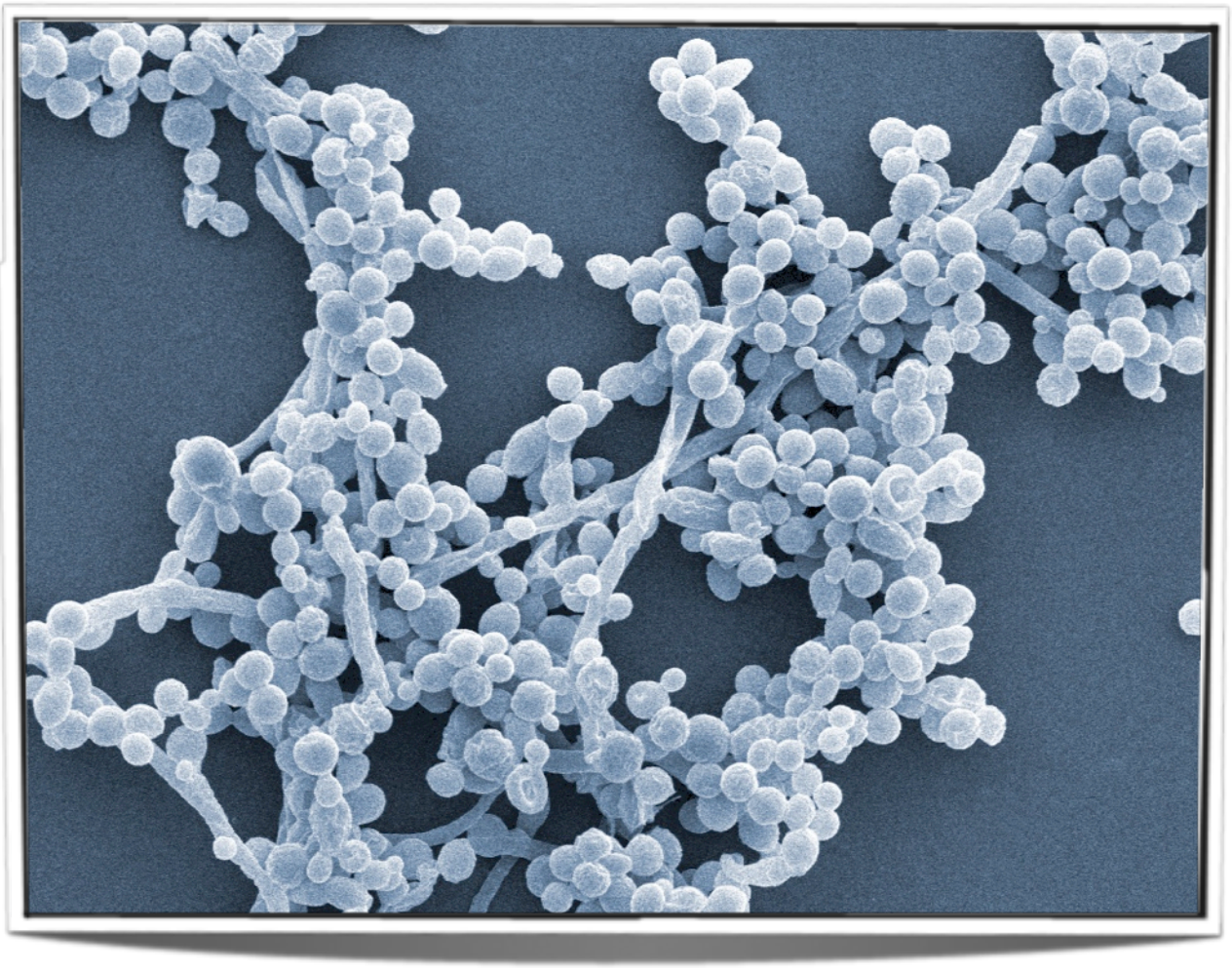
Task 2: Phosphates and yeast

"A bond between Fungi and unorganic substances"

Class 3F • Liceo Cantonale Lugano 2 • May 2013



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Part 1: Introduction

A phosphate is a salt of phosphoric acid and in organic chemistry its better called an ester of phosphoric acid. On the other hand inorganic phosphates is called the phosphorus found as a free phosphate ion in solution. A phosphate ion consists of one central phosphorous atom surrounded by four oxygen atoms in a tetrahedral arrangement. It has four distinct protonation states (Figure 1), of which the dihydrogen phosphate ($H_2PO_4^-$) and monohydrogen phosphate (HPO_4^{2-}) are the predominant forms at physiological pH (pH 4-10). In biological systems phosphate is referred to inorganic phosphate (Pi) when the valences of the central phosphorous atom are occupied with either inorganic ions, *e.g.* metals, hydrogen atoms or a combination of both.

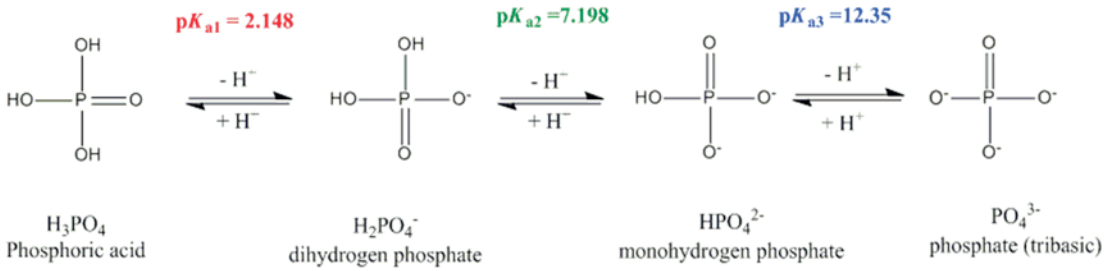


Figure (1): The four distinct protonation states of inorganic phosphate. Indicated are also the corresponding pKa values.

Phosphates find application in biochemistry, biogeochemistry and ecology. [1]

Why is phosphate important for all organisms?

Phosphates play a vital role in all organisms life and perform many important functions in theirs metabolic pathways.

- There are most commonly found in the form of adenosine phosphates, such as AMP, ADP and ATP or in DNA and RNA and can be released by the hydrolysis of ATP or ADP. These fundamental compounds are required by the great majority of metabolic reactions, for example in the glycolysis, the free energy released in this process is used to form the high-energy compounds ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). But it can only occur with the attachment of a phosphate group to the glucose molecule. Moreover, the ATP is one of the reagents necessary for the synthesis of RNA, where the phosphates indirectly affect on the ability of the cells to reproduce and acts as ″glue″ in the repair of DNA strands.

- We find phosphate in the cell membrane in the form of phospholipids, capable of forming lipid bilayers. [3]

- *Hydroxyapatite tricalcium phosphate* is a constituent of the mineral fraction of bones and teeth.
- In the blood phosphate is important for the regulation of the pH, since it constitutes an efficient buffer system.
- Phosphate can be considered as the raw material of many enzymes, proteins, nucleic acids and nucleotides, which perform various functions in our body.
- Phosphate compounds can rule important biochemical processes, such as glucose uptake, mechanisms of phosphorylation in cellular respiration, vitamin activation and, finally, his affinity to hemoglobin prevents the polymerization of this molecule.

What is well known about phosphate uptake in yeast cells?

The absorption of phosphates from the yeast cells takes place thanks to the presence of five conveyors: Pho84, Pho 89, Pho 87, Pho 90 and Pho 91. They can activate depending on solute concentration outside the cell, ensuring an optimal absorption in any condition. The transporters Pho84 and Pho89 work at high concentrations, while the remaining three at low concentrations.

How do yeast cells store phosphate?

Mankind has used yeasts during roughly 9000 years. The production of fermented beverages has occurred in China since 7000 BC, and evidence has also been found for the production of leavened bread in ancient Egypt around 3100 BC (McGovern et al., 2004; Samuel, 1996). However, it was not until 1860 that Louis Pasteur demonstrated that yeasts plays a direct role in the conversion of glucose into ethanol and carbon dioxide, a process called fermentation (Pasteur, 1860). Yeasts are recognized as unicellular fungi.

When Pi is not directly used as building blocks in Pi-rich compounds or metabolites, a large part will be incorporated into linear, large inorganic polyphosphate polymers, polyp, in the yeast cells. These polymers are composed of ten to hundreds of molecules of orthophosphate that are linked together by phospho-anhydrous bonds. Polyphosphate plays an important role in maintaining the Pi homeostasis of the cell. It confers a buffer capacity of the intracellular levels of this nutrient to assure the availability of Pi during fluctuating environmental conditions. Under favorable conditions the concentration of polyP is around 230mM and can constitute up to 20% of the cell dry weight. [2]

Why is phosphate uptake by microorganisms an important issue in our society/enviroment ?

Yeasts are one of the best-characterized eukaryotic organisms. They are more complex than bacteria, but less so than higher eukaryotes. As lower eukaryote they have many crucial features in common with cells of higher eukaryotes: metabolism, cytoskeletal organization and DNA replication.
Their ability to grow on defined media confers total control of the environmental parameters to the investigator. Yet, the main reason why *yeast cells* has become such widely used is the ease with which this organism can be genetically manipulated. This property is mainly exploited using the process of homologous recombination, which occurs at high rate and naturally in yeast. This feature can be put to work for the integration of foreign genes into the yeast genome and/or the modification of endogenous genes. Such investigations have provided invaluable insights into a large number of cellular functions as signaling pathways, regulation of the cell cycle, organelle biogenesis and

transport mechanisms.
Despite all the above mentioned advantages, one should bear in mind that it is not an absolutely ideal eukaryote experimental model system when it concerns studies of certain growth characteristics. For example, budding is a unique mode of reproduction. The asymmetry of cell division and the presence of the cell wall are not features of higher eukaryotic cells. [1] [2]

Summarizing, inorganic phosphate is an essential nutrient for all organisms. It is required for many cellular components as nucleic acids and phospholipids, and as energy-carrying compounds such as ATP. Thus, a regulated uptake of this pivotal nutrient is of outermost importance. Depending of the availability of phosphate in the surroundings the yeast make use of two different systems for transporting phosphate into the interior of the cell: a low-affinity system that is active during surplus phosphate conditions and a high-affinity system that is active when the availability becomes limited. This thesis focuses on the high-affinity system, which is comprised of the Pho84 and Pho89 transporters. Of the two transporters, Pho84 is the predominant one, responsible for almost all phosphate uptake during low phosphate conditions, and the contribution of Pho89 is of minor importance. Hence Pho84 is by far the most well characterized phosphate transporter. Even though much is known about phosphate transporters in yeast little in known about how phosphate is transported. [2]

[1] Introduction: Phosphate as an Essential Mineral - http://fipr1.state.fl.us/PhosphatePrimer

[2] **Mutational analysis of putative phosphate- and proton-binding sites in the** *Saccharomyces cerevisiae* **Pho84 phosphate:H+ transceptor and its effect on signaling to the PKA and** *PHO* **pathways**.

Samyn D, Ruiz-Pavón L, **Andersson M,** Popova Y, Thevelein J.M., Persson B.L. *Accepted for publication in Biochemical Journal.*

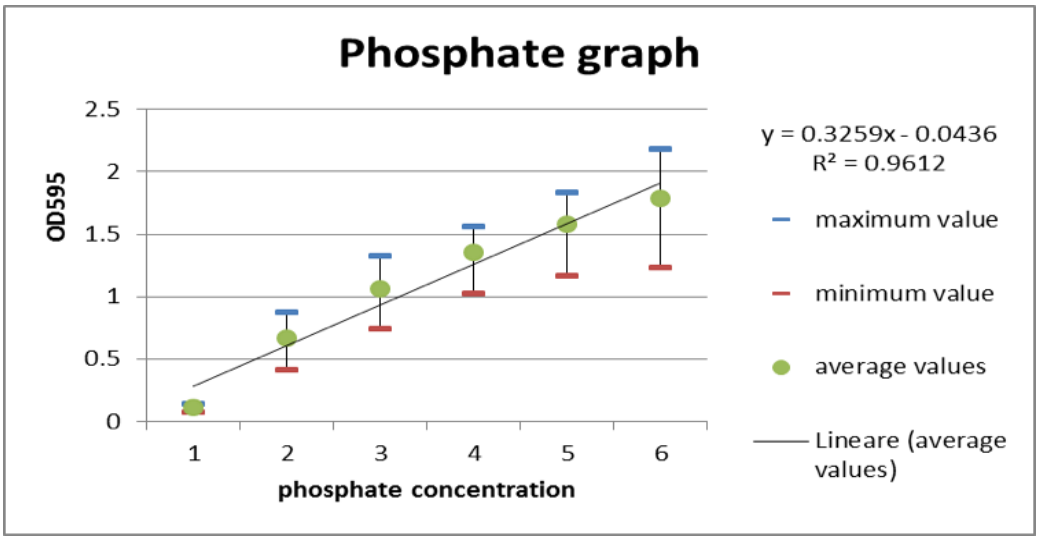
[3] Bailey, Regina. ″10 Steps of Glycolysis″ - http://biology.about.com/od/cellularprocesses/a/aa082704a.htm

Part 2: Calibrating the spectrometer

We measured the concentration of phosphates with the spectrophotometer as requested. The table below takes into account our 3 best results:

Concentration (μM)	OD595 (1)	OD595 (3)	OD595 (4)	Average	Standard deviation
0	0.074	0.127	0.14	0.113667	0.0349619
10	0.414	0.877	0.72	0.670333	0.235462
20	0.74	1.33	1.13	1.066667	0.3000556
30	1.027	1.56	1.49	1.359	0.2896429
40	1.166	1.756	1.83	1.584	0.3638846
50	1.231	2.183	1.95	1.788	0.4962449

Table (1): Data collected for the calibration



Graph (1): The calibration curve we obtained

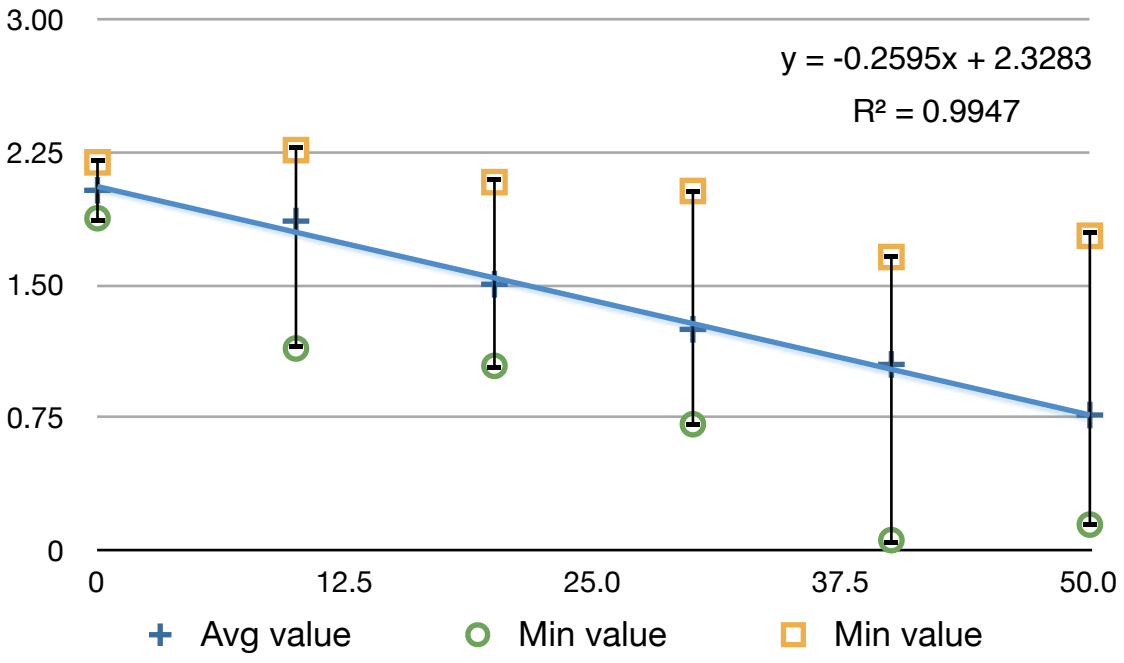
In the first measurements we found several problems, since the basic steps were wrong. But, once we understand the procedure and took familiar with the pipettes we managed to get the first right data. Unfortunately we repeated the experiment just two weeks later, and the data obtained were higher than in the first experiment. We hypothesized that in the time within the experiments, the phosphates or the distilled water had been "contaminated" by the continuous use, and consequently it was difficult to obtain accurate data.
To obtain rigorous data we split the roles. We prepared the pipettes with phosphate solutions, then 6 test tubes with the Malachitgreen Solution, where from time to time we added the Molybdat-Solution. To prevent leaving fingerprints on the cuvette we used latex gloves. We sped up the time by starting a second solution after 3 minutes. What gave us some difficulties to start was familiarity with the pipettes. Moreover we had been doing wrong two basic steps: first we used normal water and not distilled one, then we also mixed the two solutions before adding them to the test tubes containing phosphates.

Part 3: Phosphate uptake by yeast cells

In this third part we measured the absorbance of yeast cells under standard conditions: 1 g of yeast dissolved in 10 mL of 0.5 mM Sodium-Phosphate Buffer solution and 1% Glucose (pH 6.3). Every ten minutes we collected the samples which were then centrifuged, diluted with distilled water and reacted with 600 L of a solution composed of Malachitgreen-Solution and Molybdat-Solution. The table below contains our data:

Time	ODS595	ODS595	ODS595	Average	Standard deviation
0	2.192	1.876	2.034	2.034	0.158
10	2.261	1.14	2.179	1.86	0.624884789
20	1.387	2.08	1.042	1.503	0.528633143
30	0.712	2.03	1.002	1.248	0.692580681
40	0.057	1.657	1.447	1.053667	0.869501773
50	0.145	1.777	0.37	0.764	0.884467636

Table (2): The data we collected

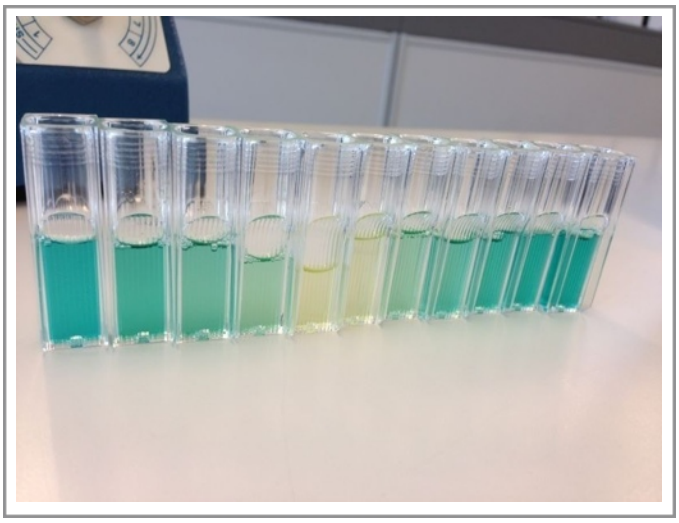


Graph (2): The values of table (2) visualized

From the graph we deduced that during the time the absorbance decreases, which means that the concentration of phosphate in the samples keep reducing. This also indicates that the yeast population has grown, consuming more and more phosphates.

Picture (1): Experiment 1 in progress at Massimiliano's home

To obtain rigorous values, we kept constantly moving the yeast samples. We also tried to carry out the operations as quickly as possible, but despite all our efforts we didn't achieve the expected results. In fact, the concentration of phosphates should decrease in relation with yeasts reproduction, but in our graphics this value increases sometimes. By now, we cannot explain it, but we hypothesized that in order to obtain correct results, we should have worked in a sterile environment, since phosphates are rudely 'everywhere', so it is much easier to "contaminate" our samples. Yeasts are also living beings and therefore is much more difficult to obtain accurate than a graph as the standard chart of phosphate that we did before. The major problems we have encountered in the measurement of the fourth and fifth point, where we found far bigger results, so we have needed to measure several times these points. Since yeasts are, as we said, living beings we used fresh yeast for each experiment.



Picture (2): picture of the cuvettes with the solutions in them

Picture (3): Our improvised chronometration station

Part 4a: Using more glucose

Aspect 1:
Knowing that the yeasts, multiplying, are usually using phosphates (they use them for DNA and phospholipids creation) we decided to try to reproduce these fungi as quickly as possible, to increase the absorption of phosphates. In the theoretical research we discovered that yeasts require a carbon source and that their ideal life conditions are at the temperatures between 30-35 ° C. Therefore we decided to add to the solution of yeasts, phosphates and glucose, a carbohydrate and raise the temperature to 30 ° C. In this way, yeasts should feed on carbohydrates, and then reproduce speeding up the absorption process.

The manipulated variable is the glucose concentration and the heat, while the fixed one is the time and the phosphate concentration.

Aspect 2:
We controlled the variables with a pair of scales, pipettes, and a spectrophotometer. The analysis procedure is analogous to that achieved in part 3, but, in the yeast solution added at the beginning of the experiment (containing phosphates, distilled water and glucose in concentration 1%) we added a glucose quantity equal to the mass of yeast, 1.0 g. We also introduced an additional variable: the temperature, which we kept constant at 30 ° C throughout the whole culturing phase, thanks to an oven. As usual, we have taken a sample every 10 minutes and the rest of the procedure is identical to that of part 3.

Aspect 3:
To try to avoid errors during the measurements we made sure to always use the same pipette and the same measurement tools. In addition, the same people almost always made the analyses, in order to avoid constant mistakes caused by inexperience.

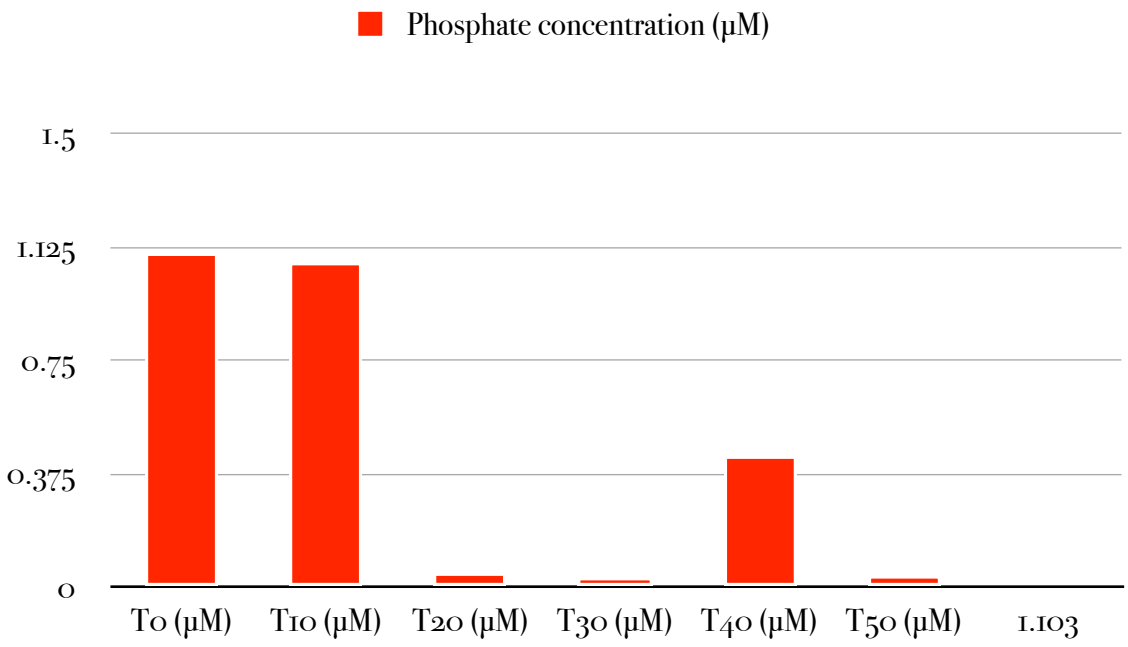
Data Measurements:

These are the measurements results of the experiment 1.

Phosphates T0 (μM)	Phosphates T10 (μM)	Phosphates T20 (μM)	Phosphates T30 (μM)	Phosphates T40 (μM)	Phosphates T50 (μM)
1.103	1.072	0.045	0.03	0.432	0.036

Table (3): data collected

With these data we detected the following graph, which shows well how phosphates have significantly decreased during the cultivation of yeasts.



Graph (3): The values of table (3) visualized

Brief comment and early considerations:

From the graph it can be well seen that the amount of phosphates decreases drastically, and reaches a very low value. The three measurement points are almost aligned. This probably happens because the yeasts have reached their maximum reproductive rate with the amount of nutrients and phosphates at their disposal, so, they do not increase more in number even as the phosphates.

Part 4b: Using different sugars

Aspect 1:

Furtheron in the our researches we found out that the absorption of various types of sugar occurs through vorious enzymes, each specialized for a specific reaction type. We reasoned as in the case of the innovative experiment 1. So, for example, glucose reactions are controlled by a specific chain of enzymes, while fructose reactions are controlled from a different one. Hence we chose to create a new experiment tby using another source of carbon: the fructose.

We have also taken the same environmental conditions compared to experiment 1 (T = 30 ° C) in order to match the absorption of phosphate with the addition of glucose either of fructose.

The manipulated variable is the concentration of fructose and the heat, while the fixed one is the time and the phosphate concentration.

Aspect 2:

We controlled the variables with a pair of scales, pipettes, and a spectrophotometer. The analysis procedure is analogous to that achieved in part 3, but, in the yeast solution added at the beginning of the experiment (containing phosphates, distilled water and glucose in concentration 1%) we added a fructose quantity equal to the mass of yeast, 1.0 g. We also introduced an additional variable: the temperature, which we kept constant at 30 ° C throughout the whole culturing phase, thanks to an oven.

As usual, we have taken a sample every 10 minutes and the rest of the procedure is identical to that of part 3a.

Aspect 3:

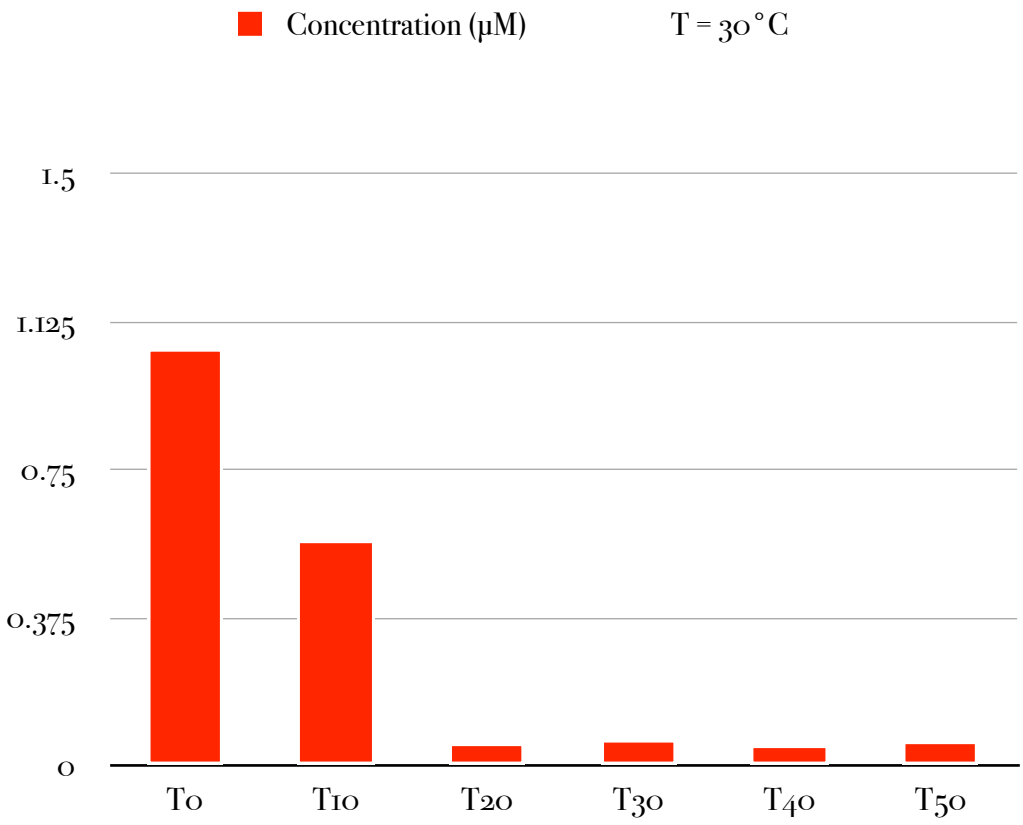
We have dealt with the same accuracy and precision of the first experiment.

Measurement results:

Phosphates T0 (μM)	Phosphates T10 (μM)	Phosphates T20 (μM)	Phosphates T30 (μM)	Phosphates T40 (μM)	Phosphates T50 (μM)
1.055	0.57	0.056	0.065	0.051	0.061

Table (4): Phosphates concentration for each measurement time

From these data, we found the following graph:



Graph (4): Visualization of the data on table (3)

From the graph it can be well seen that the amount of phosphates decreases drastically, and reaches a very low value. The first 3 points of mesurements are almost aligned. This probably happens because the yeasts have reached their maximum reproductive rate with the amount of nutrients and phosphates at their disposal, so, they do not increase more in number even as the phosphates.

Picture (4): Extreme seriousness showed by Gabriele

Part 4c: Influence of light

Aspect 1:

During our researches we read that yeasts are sensitive to UV light, so we designed to optimize their multiplication (and therefore their consumption of phosphates) by removing the light.

All the rest of the experiment is similar to that of part 3a.

Modified variables were the presence (absence) of light, while the constant variables were time and phosphate concentration.

Aspect 2:

In order to avoid that yeasts, during their culture, were exposed to sunlight or lamps, we completely covered their container with the aluminum foil. The measurements were made by a set of scales, test tubes, and a spectrophotometer.

As usual, we have taken a sample every 10 minutes and the rest of the procedure is identical to that of part 3.

Aspect 3:

We have again dealt with the same accuracy and precision of the first two experiments.

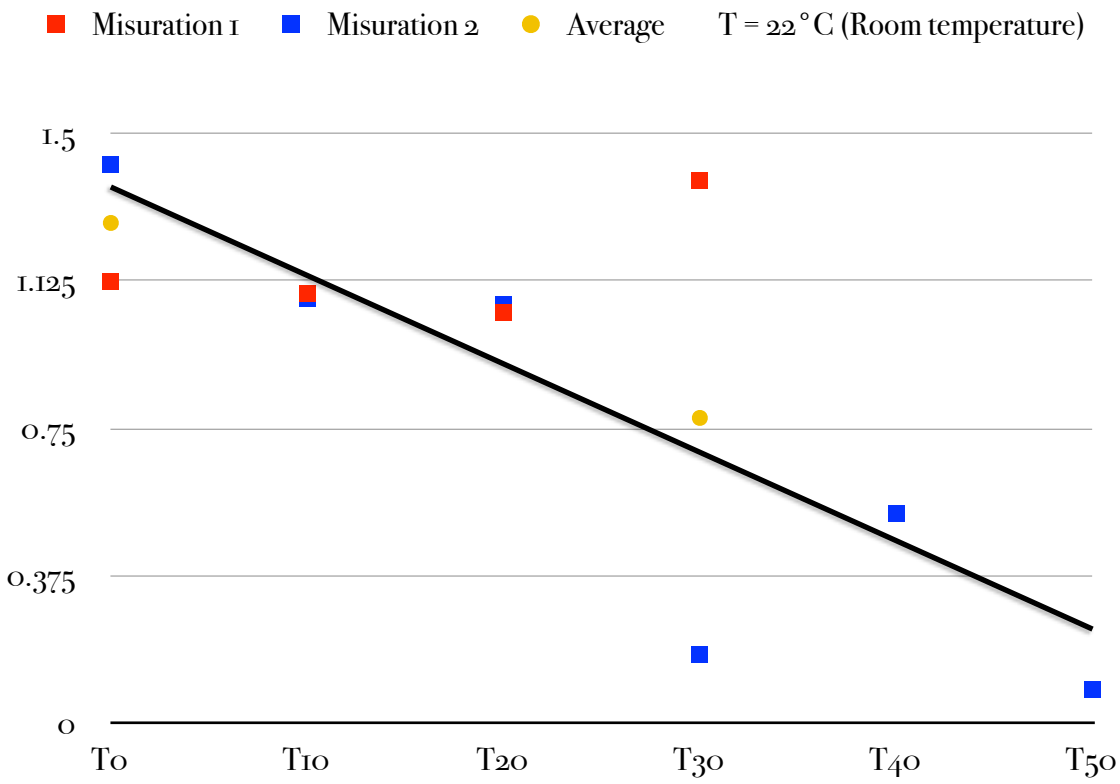
Measurements results:

These are the results of measurements of experiment 3. We conducted two tests, but the first is incomplete because we accidentally knocked the solution of yeast culture in mid-process.

	Phosphates at T0	Phosphates at T10	Phosphates at T20	Phosphates at T30	Phosphates at T40	Phosphates at T50
Misuration 1	1.124µL	1.093µL	1.043µL	1.380µL		
Misuration 2	1.420µL	1.080µL	1.065µL	0.173µL	0.533µL	0.085µL
Average	1.272µL	1.0865µL	1.054µL	0.776µL	0.533µL	0.085µL

Table (5): Data collected for the light influence experiment

By entering the data in a graph, we have obtained the result that follows. In addition to the specific points we have also included the measured average line, in order to obtain a straight line comparable to that of Part 3



Graph (5): Graph for table (5)

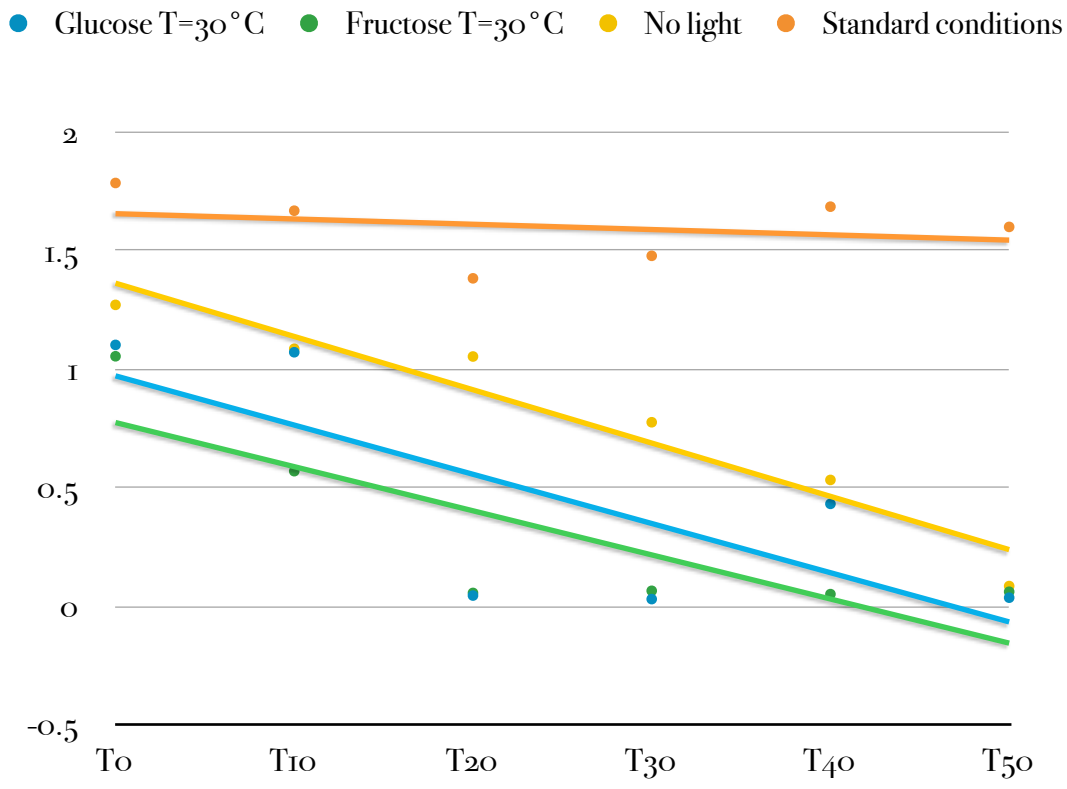
Part 5: Conclusions

Aspect 1:

The results obtained in the experiments by us proposed, suggest that all three different methods are functional, and allow a more rapid absorption of phosphate by yeasts.
Comparing the slopes of the trend lines of our experiments with the one obtained in part 3, when the yeasts were in standard conditions during their cultivation, we note that these are more "sloping", steeper. In fact their their gradient, determined by the function y = ax + b, is greater.

	Phosphates T0 (μM)	Phosphates T10 (μM)	Phosphates T20 (μM)	Phosphates T30 (μM)	Phosphates T40 (μM)	Phosphates T50 (μM)
Glucose T=30°C	1.103	1.072	0.045	0.03	0.432	0.036
Fructose T=30°C	1.055	0.57	0.056	0.065	0.051	0.061
No light	1.272	1.0865	1.054	0.7765	0.533	0.085
Standard conditions	1.7865	1.6695	1.384	1.479	1.6865	1.6011

Table (6): Comparison between the experiments



Graph (6): Complete overview of all the experiments (trend lines shown)

As can be seen from this graph, the more efficient method of the three by us experimented was the one with the fructose. It has well increased the absorption capacity of the phosphate combined with the temperature increase. This is probably due to the fact that the enzyme that converts fructose is faster than the one of glucose. However, to be certain of these results we should repeat this experience. Considering the efficiency of the method, at the second place comes the experiment with the glucose (always related with the temperature increase), and finally that the darkness experiment.

In the cases of glucose and fructose,
the absorption has been so rapid that after 30 minutes the yeast had almost exhausted their reserves of phosphate and therefore they practically stopped their assimilation. It may also be possible that the amount of phosphates is not likely became 0 because the fungi had finished the carbon source and therefore they stopped their assimilation.

Aspect 2 and aspect 3:

- In order to actually determine how each variable has affected the increase of phosphates absorption, we would have to do the measurements entering one change at a time, for example, by adding only glucose, or only by heating the yeast. Then, in order to se a higher increase we could have done a combination of two variables (for example, the darkness condition and the fructose addition). So we would have more certainty of the role of each. We didn't do like that because doing more than 3 experiments would have been impossible, for time reasons and materials. However, by introducing two variables we were pretty sure to achieve a good result.

- To be sure of our conclusions we should have repeated the experiences several times, in order to have more results and be able to determine a plausible average, but the time and the material was not sufficient to do that. However, the huge differences of our variations from the standard conditions permit to hazard a pretty sure truth in our hypothesis.

- To further improve the quality of data we would have to be much more precise with the times of the measurements, but the time to pick up the liquid with pipette, close the tubes, etc. we were always losing a few seconds. For example, the results of the analysis of glucose are quite inaccurate, since the value of $R^2 = 0.5697$.

- However, we can be satisfied with the work we did; we tried to be accurate as far as possible, but we lack of experience with using the tools. At the end the results were better than at the beginning because we improved our dexterity with tools. The material we used it seemed appropriate for what concerns our measurements, the spectrophotometer had a good accuracy, which is sufficient to determine the presence of significant changes in the amount of phosphate, despite the very small values. The fact that always the same people have made the measurements, has avoided lots of mistakes and incomprehension. The measurements were also made usually in the same way; although in the end the process was already established you might have slight differences in the times.

- In the case of glucose and fructose addition, we wanted to add an amount of sugar equal to the mass of yeast, therefore, in order to maintain this variable constant over time, we would have to add sugar during the yeast reproduction, but this would result particularly complicated. In this case we would have discovered the reason of why the amount of phosphates is stabilized at very low levels without reaching the 0, and if it is due to lack of nutrients or not.

- As can be seen from the parameter R2 (which should be equal to -1 / +1) our trend lines are not always very reliable. They are often far from this value, due to the fact that there are peaks that off settle a bit the line.

- We started to work very late, and we still consider one month a very poor time for such a Task.

Considerations written above are clearly both critics to our work and idea of possible ways to improve the experiments.

Data analysis bibliography:

http://bcs.whfreeman.com/thelifewire9e/default.asp#542578__595681__

Activity list:

Introduction by: Adriana Crovetto, Maya Brignoni, Valentina Bubba, and other classmates

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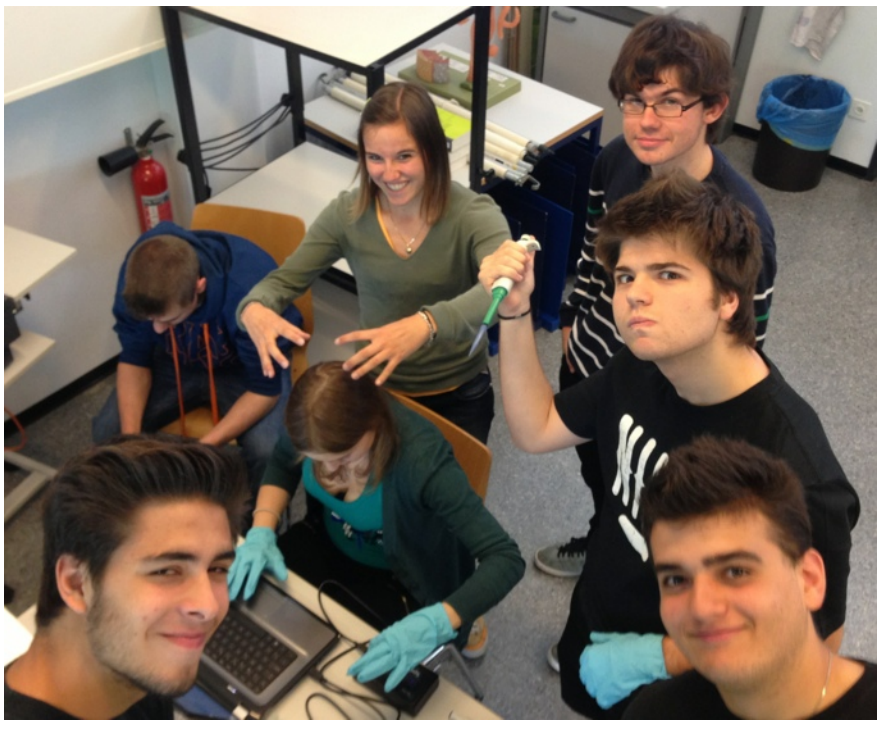

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Michele Calzana

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*An edited image of the yeast Spathaspora passalidarum taken by electron microscopy. Credit: Dana Wohlbach and Thomas Kuster.