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Characterization of the activity and stability of amylase from saliva and detergent

LABORATORY PRACTICALS FOR STUDYING THE ACTIVITY AND STABILITY OF AMYLASE FROM SALIVA AND VARIOUS COMMERCIAL DETERGENTS

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Abbreviations:

Enzymes play important roles not only in physiological fields but also in industrial performance and commercial applications.

This article presents two integrated laboratory exercises intended to show students the role of α-Amylases (AAMYs) in saliva and detergents. These laboratory practicals are based on the determination of the enzymatic activity of amylase from saliva and different detergents using the Phadebas test (quantitative) and the Lugol test (qualitative) under several different conditions (e.g., variations in temperature and alkalinity). It also proposes the study of enzyme stability in the presence of several surfactants and oxidizing agents using the same technical approach. These laboratory practicals promote understanding of the physiological function of this enzyme and the biotechnological applications of AAMYs in the detergent industry. They also promote understanding that enzymatic stability/performance is dependent on the organism of

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origin, and if necessary, these properties could be artificially modified by genetic engineering. Additionally, this paper reinforces the development of laboratory skills, problem-solving capabilities, and the ability to write a laboratory report. The exercises are proposed primarily as an undergraduate project for advanced students in the biochemical and biotechnological sciences. Finally, these laboratory practicals are complementary to the previously published BAMBED article (Biochemistry and Molecular Biology Education Vol. 39, No. 4, pp. 280-290, 2011) referring to detergent proteases.

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INTRODUCTION

AAMY (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) is an enzyme present in microorganisms and in tissues from animals and plants. It catalyzes the hydrolysis of α-1,4-glucosidic bonds in glycogen, starch, related polysaccharides (such as the mainly linear amylose and the branched amylopectin), and some oligosaccharides. The enzyme liberates α-maltose, α-glucose, and α-limit dextrins in a stepwise fashion.

Bacterial AAMYs, specifically those from *Bacillus* and *Aspergillus* species, have been successfully used in detergent formulations. Detergent AAMYs boost overall detergent performance at lower wash temperatures and with milder detergent chemical systems.

In a physiological context, AAMY is found in human saliva and pancreatic secretions. Ptyalin, the enzyme present in the saliva, cleaves the glucose linkages present in insoluble starch and releases soluble starches (amylodextrin, achrodextrin, and erythrodextrin) that are successively converted to smaller starches and finally to maltose; thus, this AAMY initiates the process of digestion of starch to sugar in the mouth. Ptyalin is a metalloenzyme and requires calcium ions for function. When food is

ingested, the saliva secreted by the salivary glands softens the food, and the tongue mashes the food as it is chewed and converts it to a bolus.

Ptyalin does not have much time to act on the food in the mouth. However, its action is quick, and it continues to digest the starch within the bolus until it reaches the stomach. Salivary AAMY is inactivated by the low pH (3.3) in the stomach caused by the secretion of gastric juices. The starch and glucose present in the bolus provide some protection for the enzyme from stomach acid, and the salivary AAMY continues starch digestion for a short period of time until it is completely inactivated.

Optimum conditions for ptyalin activity are a pH of 5.6–6.9, a temperature of 37ºC, and the presence of certain anions and activators, such as chloride, bromide, and iodide.

The amount of salivary AAMY differs in people of different ethnic backgrounds. Duplication of the gene during evolution has led to gene copy number variation. The number of gene copies is directly related to the levels of salivary amylase. Additionally, smoking habits clearly modify the enzymatic activity of AAMY [1].

In the industrial context, rapid development, seen primarily over the past four decades thanks to the evolution of modern biotechnology, has shaped the enzyme industry as we know it today. Enzymes found in nature have been used since ancient times in the production of food products, such as cheese, sourdough, beer, wine, and vinegar, and in the manufacture of commodities, such as leather, indigo and linen. However, the enzymes were not used in the pure or well-characterized forms. The development of the fermentation process during the latter part of the last century, which was aimed specifically at the production of enzymes through the use of selected production strains, made it possible to obtain purified, well-characterized enzymes on a large scale. This development allowed the introduction of enzymes into true industrial products and processes, such as within the detergent, textile and starch industries [2].

A detergent is a mixture of substances primarily used for laundry and dish washing. The core components of most modern detergents are surfactants, a builder and

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cobuilder, bleach and a bleach activator, and special additives, such as fluorescent brightener, filler, a corrosion inhibitor, an antifoaming agent and enzymes [3].

Enzymes are now an essential component of detergents, and their use provides consumers with thoroughly proven advantages over non-enzymatic detergents, both in the washing process itself and in terms of the environment. Enzyme detergents require a low workable temperature and low mechanical energy; are cost-effective, eco-friendly, less toxic and non-corrosive; and have enhanced stability in different formulations compared with non-enzymatic detergents [3].

The compositions of detergents depend on the application for which they are designed, so their enzymatic content may differ. Currently, the most common enzymes found in detergents are proteases that assist in the removal of protein-based stains, amylases that decompose starch to small sugars (e.g., in the removal of starch sizing from textiles and in the liquefaction of starch), lipases that facilitate the removal of fat and oil-based stains and cellulases that remove microfibrils formed during the washing and wearing of cotton and cotton blends and brighten and soften colors [4]. However, there is a continuous need to formulate detergent compositions that provide improved cleaning performance, and several other enzymes, such as peroxidases, hemicellulases and mannanases, can be found in new detergent compositions [5].

The use of enzymes as detergent additives still represents the largest application of industrial enzymes, both in terms of volume and value. New and improved engineered versions of the “traditional” detergent enzymes, such as proteases and amylases, are constantly in development. These new second- and third-generation enzymes are optimized to meet the requirements for performance in detergents, the composition of which is also constantly developing [2].

Second-generation detergent proteases, amylases and lipases were developed using protein engineering and genetic techniques to (1) improve performance under difficult washing conditions [e.g., low temperature and single wash effects (cleaning, stain

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removal, whitening, softening, etc., achieved with one single wash treatment)], (2) improve compatibility with new detergent technologies (e.g., improved activated bleach) and (3) broaden application (e.g., in automatic dish washing, liquid detergents, tablets, gels and pads) [6].

The important role of enzymes in several common products has usually only been described to students from a theoretical point of view. To provide a practical approach to this issue, we have introduced a series of easy laboratory exercises that reinforce knowledge of the utility and the impact of the biotechnological applications of enzymes in normal life. Additionally, these exercises can be used to revisit several elementary enzymology concepts, such as the influence of temperature, the influence of pH and the effect of surfactants and oxidants on AAMY activity. The laboratory exercises presented here are designed to get students involved in nearly every aspect of the teaching process. Students are asked to prepare the project by reading the primary literature on the physiological role of enzymes as well as their industrial applications [7, 8, 9]. The first lab session is also used to discuss detergent-specific formulations, the influence of several parameters (substrate, pH and temperature) on ptyalin and detergent enzymatic activities and quantitative and qualitative methods for evaluating enzymatic activity [10]. The next four lab sessions are used to evaluate ptyalin and three commercial household laundry detergents (two standard detergents that contain AAMYs and a detergent for delicate clothes that contains no enzymes) by both measuring the influence of temperature on AAMY activity and studying the influence of pH and surfactants/oxidizing agents on detergent AAMY activity. These exercises are evaluated by quantitative and qualitative methods that are performed quickly and easily.

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EXPERIMENTAL PROCEDURES

Equipment

The following equipment is necessary to carry out the practical exercises: a table spectrophotometer, a thermostatic water bath, microwaves, an electric heater, micropipettes, and consumables, such as cuvettes, tips, plates and tubes.

Materials and solutions

The following solutions are needed: Triton X-100 (5% and 1% v/v), SDS (0.1% and 1% w/v), H_2O_2 (1% and 5% v/v), 0.5 M NaOH, 1 M KH_2PO_4, and 1 M K_2HPO_4.

For the starch-agar plates, the required quantities of 1.5% agar and 0.5% starch were weighed and added to 250 ml of distilled water. The mixed solution was heated using a microwave for 3-4 minutes and stirred every 30 seconds. The solution was left on the bench until it cooled, and the plates were filled to 75% of their volume. The solidified starch-agar plates can be stored in the fridge until needed.

Products

The following products are required: sodium hydroxide pellets (Panreac Quimica), hydrogen peroxide (33% w/v, Panreac Quimica), sodium dodecyl sulfate (SDS) (Merck), Triton X-100 (Fluka), bacteriologic agar (Cultimed, Panreac), soluble potato starch (Panreac), Lugol (iodine solution, QCA Química Clínica Aplicada), and Phadebas Amylase test tablets (Magle Life Sciences).

Hazards

SDS and hydrogen peroxide are irritating and toxic, respectively. Gloves and a lab coat should be worn when handling the solutions, and solutions must not be pipetted by mouth.

Samples

The samples consist of three tubes, each with 10 ml of one of following household liquid detergents: two standard detergents (**A** and **C**) that contain AAMYs and a

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detergent (**B**) for delicate clothes that contains no enzymes. If desired, these detergents can be brought by the students. It is important to note that specific AAMY activity can vary between different detergent brands or uses. This means that the appropriate dilution factors for each detergent must be assayed prior to the enzymatic activity assays. Different detergents may also have a different optimal temperature for enzyme activity; therefore, a preliminary temperature test must be developed to determine the temperature range that should be tested in further analyses.

For the biological samples, 1-2 ml of saliva from each student is needed. This can be obtained by chewing a clean rubber band or by drooling into a clean test tube. Saliva samples must be centrifuged at 5000 rpm for 5 min to eliminate solid particles.

LABORATORY SESSIONS

As depicted in figure 1, the laboratory practical took place over five sessions of approximately 3 hours each. The first was a theoretical and experimental session. The four additional sessions were used to carry out the laboratory practicals.

We divided the experiment into two blocks. The first block (sessions 2 and 3) involved screening the detergent(s) that show AAMY activity and determining what temperature is most appropriate to study the enzymatic activity. The second block (sessions 4 and 5) involved studying the effect of pH on the amylase activity of detergent **C** (AAMY positive) at 40ºC and 60ºC and the amylase activity of saliva at 40ºC, as well as the effects of surfactants/oxidizing agents on the amylase activity of detergent **C** at 60ºC.

Laboratory session 1.

We used the first half of the first session in the laboratory (1.5 hours) for the theoretical discussion of several aspects related to the physiological and biochemical role of enzymes (e.g., digestive enzymes) and their industrial use (focusing on AAMYs). The aspects under discussion included the following topics: the physiological role of human

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AAMYs, the biological role of bacterial AAMYs, detergent-specific formulations, the influence of various parameters (e.g., substrate, pH, and temperature) on detergent enzymatic activities and technical methods for evaluating enzymatic activity (both quantitative and qualitative). After this discussion, the students were asked to do the following:

1. Calculate the saliva and detergent AAMY activities at the temperature used in the test exercises. Make a graphical representation.
2. Describe the results obtained in the Lugol test for the three types of detergents and saliva in the temperature test exercises.
3. Determine which detergent had the most AAMY activity.
4. State what they thought was the optimal temperature for ptyalin and the detergent AAMYs.
5. Explain why one of the detergents had no AAMYs in its composition.
6. Determine whether the quantitative and qualitative evaluations of the enzymatic activity were correlated and justify the answer.
7. State which pH had more influence on AAMY activity and whether there was a pH that could inhibit the amylase activity.
8. State whether they obtained the same results regarding the influence of pH on AAMY activity from the quantitative and qualitative methods.
9. Describe which surfactant/oxidizing agent(s) had a stronger influence on AAMY activity and state whether there was any surfactant/oxidizing agent(s) that could inhibit AAMY activity.
10. State whether they obtained the same results regarding the influence of oxidizing agents on AAMY activity from the quantitative and qualitative methods.

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11. Discuss all of the results obtained regarding saliva and the three detergents in general.

Students had to perform these exercises after the end of the fifth laboratory session and hand in their answers to teachers one week later as their final laboratory reports.

The second half of the session was used to prepare the different buffers and surfactant/oxidizing agents. The students made plates of agar-starch medium (0.5% and 1.5%), which were stored at 4°C until needed in laboratory session 2.

Laboratory session 2.

Quantitative analysis of the effect of temperature on AAMY activity (Phadebas test)

The second laboratory session was used to measure AAMY activity at different temperatures. For this purpose, we used the Phadebas test, which is a method for quantitatively assaying AAMY activity in fluids. A Phadebas tablet consists of homogeneously interlinked starch polymers that take the form of globular microspheres of a defined size. These bio-degradable starch microspheres (DSMs) are insoluble in water. A blue dye is covalently bound to the DSMs. When the dye is bound to the microspheres, it remains water insoluble. However, in the presence of amylase, the DSMs are degraded at a speed proportional to the AAMY activity, and the blue dye is liberated (figure 2a). The characteristics of the liberated blue dye molecule are key for the action of Phadebas. The free dye molecule is water-soluble, and its concentration is measurable at 620 nm. With a fixed assay time, the solution's amylase activity can be determined from the absorbance determination and the standard curve supplied with the product.

Students prepared a 1/40 dilution of each detergent and a 1/1000 dilution of the saliva sample and pipetted 200 µl of these diluted samples into 10-12 ml centrifuge tubes.

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Next, 4 ml of distilled water was added to each tube. A blank (distilled water) of 4.2 ml should also be subjected to the entire procedure. The tubes were pre-incubated for 5 minutes in a water bath at 4ºC, 40ºC or 60ºC. One tablet was then added to each tube, and the tubes were immediately vortexed for 10 seconds and placed again in the corresponding water bath. The reaction was stopped exactly 15 minutes after tablet addition by pipetting 1 ml of 0.5 M sodium hydroxide into the tube and vortexing immediately. The samples were then centrifuged for 5 minutes at 1500 g, and the supernatant was pipetted into a cuvette. The absorbance was then measured at 620 nm, and the AAMY (U/L) was obtained by plotting the absorbance against the standard curve supplied with the Phadebas test product. The dilution factor of the sample should be taken into account to obtain the correct AAMY activity in each sample.

In laboratory session 2, students must also begin preparing for the qualitative method (Lugol test) that will by used in the following session by preparing starch-agar plates.

Laboratory session 3.

Qualitative measurement of the effect of temperature on AAMY activity (Lugol test).

In laboratory session 3, the students used a qualitative assay to measure the effect of temperature on amylase activity.

Lugol iodine, also known as Lugol solution, is often used as a reagent for starch detection in routine laboratory and medical tests. This solution is used as an indicator for the presence of starches in organic compounds. It reacts with starch to give a dark blue/black color. Elemental iodine solutions like Lugol stain starches due to the interaction between iodine and the coil structure of the polysaccharide. Detectable starches include the plant starches amylose and amylopectin as well as glycogen in animal cells. Lugol solution will not detect simple sugars, such as glucose or fructose. The proposed method consists of the incubation of different dilutions of each detergent

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or saliva in a plate with 0.5% starch - 1.5% agar. We worked with the following samples: (a) dilutions of 1/10, 1/20, 1/40, 1/60, and 1/80 and negative (water) and positive (ptyalin 1/500) controls for the detergent samples, and (b) dilutions of 1/250, 1/500, 1/1000, 1/2000, and 1/5000 and a negative control (water) for the saliva samples. An aliquot (80 µl) of each dilution was put into the corresponding hole (previously made with a cork borer), and each plate was incubated at 4ºC (fridge), 40ºC or 60ºC (electric heater) for 48 hours. To observe whether the detergents had amylase activity, the plates were dyed by the addition of Lugol solution and rinsed with distilled water after 10 minutes. In regions where starch has been degraded by AAMY, clear halos will appear around the hole. The halo's size is proportional to the AAMY activity (figure 2b). Students took photos of the plates and also measured the diameter of each halo.

Laboratory session 4.

Quantitative and qualitative analysis of the influence of pH on AAMY activity (ptyalin and detergent).

At this point in the practicals, the optimal temperature and the activity for each initial enzyme source (ptyalin or detergent) had already been determined. Therefore, we studied the hypothetical pH influence (positive or negative) on the amylase activity at the optimal temperature. This assay was developed using both quantitative and qualitative methodologies. For the quantitative assay, two buffers were prepared: 1 M KH_2PO_4 (pH 3.7) and 1 M K_2HPO_4 (pH 9). By mixing these buffers appropriately, we obtained solutions with different pH values. Students worked with solutions with four different pH values (5, 7, 8 and 9). An aliquot (4 mL) of each solution was put into a glass tube, and 200 µl of detergent **C** (diluted 1/40) or 200 µl of saliva (diluted 1/1000) was added to the same tubes. The samples were pre-incubated for 5 minutes at 40ºC or 60ºC for samples containing detergent **C** and at 40ºC for samples containing saliva.

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Phadebas tablets were then added, and the tubes were mixed using a vortexer. After 15 minutes of incubation, 1 ml of NaOH was added to stop the reaction. After a centrifugation step (1500 g for 5 minutes), the supernatant was pipetted into a cuvette, and the absorbance was measured at 620 nm.

For the qualitative assay, pH gradient plates (used to analyze the effect of pH) were prepared the day before (instructions can be found in figures 3a and 3b). We obtained pH gradient plates with four different pH zones (5, 7, 8 and 9) as shown in figure 3c. Aliquots (80 µl) of detergent **C** (1/40 dilution) or saliva (1/1000 dilution) were aliquoted into each hole, and the plates were incubated at the desired temperatures for 48 hours. Next, the starch degradation halos in each condition were measured and photographed.

Laboratory session 5.

Quantitative and qualitative analysis of the influence of surfactants/oxidizing agents on detergent AAMY

In this part of the practicals, the hypothetical influence of several surfactants/oxidizing agents on the amylase activity in was studied. Three different compounds were prepared at two different concentrations: Triton X-100 (5% and 1%), SDS (0.1% and 1%) and H_2O_2 (5% and 1%). Students prepared these solutions, and detergent **C** was diluted 1/40. The detergent and the surfactant/oxidizing agent were mixed, and the Phadebas test at 60ºC was performed as explained above.

For qualitative analysis of the oxidant/surfactant effects, 0.5% starch - 1.5% agar plates were prepared as in laboratory session 3. Students also prepared the previously mentioned 1/40 dilutions of detergent **C** in the three different surfactant/oxidant agents. An 80-µl aliquot of each dilution was put into each hole, and the plates were incubated at 60ºC for 48 hours. After 48 hours, the plates were processed as in laboratory session 3. Students took photos of the plates and measured the diameter of each halo.

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RESULTS AND LABORATORY REPORTS

Previous tests of starch concentration using saliva samples

The starch concentration was tested in advance by the teachers to establish the optimal conditions for the qualitative analysis of saliva samples. Saliva obtained from the teachers (1-2 ml) was used. Three starch concentrations (0.5%, 1% and 1.5%) were checked. The plates were incubated at 4ºC, 40ºC or 60ºC for 48 hours. The results in figure 4a show the appearance of larger starch-degradation halos in the 0.5% starch plate compared with the other plates (1% and 1.5%). Additionally, in the 0.5% starch plate, the halos were observed at lower saliva dilutions than in the other two plates.

These preliminary results suggest that starch concentration is an important parameter for the sensitivity level (in terms of AAMY detection capacity) of the qualitative assay. We hypothesize that this could be related to the level of agar fluidity due to the starch concentration, making the 0.5% starch media more suitable in terms of the optimal dispersion of the enzyme through the plate and the minimal substrate concentration required.

Quantitative AAMY analysis

Three currently available laundry detergents were studied, and only detergents **A** and **C** had AAMY activity (figure 5). Detergent **C** showed higher AAMY activity than detergent **A**. Of the temperatures tested, the optimal temperature for AAMY activity in detergent **A** was 60ºC, but the best temperature for detergent **C** was 40ºC.

Although detergent **C** has its highest activity at 40ºC, it also has significant activity at 60ºC, reinforcing the concept that industrial enzymes are thermostable.

We also studied the saliva AAMY activity quantitatively (figure 4b). We observed that the best temperature for ptyalin was 40ºC, and no activity was observed at either 4ºC or 60ºC. It is noteworthy that the absolute amylase activity was nearly 18 times higher

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in the saliva samples (ptyalin at 40ºC) than in the detergents (detergent **C** at 40ºC), but the human enzyme was less resistant to significant temperature changes.

Qualitative AAMY analysis

The qualitative analysis (Lugol test) was done using the same three detergents (**A**, **B**, and **C**) and three temperatures (4ºC, 40ºC and 60ºC) as were used in the quantitative analysis. Although we had already determined that the best temperature for ptyalin was 40ºC, saliva samples were used as positive controls in all of the assays. This allowed us to confirm the specificity of the negative and positive results for the detergent amylases.

We tested the qualitative assay using two different incubation times (24 and 48 hours). Using this methodology, we were able to obtain AAMY activity halos only with detergent **C** after 48 hours of incubation and surprisingly only at 60ºC. This activity was seen as specific degradation halos that decreased during detergent dilution; detergents **A** and **B** had no AAMY activity at any temperature (figure 6).

The qualitative test was also done with saliva samples (plate distribution shown in figure 7b) using a starch concentration of 0.5%. It was clear that of the tested temperatures, the best temperature for saliva samples was 40Cº, which is consistent with the physiological context of the enzyme (figure 7a).

Correlation between qualitative and quantitative analysis

The quantitative test (Phadebas) seems to be more sensitive than the qualitative test (Lugol). We were able to observe AAMY activity at 40ºC and 60ºC in detergent **A** with the quantitative method but not with the qualitative method. Additionally, detergent **C** showed activity at both 40ºC and 60ºC using the Phadebas test; however, when the Lugol test was used, we saw clear starch-degradation halos only at 60ºC (with slightly transparent halos at 40ºC). Initially, we believed this result to be incorrect (e.g., due to bad manipulation or bad assay development), but we (students and teachers) obtained

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the same final result in all of the replicates performed. As a consequence, a result initially believed to be an error actually gave hypothetical information about the final structure of two enzymes with the same activity. This observation could be due to a different enzyme presentation/configuration of amylase from detergent compared with that from ptyalin (which was clearly positive in this assay).

However, this effect (i.e., no restriction due to hypothetical rigidity changes) was not evident in the quantitative assay, possibly because the liquid media does not present the same hypothetical accessibility restrictions as the solid media. The results of the quantitative and qualitative tests of the saliva samples were absolutely concordant.

pH influence on the activity of AAMY

We studied the influence of pH on the activity of AAMY in detergent **C** at both 40ºC and 60ºC because we observed significant enzymatic activity and unexpectedly better starch-degradation halos at 60ºC in the qualitative test. This observation facilitated the correlation between the quantitative and qualitative assays.

For saliva samples, we used incubation at 40ºC, which was the best temperature of those tested.

In the quantitative assay of detergent **C** (figures 8 and 9), a similar effect of pH was observed at both tested temperatures. The amylase activity was higher at pH 7, and there was a slight decrease in activity when the pH was increased (i.e., when the solution was made more alkaline). When a more acidic pH was used (pH 5), the activity was significantly affected compared with that at pH 7. This observation reinforces the concept that laundry detergent enzymes must be adapted to washing conditions with high alkalinity. The results of the qualitative assay for detergent **C** followed the same profile as previously observed: there were no clear starch-degradation halos at 40ºC (no evaluable results), but apparent degradation halos were seen at 60ºC. At this temperature, the clear differences obtained using the quantitative assay were less

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evident in the qualitative test, where only slight differences in the halo diameters were observed between different pHs.

In contrast, the quantitative test showed that the optimal pH for ptyalin activity at 40ºC (figure 10) was between 7 and 8. The results of the qualitative test suggested that of the pH values tested, the best pH was between 5 and 7, and a pH of 9 slightly affected ptyalin starch degradation activity. Although the differences obtained from the quantitative test were generally less evident, taken together, these data show that the optimal pH for ptyalin is around 7.

We have to keep in mind that the absolute activity of the detergent enzyme is nearly 18 times lower than that of the saliva enzyme. This fact could influence the results obtained in the detergent qualitative assays, which have been shown to be less robust during the pH test assays.

The influence of surfactants and oxidizing agents on the activity of detergent AAMY

The stability of the amylase enzyme from detergent **C** was assessed by exposing this enzyme to different surfactants and oxidizing agents. This assay also used both quantitative and qualitative methodologies.

Triton X-100 (1% and 5%) seemed not to affect AAMY activity. In contrast, the higher concentration (1%) of SDS decreased the enzymatic activity in both the quantitative and qualitative assays. The oxidizing agent hydrogen peroxide strongly affected the AAMY activity: we saw a low activity in the quantitative analysis, and we were not able to see halos in the qualitative analysis.

In this case, we were able to obtain a good relationship between the quantitative (figure 11a) and qualitative tests (figures 11b and 11c).

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DISCUSSION AND CONCLUSIONS

In this article, we describe our studies of the presence, activity and stability of AAMY in various commercially available detergents and saliva samples (ptyalin). Our results reinforce the knowledge of the optimal physiological conditions for the activity of salivary AAMY (in terms of temperature and pH) and demonstrate the importance of AAMYs, especially in the starch and food industries. The demand for liquefying AAMYs for use in laundry and automatic dishwashing detergents has been growing for several years [11]. In fact, detergent enzymes have to function in washing machines or dishwashers under conditions that are unfavorable for the stability of the enzyme. Therefore, this paper also emphasizes the need for detergent AAMYs to be thermostable. The pH is high (alkaline) in washing conditions, and the high temperature (55-60℃) in a dishwasher requires thermostable enzymes. Enzymes should also be resistant to various detergent ingredients, such as surfactants, chelators, and oxidants (bleach). However, most bacterial liquefying AAMYs are not suitable for use in laundry and dishwashing detergents with high alkalinity and temperature. As shown in our results, enzyme activity varies with temperature in both detergent and salivary AAMYs. Our results also suggest that, depending on the origin of the enzyme (human or bacteria), modifications may be made through genetic engineering to stabilize the enzyme for the demanding washing conditions. This possibility is shown in figures 5 and 6, where we could see differences in AAMY activity between the quantitative and qualitative assays of detergent **C** at 40ºC. Furthermore, the activity of ptyalin is positive in a qualitative assay context, whereas the detergent enzyme activity is negative.

It has often been reported that increased thermal stability of engineered enzymes is accompanied by a decrease in their catalytic activity at moderate temperatures, presumably as a consequence of the overall protein rigidity [12,13]. We therefore hypothesize that the amylase from detergent **C** may have a more rigid structure than the salivary amylase, leading to different accessibility to the substrate (starch). When

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we exposed detergent **C** to a temperature of 60ºC, where the starch-agar media may have a higher degree of fluidity, the detergent AAMY could easily access and degrade the substrate, creating starch-degradation halos. However, this hypothesis is only one possible explanation of the difference in detergent **C** activity between 40ºC and 60ºC in a qualitative assay; we cannot rule out the possibility that other factors could be wholly or partially responsible for this difference. For example, ptyalin is a more pure enzymatic form of AAMY compared with the complex matrix of components that go along with detergent AAMYs. Therefore, it is possible that one or several matrix components could have a restrictive effect on detergent AAMY activity in the qualitative assay at 40ºC but not at 60ºC.

The results obtained in the quantitative pH assays suggest that the activity of the enzyme from detergent **C** is higher at a neutral pH but is also consistent at alkaline pHs. Ptyalin has an optimal activity around pH 7, although it is also resistant to pH variations. In general, although there were minor weaknesses in the correlation with the quantitative assay (as for example at pH 5), the qualitative test reinforced the importance of neutrality (pH 7) for both enzymes, despite their different origins.

Regarding the different activity levels of the enzymes under different temperature and alkalinity conditions, it is interesting to note that ptyalin is affected to a greater extend by temperature than by pH, and the AAMY from detergent **C** seems to be more resistant than ptyalin to temperature changes.

If we compare the absolute enzymatic activity of both enzymes, it becomes evident that ptyalin has higher activity than detergent amylases. Although the enzyme concentrations in the different samples (saliva and detergent) are not known, we can speculate that ptyalin is in a more concentrated form; therefore, it seems logical that the saliva is 18 times more active than the detergent samples. Nevertheless, it is also possible that a hypothetical thermal stabilization (suggested by the similar activity levels of detergent **C** at 40ºC and 60ºC) of the detergent enzyme, which has been

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shown to be related to a decrease in catalytic activity, could also be responsible for part of the difference in the absolute activities of the different enzymes.

We also studied the influence of surfactants and oxidizing agents on the activity of detergent amylases. Detergent **C** AAMY was extremely stable in the presence of non-ionic surfactants, such as Triton X-100, and mildly stable in the presence of anionic surfactants, such as SDS. In general, SDS is known to be a strong denaturant of proteins, including AAMYs. SDS can unfold most proteins through the interactions between its charged head group and the positively charged amino acid side chains of proteins, as well as through the interactions between the alkyl chain of SDS and the non-polar regions on the surface and in the interior of proteins [14]. Stability in the presence of SDS is considered to be a very important factor in industrial enzymes because few SDS-stable enzymes have been reported thus far [15]. In contrast to SDS, Triton X-100 had no effect on the activity of detergent enzymes. Interestingly, it has previously been shown that this non-ionic detergent activates and stabilizes porcine pancreatic AAMYs. The activation and stabilization of 10 starch-degrading enzymes by Triton X-100 is thought to occur through binding of the detergent to the enzymes, which promotes an optimally folded and compact barrel motif structure and produces the maximum possible amount of activity [16].

One of the primary sources of protein instability is susceptibility to oxidation and subsequent inactivation or denaturation. This is especially true for proteins containing methionine, cysteine, or tryptophan residues in or around the active site. While methionine sulfoxide in proteins can be reduced *in vivo*, industrial applications using enzymes and proteins can be hampered by oxidative inactivation. Oxidation has been shown to have a negative effect on the stability of AAMYS. Cysteine 362 has been identified as the oxidation-prone residue in *Bacillus stearothermophilus* AAMY [17], and methionine 197, which is situated close to the active site, has been shown to be responsible for the inactivation of *Bacillus licheniformis* AAMY. Several attempts have

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been made to enhance enzyme stability by protein engineering because it is important to obtain enzymes with high stability against surfactants and oxidants for industrial applications. For example, the introduction of any non-sulfur-containing amino acid at position 197 has been shown to greatly reduce the sensitivity of *Bacillus licheniformis* AAMY to oxidation [18].

Detergent **C** AAMY was not stable in the presence of the oxidizing agent H_2O_2, as it showed a 3.5-fold decrease in activity after incubation with 1% H_2O_2 and a 7-fold decrease after incubation with 5% H_2O_2. These results suggest that the AAMY in detergent **C** has not been artificially modified to protect against oxidative insult.

It is also noteworthy that there are clear differences in the AAMY activities between smokers and non-smokers. Thus, the protocol presented here (both the quantitative and qualitative approaches) is also suitable for the study of ptyalin activity differences between smoking and non-smoking students.

The two methodologies described in this paper have different advantages. Generally, the qualitative test is good for screening samples for the presence or absence of AAMY activity and for determining which temperature or incubation time is more suitable. The quantitative test is better than the qualitative test for comparing the enzyme activity between different specific conditions (e.g., different temperatures and pHs and the presence or absence of surfactants/oxidizing agents).

The methodologies developed in these practicals are cheap and easy. They can be performed with several initial samples, and, in the case of the detergent enzymes, are complementary to the experiments presented in the previously published BAMBED article about detergent proteases [19].

The goal of the experiments presented in this article is to make students aware, both theoretically and practically, of a major industrial application of enzymes and of how various parameters affect the action of the final industrial product. Students learn that in addition to temperature and pH, there are other factors, such as ionic strength,

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surfactants and oxidizing agents, that can affect the enzymatic reaction. They also learn how each of these physical and chemical parameters must be considered and optimized in order for an enzymatic reaction to be accurate and reproducible. Furthermore, students learn how to apply and synthesize biotechnological concepts and biochemical principles and how to communicate ideas and information about what they have learned in the course. Discussions led by the course teacher and instructors, accompanied by supplementary lectures and bench experiments, help to give the problem a context and conceptual framework.

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FIGURE LEGENDS/LIST

Figure 1. Details of the distribution of student tasks for each laboratory session. **A** and **C** correspond to two standard detergents; **B** corresponds to a detergent for delicate clothes that contains no enzymes.

Figure 2. a: Quantitative amylase test (Phadebas test). b: Qualitative amylase test (Lugol test).

Figure 3. Preparation of pH agar-starch gradient (1.5%-0.5%) plates. a: The first portion of agar-starch (15 mL) and KH_2PO_4 (1 mL) is poured into a petri plate placed on an inclined board. The layer is allowed to solidify while the plate is held in the inclined position. b: The second portion of agar-starch (15 mL) and K_2HPO_4 (1 mL) is poured into a plate placed on a level surface. The layer is allowed to solidify while the plate is held in the level position. c: The final pH distribution along the starch-agar plate.

Figure 4. a: Lugol test using saliva samples to determine the most suitable starch concentration. b: Representation of the amylase activity (determined using the Phadebas test) in saliva at different temperatures**.**

Figure 5. Representation of the amylase activity in different detergents at different temperatures.

Figure 6. Comparison of the results of the Lugol test (qualitative test) with different incubation times. a: Lugol test, 24 h incubation. b: Lugol test, 48 h incubation.

Figure 7. a: Lugol test using saliva samples to determine the most suitable incubation time (24 or 48 h). b: Image depicting the plate locations of the different dilutions of saliva.

Figure 8. Representation of detergent **C** amylase activity in solutions of different pHs at 40ºC. a: Results of the quantitative test. b: Image of the qualitative test. c: Measurement of halos in the qualitative test was not possible.

Figure 9. Representation of detergent **C** amylase activity in solutions of different pHs at 60ºC. a: Results of the quantitative test. b: Image of the qualitative test. c: Measurement of halos in the qualitative test.

Figure 10. Representation of saliva amylase activity in solutions of different pHs at 40ºC. a: Results of the quantitative test. b: Image of the qualitative test. c: Measurement of halos in the qualitative test.

Figure 11. Representation of detergent **C** amylase activity in solutions of different oxidant/surfactant agents at 60ºC. a: Results of quantitative test, b: Image of qualitative test, c: Measurement of halos in qualitative test.

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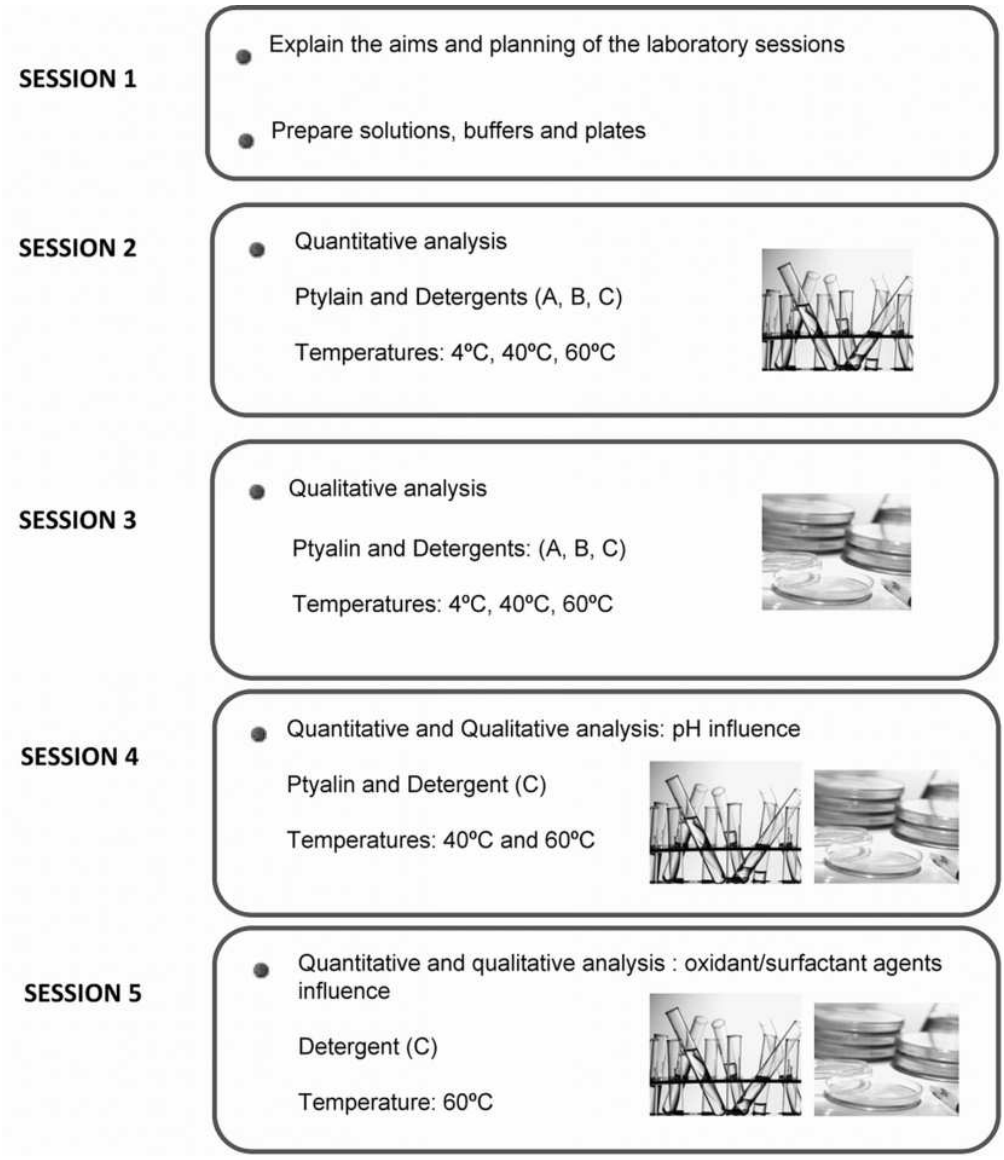

Figure 1. Details of the distribution of student tasks for each laboratory session. A and C correspond to two standard detergents; B corresponds to a detergent for delicate clothes that contains no enzymes.
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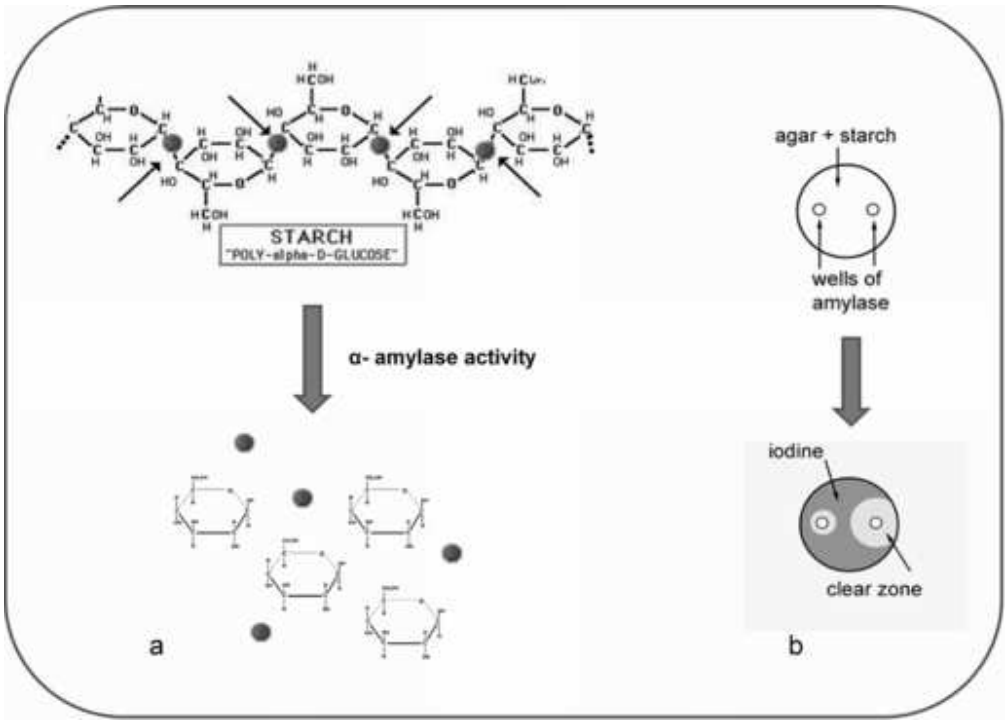

Figure 2. a: Quantitative amylase test (Phadebas test). b: Qualitative amylase test (Lugol test).
43x31mm (300 x 300 DPI)

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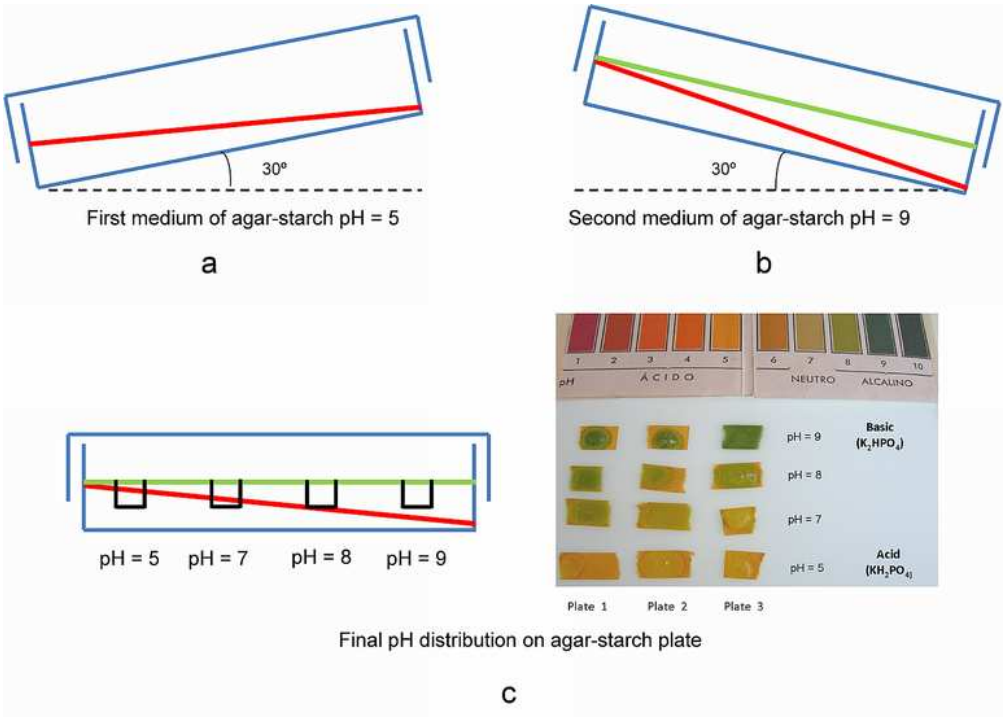

Figure 3. Preparation of pH agar-starch gradient (1.5%-0.5%) plates. a: The first portion of agar-starch (15 mL) and KH2PO4 (1 mL) is poured into a petri plate placed on an inclined board. The layer is allowed to solidify while the plate is held in the inclined position. b: The second portion of agar-starch (15 mL) and K2HPO4 (1 mL) is poured into a plate placed on a level surface. The layer is allowed to solidify while the plate is held in the level position. c: The final pH distribution along the starch-agar plate.
65x46mm (300 x 300 DPI)

View

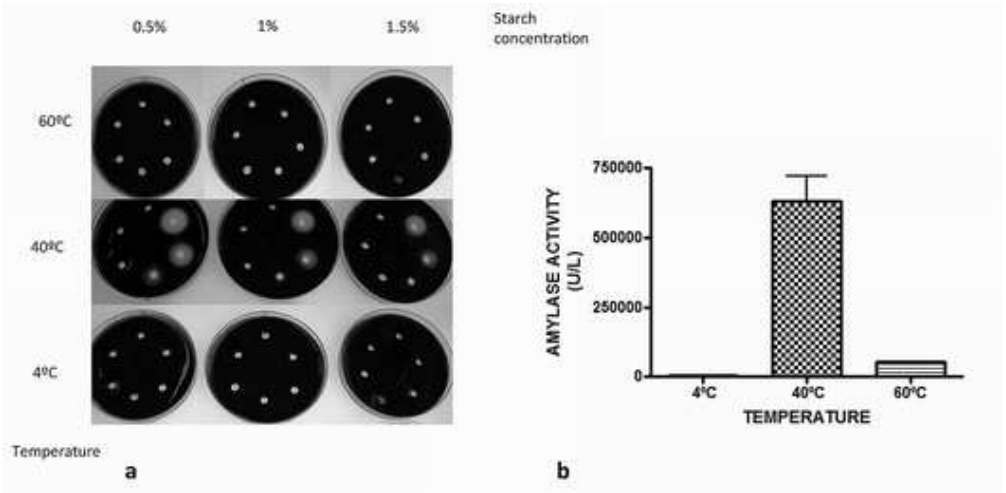

Figure 4. a: Lugol test using saliva samples to determine the most suitable starch concentration. b: Representation of the amylase activity (determined using the Phadebas test) in saliva at different temperatures.
44x21mm (300 x 300 DPI)

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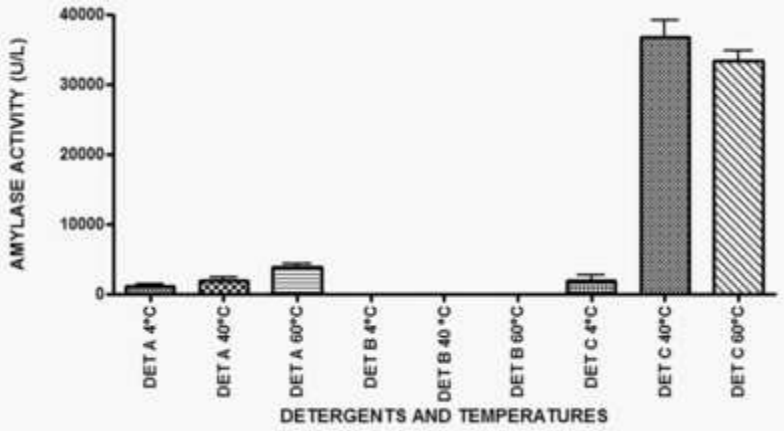

Figure 5. Representation of the amylase activity in different detergents at different temperatures.
33x18mm (300 x 300 DPI)

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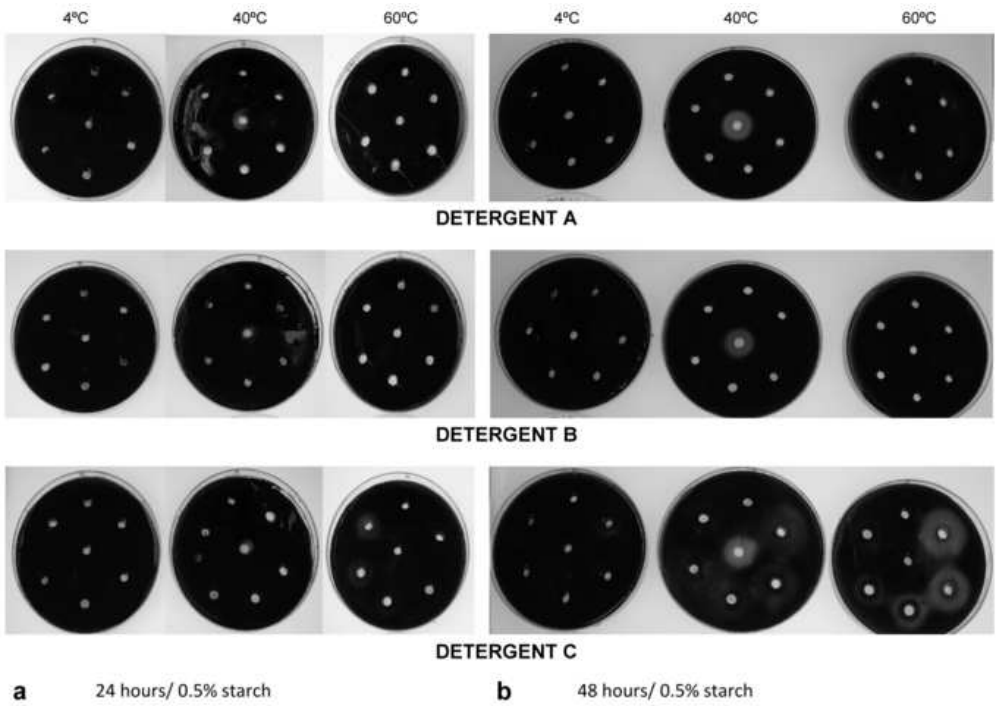

Figure 6. Comparison of the results of the Lugol test (qualitative test) with different incubation times. a: Lugol test, 24 h incubation. b: Lugol test, 48 h incubation.
57x40mm (300 x 300 DPI)

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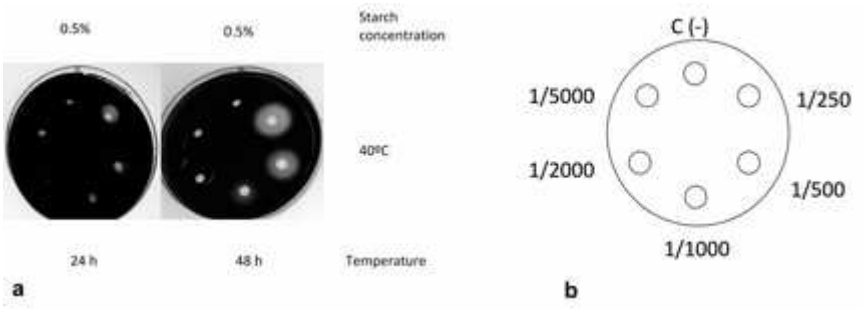

Figure 7. a: Lugol test using saliva samples to determine the most suitable incubation time (24 or 48 h). b: Image depicting the plate locations of the different dilutions of saliva.
36x12mm (300 x 300 DPI)

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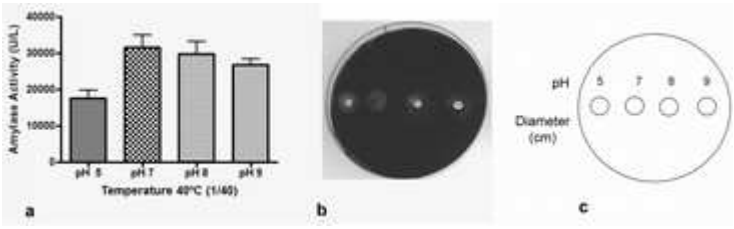

Figure 8. Representation of detergent C amylase activity in solutions of different pHs at 40ºC. a: Results of the quantitative test. b: Image of the qualitative test. c: Measurement of halos in the qualitative test was not possible.
30x9mm (300 x 300 DPI)

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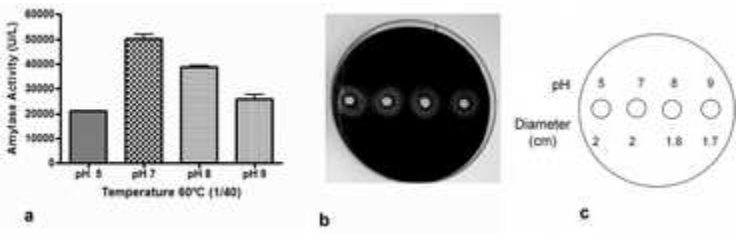

Figure 9. Representation of detergent C amylase activity in solutions of different pHs at 60ºC. a: Results of the quantitative test. b: Image of the qualitative test. c: Measurement of halos in the qualitative test.
31x9mm (300 x 300 DPI)

For Peer Review

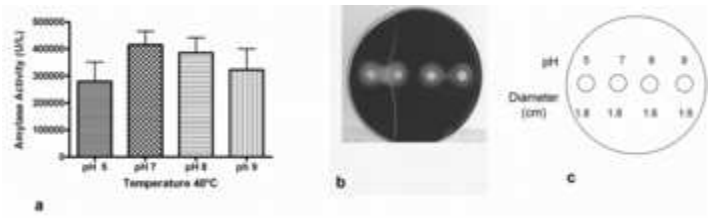

Figure 10. Representation of saliva amylase activity in solutions of different pHs at 40ºC. a: Results of the quantitative test. b: Image of the qualitative test. c: Measurement of halos in the qualitative test.
29x8mm (300 x 300 DPI)

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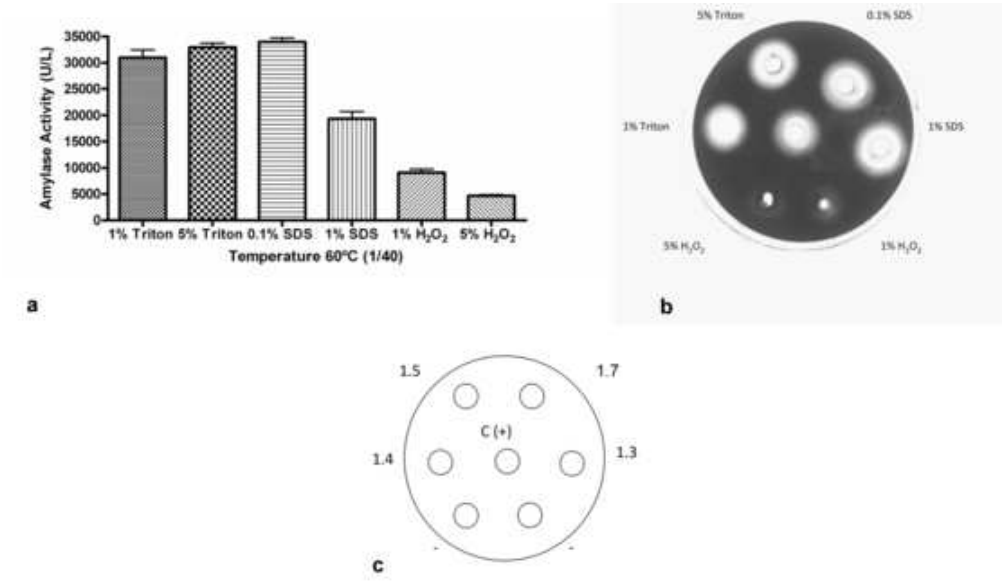

Figure 11. Representation of detergent C amylase activity in solutions of different oxidant/surfactant agents at 60ºC. a: Results of quantitative test, b: Image of qualitative test, c: Measurement of halos in qualitative test.
46x27mm (300 x 300 DPI)

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