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Physiological adaptive mechanism to oxidative stress in *Lactobacillus reuteri* DSM 17938

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Abstract

Probiotics are living microorganisms which could induce a potential health benefit to the host when consumed in adequate amounts. Among the different probiotics in the market, *Lactobacillus reuteri* strain DSM 17938 is a microaerophilic and heterofermentative organism that is able to tolerate high oxygen conditions compared with another *L. reuteri* and lactic acid bacteria strains. This study investigated the enzymatic and metabolic responses to oxidative stress by *L. reuteri* DSM 17938 and its effects on the enzymatic activities, the metabolism and survivability of the cells. Cultivation under aerobic conditions at different levels of aeration, 0%, 33%, 66% and 100% of air at 0.5 L/L/min, did not trigger a significant increase on the concentration of hydrogen peroxide (H₂O₂) accumulated that could affect generating an inhibitory effect. Specific enzymes activities were evaluated for NAD(P)H oxidases, NAD(P)H peroxidases and glutathione peroxidase; characteristic enzymes associated with the enzymatic oxidative stress mechanisms. A ten to thirty-fold increase in the activity of the NAD(P)H oxidases, NAD(P)H peroxidases, and glutathione peroxidase, proportional on the level of oxygen supplied to the system after a threshold of 33% of air at a rate of 0.5 L/L/min. The enzyme assays suggest that the lack of accumulation of H₂O₂ in the media was absorbed by the NAD(P)H peroxidases and glutathione peroxidase which were constitutively-active under anaerobic conditions but with a higher induction at higher aeration rates in contrast to NAD(P)H oxidase. The different levels of aeration were sufficient for a significant reduction in end-products typically found during anaerobic growth, ethanol and lactate, and an increase of the concentration acetate. At different levels of oxygen, NAD(P)H is mainly regenerated by the NA(P)DH oxidase - peroxidases system rather than through the production of ethanol and lactate allowing *L. reuteri* to produce an extra ATP by the production of acetate. These observations indicated that a coupled NAD(P)H oxidase – NAD(P)H peroxidase – glutathione peroxidase system was the main oxidative stress resistance mechanism in *L. reuteri* DSM 17938, and was regulated by oxygen availability.

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Introduction

Probiotics can be defined as living microorganisms which could induce a potential health benefit to the host when consumed in adequate amounts by maintaining or improving the microbial balance in the gut (Quigley, 2010). Their health benefits comprise the improvement of nutrients bioavailability (Pokusaeva, Fitzgerald, & van Sinderen, 2011), the treatment of colonic disorders (Dieleman et al., 2003; Madsen, Doyle, Jewell, Tavernini, & Fedorak, 1999), constipation (Huang & Hu, 2017) and acute rotoviral diarrhea (Ahmadi, Alizadeh-Navaei, & Rezai, 2015). Moreover, it has been reported that probiotics have also anti-osteoporosis, antihypertensive, and anticarcinogenic effects, among others (Chiang & Pan, 2012). Considering their potential health beneficial effects, probiotics have been increasingly incorporated during the last two decades to several supplements and food products.

Different fermentation technologies, such as fed-batch, continuous fermentation, membrane bioreactors, and immobilized cell technology are applied to produce probiotics with high cell yield and productivity. These technologies are considered convenient to propagate probiotics; however, they are limited by the susceptibility of the cells to toxic by-products (e.g. organic acid, hydrogen peroxide), resistance to oxidative stress and changes in their morphology and functionality. Therefore, the obtention of all the aforementioned beneficial health effects require the probiotic should have good technological properties that allow its production on a large scale without losing its functionality and viability (Lacroix & Yildirim, 2007).

Among the probiotics commercially available, lactic acid bacteria (LAB) are the most common probiotic bacteria and among them *Lactobacilli* such as *L. delbreuckii* ssp. *Bulgaricus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. lactis*, and *L. reuteri* are the most used probiotic microorganisms (Naidu, Bidlack, & Clemens, 1999; Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). *Lactobacilli* are Gram-positive, microaerophilic, rod-shaped bacteria, meaning that they thrive in environments with little or no oxygen depending on the oxidative stress resistance of each strain (Actor, 2012). During their production, manufacturers face difficulty preserving the viability of probiotics as strict anaerobic or low oxygen conditions are difficult to maintain during their production and storage (Anandharaj, Parveen Rani, & Swain, 2017). Consequently, due to economical and health reasons, it is crucial to overcome the problem of oxidative stress to facilitate high yield cell production with high stability in the final products; e.g. food, supplements, tables (Lacroix & Yildirim, 2007).

Oxidative stress is defined as the disproportion in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses

(Betteridge, 2000). In LAB, most of the ROS is generated in the form of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) due to their ability to tolerate oxygen and use it as electron acceptor during the regeneration of the enzyme cofactor NADH. To date it remains still unclear what mechanisms are present in oxidative stress' tolerant strains that mitigate the deleterious effects of these species; however different mechanisms like the reduction of the intracellular potential and the repair of oxidative damage by chaperones have been proposed as some of the mechanisms to explain their tolerance (Anandharaj et al., 2017).

In the case of *Lactobacillus*, with the exception of few reports on *L. plantarum* (Zotta, Ianniello, Guidone, Parente, & Ricciardi, 2014), *L. brevis*, *L. paralimentarius* (Brooijmans et al., 2009) and *L. panis* (Kang, Korber, & Tanaka, 2013); reports on the aerobic metabolism in other species are scarce. Nevertheless a series of studies performed in the laboratory of Applied microbiology at Lund University using different strains of *L. reuteri* have found that the strain DSM 17938 can tolerate H_2O_2 concentrations as high as 450 μM and most important, it seems to adapt to high oxygen levels (1.0 L/L/min) by growing faster than under anaerobic conditions with no apparently deleterious effects. The later finding is of crucial importance as the confirmation of aerobic growth by this strain may result in physiological and technological advantages that could be exploited to reduce the cost of production and to expand their use in several food products. Therefore, this study will focus to prove if the oxidative stress resistance of this strain is associated with an overexpression of the NAD(P)H (per)oxidases and its effects on the metabolism and survivability of the cells.

Background

Oxidative stress

Stress in microorganisms is defined as a physiological perturbation, originated by chemical, nutritional and/or physical alterations that could hamper the cell growth or even cause cell death (Fridovich, 1998; Giuliadori, Gualerzi, Soto, Vila, & TavíO, 2007). Among the different types of bacterial stress, the condition in which the production of free radicals, or ROS, cause a deleterious effect upon cell fitness is described as oxidative stress (Betteridge, 2000). Oxygen in its' free state is incapable to generate any physiological damage to the cell; nonetheless, in the different metabolic pathways, its reduction to water produces ROS as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\bullet$) (Figure 1.) (McCormick, Buettner, & Britigan, 1998). These species are characterized for their high oxidizing potential, which make them especially prone to react with nucleic acids and proteins causing metabolic path disruptions, spontaneous mutations, and bacteriostatic or bactericidal effects (Sies, 1997; Storz & Imlay, 1999).

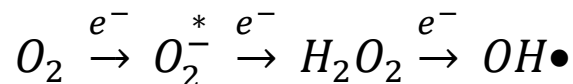


Figure 1. Oxygen and the reactive species produced during the incomplete oxygen reduction. hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\bullet$) and superoxide (O_2^-).

In the case of *L. reuteri*, it is a heterofermentative, aerotolerant bacterium that lacks a proton translocating electron transport chain but possesses oxidases which use oxygen to oxidize different substrates such as pyruvate, or NADH/NADPH (Sedewitz, Schleifer, & Götz, 1984; Stolz, Vogel, & Hammes, 1995). The aforementioned system converts oxygen into water using 4 electrons; however, during the intermediate steps of the reduction, ROS can be produced, depending on the type of oxidase produced by the microorganisms, e.g. NAD(P)H:H₂O oxidase uses 2 molecules of NADH or NADPH (4 e⁻) to react with one O₂ molecule to produce 2 molecules of water. However, other lactobacilli possess both or only a NAD(P)H:H₂O₂ oxidase producing H₂O₂ that accumulates, due to lack of electron donors; 2e⁻ instead of 4e⁻, which combined with high oxygen concentration that overflows the availability of electron donors generates the accumulation of ROS in the system (Figure 2.) (Condon, 1987; van Niel, Hofvendahl, & Hahn-Hägerdal, 2002).

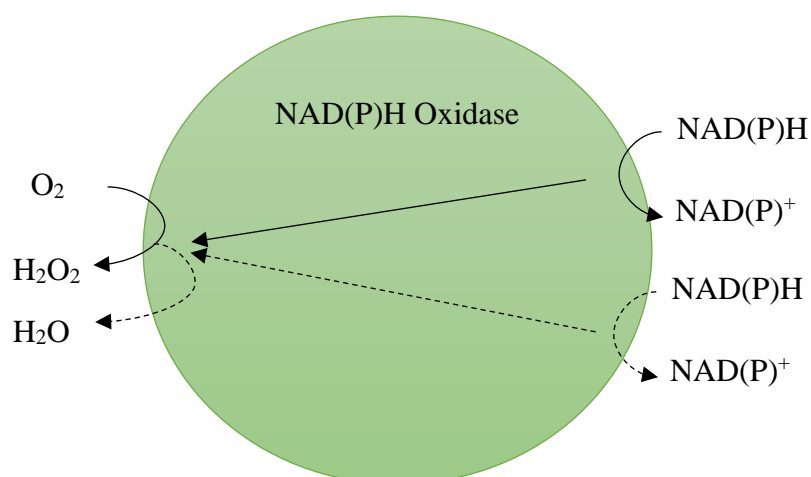


FIGURE 2. Schematic representation of the NAD(P)H oxidase:H₂O₂ (-) and NAD(P)H oxidase:H₂O (- -)

Oxidative stress effects at molecular level

ROS are intrinsically unstable and extremely reactive molecules due to their unpaired electrons. However, the level of their reactivity differs among the radicals by their capacity to accept or donate electrons to other molecules to acquire a more stable state (Sonomoto & Yokota, 2011). Since the majority of the intracellular molecules are non-radicals (e.g. proteins, nucleic acids, lipids and biologicals macromolecules), most of the free radicals tend to react with a non-radicals to acquire a more stable form. However, in the process a free radical chain reaction is generated producing new radicals which react with additional macromolecules (Sies, 1997).

In general, ROS tend to react with biological molecules, such as nucleic acids and proteins; nonetheless every free radical reacts with specific targets according to their oxidizing power. H₂O₂ is able to oxidize, without any intermediate step, protein cysteinyl residues, which inactivates the enzymes of the organism. It also reacts with cations through the Fenton and Haber-Weiss reactions (e.g. Fe²⁺ and Cu⁺) producing OH•, which can oxidize most of the organic compounds and break or modify the spectrum of bases in nucleic acids due to its' strong oxidizing potential (Duwat, Ehrlich, & Gruss, 1995). Also, it is able to affect the osmoregulation and permeability of the cell affecting the stability of the cell membrane lipids via peroxidation (Czapski, 1984). On the other hand, O₂⁻ has a moderate oxidizing potential which allows it to attack compounds such as ascorbate, catecholamines and polyphenols (Farr & Kogoma, 1991; Fridovich, 1998).

Oxidative stress effects on the metabolism

Over the years, the shift in the metabolisms caused by the presence of oxygen in the environment has been largely investigated in homofermentative but not in heterofermentative LAB. In homofermentative LAB, the hexoses are metabolized through the Emden Meyerhoff Pathway (EMP) to pyruvate, which in presence of oxygen is transformed into acetate and acetoin instead of lactate. In the case of heterofermentative LAB, recent investigations have found that in presence of oxygen or other electron acceptor, the metabolization of hexoses via the phosphoketolase pathway (PKP) shift the metabolism from ethanol to acetate as the major product of the transformation of acetyl phosphate and diminish the production of lactate, favoring the production of acetate. This shift is generated by the utilization of oxygen in the cofactor regeneration via NAD(P)H oxidases, which freed pyruvate to be also converted to acetate, enabling the microorganism to produce an extra ATP and regulate the pH through the production of acetate. However, the activity of the oxidases also produces ROS, as stated before, which induce a specific protective response in the cell (Ianniello, Zheng, Zotta, Ricciardi, & Gänzle, 2015). In the case of *L. reuteri*, Årsköld et al 2008 found that the strain ATCC 55730 is able to metabolize glucose through both pathways, EMP and PKP; using the former one as the main carbon metabolic pathway while the last one is PKP, while the EMP is used as a bypass. Therefore, it would be expected that the use of oxygen as electron acceptor by *L. reuteri* DSM 17938 have the same effect than in others heterofermentative LAB. However, the presence of both metabolic pathways could produce a different effect that should be evaluated.

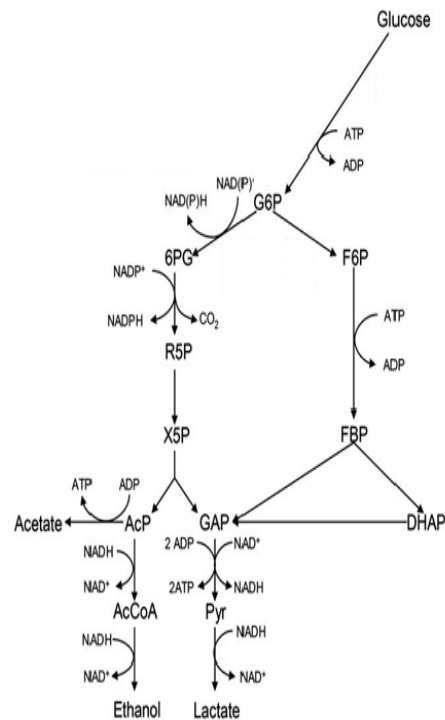


Figure 3. Glycolytic pathways in lactic acid bacteria. PKP (left) and EMP (right)

Oxidative stress resistance mechanisms in lactic acid bacteria

LAB is one of the few organisms that can thrive in the presence of oxygen but lack of part or a complete set of ROS scavenging enzymes. Instead, they use non enzymatic compounds (e.g. ascorbate, cysteine, Mn^{2+}) or enzymatic systems (oxidases, peroxidases, superoxide dismutases SOD) to diminish oxygen radicals. Experimental data indicate that the mechanisms used to handle ROS varies among different groups of *Lactobacilli* depending on the expression and types of mechanism as it has been found that some of them can thrive in media with high concentrations of H_2O_2 that could kill catalase-positive microorganisms, which has been hypothesized to be a mechanism of defense against other microorganisms (Sonomoto & Yokota, 2011).

Among the non-enzymatic mechanisms, manganese ions have been reported as superoxide scavengers used by LAB. The evidence shows that *L. plantarum* thrive well under aerobic conditions despite its lack of a SOD enzyme. Investigations on the reasons behind this behavior found that its' growth is positively affected by the availability of the ion in the medium under aerobic conditions. Also, it has been reported that *L. plantarum* can accumulate manganese ions intracellularly, up to 30 mM, which suggest that intracellular manganese ion might scavenge superoxide (Archibald & Fridovich, 1981).

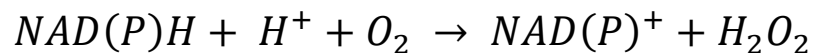
Cysteine have been reported as effective preventing the formation of H_2O_2 in *L. fermentum* (Hung, Cooper, Turner, Walsh, & Giffard, 2003). Hung et al, found that mutants of the surface protein responsible for normal cysteine uptake (BspA) are more sensible to oxidative stress. The addition of this amino acid to the medium showed to prevent the formation of H_2O_2 in aerobic cultures; however, its influence was more effective in the non-mutant strain, which indicates that *L. fermentum* uses exogenous cysteine to breakdown H_2O_2 (Hung et al., 2003).

Among the enzymatic mechanisms, it has been reported that *L. delbruekii subsp. bulgaricus* and *L. plantarum* among others LAB are able to reduce the concentration of O_2 through its reduction with a NADH-oxidase. The continuous detoxification through this mechanism led to overproduction of H_2O_2 (Marty-Teyssset, de la Torre, & Garel, 2000); however, it has been reported that some strains of *L. plantarum* and *L. casei* are able to reduce O_2 to water in a single step via a NADH: H_2O oxidase (Condon, 1987). On the other hand, the LAB that lacks this enzyme breaks down H_2O_2 and other ROS using supplementary enzymes to complete the reduction. *L. sanfranciscensis* reduces the H_2O_2 accumulated in the medium by a NADH-dependent peroxidase (Götz, Sedewitz, & Elstner, 1980; Stolz et al., 1995). Moreover, some strains of *L. sake* exhibit the capacity to diminish superoxide by a set of superoxide dismutase – Haem-dependent

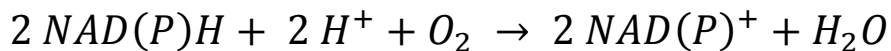
catalase enzymes that scavenge the ROS with H₂O₂ as an intermediate, and allows them to grow at a 90% higher growth rate than those with ROS sensitive enzymes (Knauf, Vogel, & Hammes, 1992).

Other H₂O₂-degrading enzyme system is the one used by *L. fermentum* which uses a glutathione (GSH) based system to diminish H₂O₂ into water. The experimental data had shown that *L. fermentum* ME-3 is able to use GSH from the medium to reduce H₂O₂ via glutathione peroxidase and glutathione reductase to diminish the oxidative stress in the medium. Also, the same investigation evidences that the strain is able to translocate GSH from the environment and synthesize it after a complete reduction process which makes *L. fermentum* ME-3 a perfect strain against oxidative stress (Kullisaar et al., 2010).

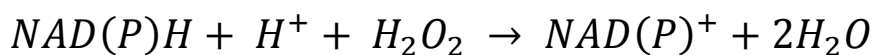
There are other enzymatic mechanisms described in the literature; however, they have been discarded as potential oxidative stress mechanisms as their components haven't been used during the experiments (e.g. Thioredoxin, Haem, α-glycerophate, among others). Therefore, it would be the purpose of this study to show that the mechanisms used by *L. reuteri* DSM – 17938 to tolerate oxidative stress are either by its NAD(P)H oxidase-peroxidase and/or glutathione reduce systems:



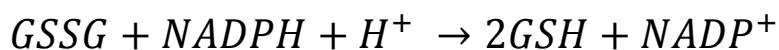
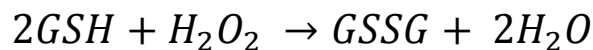
NAD(P)H: H₂O₂ oxidase



NAD(P)H: H₂O oxidase



NAD(P)H peroxidase



Glutathione peroxidase – reductase

Figure 4. Enzymes and reactions potentially involved in the oxidative stress protective mechanisms of *L. reuteri* DSM 17938

Materials and Methods

Bacterial strain, media and culture conditions

The *L. reuteri* strain DSM 17938, obtained from BioGaia AB, Stockholm, Sweden, was grown under anaerobic conditions at 37°C for 18 h in a 50 mL Falcon tube with 50 mL MRS medium (Merck, Germany). After incubation, the culture was used as inoculum (5% vol/vol) in a 3 L single wall bioreactor (Applikon, Netherlands) equipped with an ADI 1025 Bio-Console and an Applikon ADI 1010 control unit with a 1 L working volume consisting of SD4 medium (Table 1, for protocol see appendix 'SD4 Medium'). The culture was grown in an anaerobic pH-controlled batch mode until the optical density (OD_{620}) was greater than 0.6 and then the continuous mode was started with a dilution rate of 0.05 h^{-1} . The pH was maintained at 5.5 ± 0.1 by addition of 3 M KOH, the stirring speed was set to 200 rpm and the temperature was maintained at $37 \pm 1^\circ\text{C}$. Anaerobic conditions were obtained by sparging nitrogen gas through the medium before inoculation.

During the continuous cultivation, air was sparged (0.5 L/min) through the system at 4 different composition ratios (0% Air - 100% N_2 , 33.3% Air - 66.6% N_2 , 66.6% Air - 33.3% N_2 , and 100% Air - 0% N_2). The aeration was modified once the system reached steady state which was obtained once the optical density (OD_{620}) did not vary more than 10% after 3 residence times, during 2 consecutive residence times. Growth (OD_{620} and DCW), fermentation products (acetate, ethanol and lactate), hydrogen peroxide, and glucose were followed during the fermentation.

Samples (50 mL) were collected every 24 hours and centrifuged at $4000 \times g$ for 10 min at 4°C (Centrifuge 5810 R, Eppendorf). The supernatant was collected for the H_2O_2 quantification while the pellet was washed with TEA buffer (50 mM triethanolamine, pH 7.2). After a second centrifugation, the pellet was dissolved in 3 mL TEA buffer and stored at -20°C .

Dry cell weight and H_2O_2 quantification

The production of H_2O_2 was quantified by an enzymatic assay with ABTS (10.20 g/L) and horseradish peroxidase (0.35 mg/mL). The assay was done using 1 mL of supernatant mixed with 5 μL of ABTS and 5 μL of peroxidase per triplicate. The absorbance of the solution was measured at 433 nm until stabilization and the quantification was done with a calibration curve drawn using dilutions of pure H_2O_2 30% (range 0-15 μM).

The dry cell weight (DCW) was quantified by vacuum filtering 5 mL of cell-suspension solution with a pre-weighted filter paper per triplicate. The filter residue was washed three times with distilled water (5 mL), dried in a microwave during 8 min at 350 W and cooled down in a desiccator. The weight of the dried cells was calculated by subtracting the weight of the filter paper from the weight of the filter paper with the dried cells and dividing the value obtained by the volume of the sample.

Enzyme assays

Cell extracts (CE) were prepared from the pellets by disruption using the Precellys® 2 mL Bacteria Lysing kit (VK01) tubes filled with 1.2 mL of the harvested cells in a Cryolys Precellys® bead beater (Bertin Instruments, Montigny-le-Bretonneux, France) by 6 cycles of shaking during 30 s with 30 s interval of cooling on dry ice. The cytosolic CE content was obtained through removing cell debris by centrifugation at 13,200 × g for 2 min.

All enzyme assays were evaluated spectrophotometrically by measuring the oxidation of NAD(P)H. The activity assays were measured over 1 min using a spectrophotometer (Ultrospec 2100 pro, Amersham biosciences) at 340 nm ($\epsilon = 6.22/\text{mM cm}$) and 25 °C. The assays were done in a quartz cuvette with μL 900 of TEA buffer (50 mM triethanolamine, pH 7.2), 50 μL NAD(P)H (10 mM), CE (50 μL), 50 μL H₂O₂ (0.3%, peroxidase assay), 50 μL L-glutathione reduced (2 mM, glutathione peroxidase assay) and 5 μL glutathione reductase (100 U/ml, glutathione peroxidase assay; Sigma). The background activity of the assays were determined by replacing the CE with the reaction buffer in the case of the NAD(P)H oxidase assays while for the other two assays (NAD(P)H peroxidase and glutathione peroxidase) the H₂O₂ was the one replaced. The NAD(P)H peroxidase and glutathione peroxidase assays were performed with degassed TEA buffer supplemented with cysteine to reduce the oxidase activity of the CE. The assays were carried out in duplicate to estimate the mean values and standard deviations for the reduction activities. One unit of enzyme activity (U) is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per min. Protein concentrations were measured by the Bradford method (Bradford, 1976), quantifying with a calibration curve made with bovine serum albumin as standard (range 125-2000 $\mu\text{g/mL}$).

Measurement of substrate components and metabolites

Glucose, acetate, lactate, and ethanol were measured using HPLC. Separation proceeded at 30°C on 2 consecutive HPX-87H columns (300 mm 7.8 mm, Bio-

Rad, Richmond, CA) at an eluent flow-rate of 0.6 ml/ min. 5 mM H₂SO₄ was used as eluent. Components were identified and quantified using a Shimadzu™ SPD-6AV UV-VIS spectrophotometric detector. Product identification was based on comparison of retention times with those of standards prepared in fresh deionized water.

Statistical Analysis

Values were expressed as means (from 2 replicates) ± standard deviation (SD). Differences between groups were determined by one-way analysis of variance (ANOVA) and a t-test. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Continuous Fermentation

The experiment of this project consisted in running a continuous fermentation of *L. reuteri* at 4 different levels of air (0%, 33%, 66% and 100%) under the conditions stated in the previous section. This technique of cultivation due to its intrinsic characteristic guarantee stable growth rate, cell density and internal conditions once the steady state has been achieved (Lacroix & Yildirim, 2007). In the particular case of this project, a stable cell density and precise control of the oxygen (air) is essential to study the effect of the oxidative stress on protective mechanism of the strain and its metabolism.

In this project, only *L. reuteri* DSM 17938 was tested as previous investigations showed that this strain is particularly resistant to oxygen compared to other stress (Artsanchorn, 2018). Artsanchorn found that this particular strain is able to resist air at rates as high as 1 L/L/min while the strain PTA 4659 was only able to tolerate air at rates of less than 0.1 L/L/min. Therefore, it became of interest to know if the resistance of the *L. reuteri* DSM 17938 strain was associated with a set of NAD(P)H oxidases that do not produce H₂O₂ and/or system of NAD(P)H and glutathione peroxidases that are able to detoxify the medium to non-harmful levels for the microorganism despite the relative high amounts of oxygen supplied to the system.

Initially the system was set to operate with an aeration rate of 1 L/L/min; however, this condition generated an excessive amount of foam that could not be controlled by variations of the stirring rate, modification of the impeller location and cooling of the gases expelled through the condenser. As the foam in the system was beyond control despite all the solutions mentioned before, it was decided to run the fermentation with an aeration of 0.5 L/min, which reduced the risk of wash-out of the system and contamination of the medium feeding stream. Furthermore, it is important to point out that the rearrangement on the cultivation conditions should not have a negative impact on the results of the study as the main purpose was to check the response of the protective mechanisms at different levels of aeration regardless whether the extreme conditions were or were not tested. However, the variation on the conditions had an impact on the levels of H₂O₂ that were detected throughout the fermentation.

The fermentation was run during a period of 288 h with constant conditions except for the mix of gases sparged through the culture. The sampling was initially intended to be done every 3 hours during the first 9 hours of each air mix and then every 24 hours until the steady state was reached. However, this wasn't possible as the amount of sample necessary to be taken for the analysis; more

than 10% of the working volume, could generate big perturbances on the system and delay reaching a steady state. On the other hand, the H₂O₂ production and the enzyme activities of the samples taken during those first 18 hours were significantly the same which showed that extra sampling was unnecessary.

After growing cells under anaerobic condition until the system reached steady state, air was sparged through the system and samples were taken following the procedure described in the previous section. The measurement of the OD₆₂₀ was an indicator used to establish if the steady state has been reached but not a marker of the cell density of the system as it was done by a direct measurement of the DCW (Figure 5.). The results of this measurement showed that the cell density in the system is significantly constant during the whole fermentation, which indicates that the levels of aeration evaluated did not have any harmful effect on the growth of the strain. During this phase of the experiments, the measurements of H₂O₂ concentration showed that, under the conditions of the experiment, the production of H₂O₂ was reduced as it never reached a concentration greater than 9 uM. However, the results showed its concentration was significantly greater than the baseline; except at 48 h, and a positive correlation with the amount of air in the system (Figure 5).

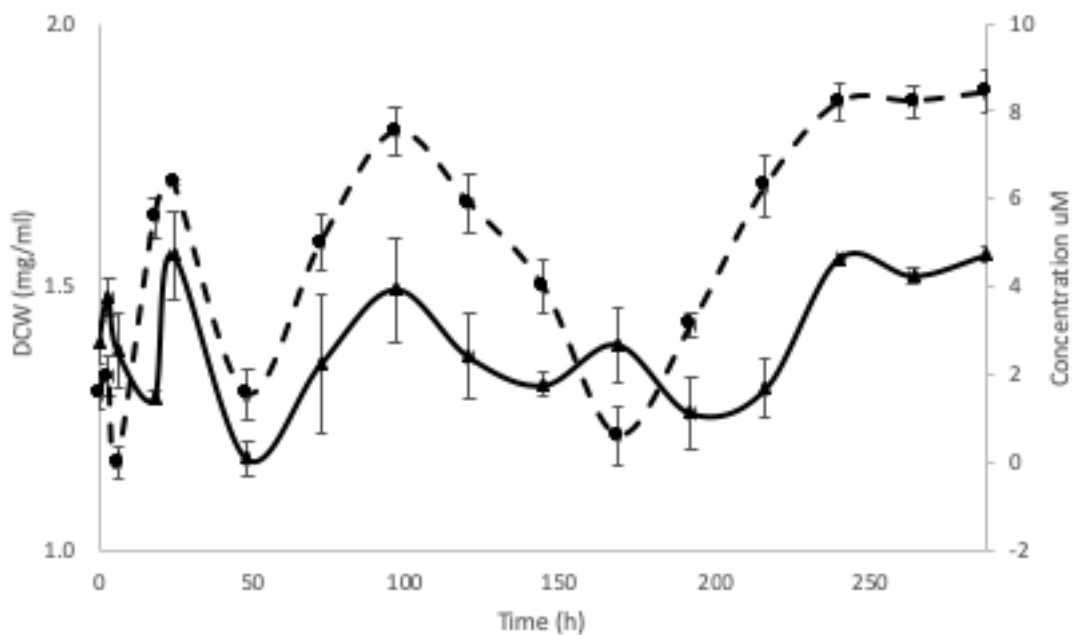


FIGURE 5. DCW (▲) and the production of H₂O₂ (●) of *L. reuteri* DSM 17938 over time when aerated with 500 mL/min of 4 different mixtures of air (0% at 0h, 33% from 0 to 120 h, 66% from 120 to 216 h and 100% from 120 to 216 h) together with stirring speed of 200 rpm.

Microscopy

Microscopy images were also taken at every sample point to check whether there was any detectable change in the shape and the distribution of the cells in the system throughout the fermentation. The images showed that there was no apparent change of shape and size of the cells; however, this impression is not conclusive as no measurement or any systematical and standardized analysis was performed on the images. On the other hand, the images revealed that the cells tend to aggregate and generate clumps, which became greater and denser as the amount of oxygen in the system increased.

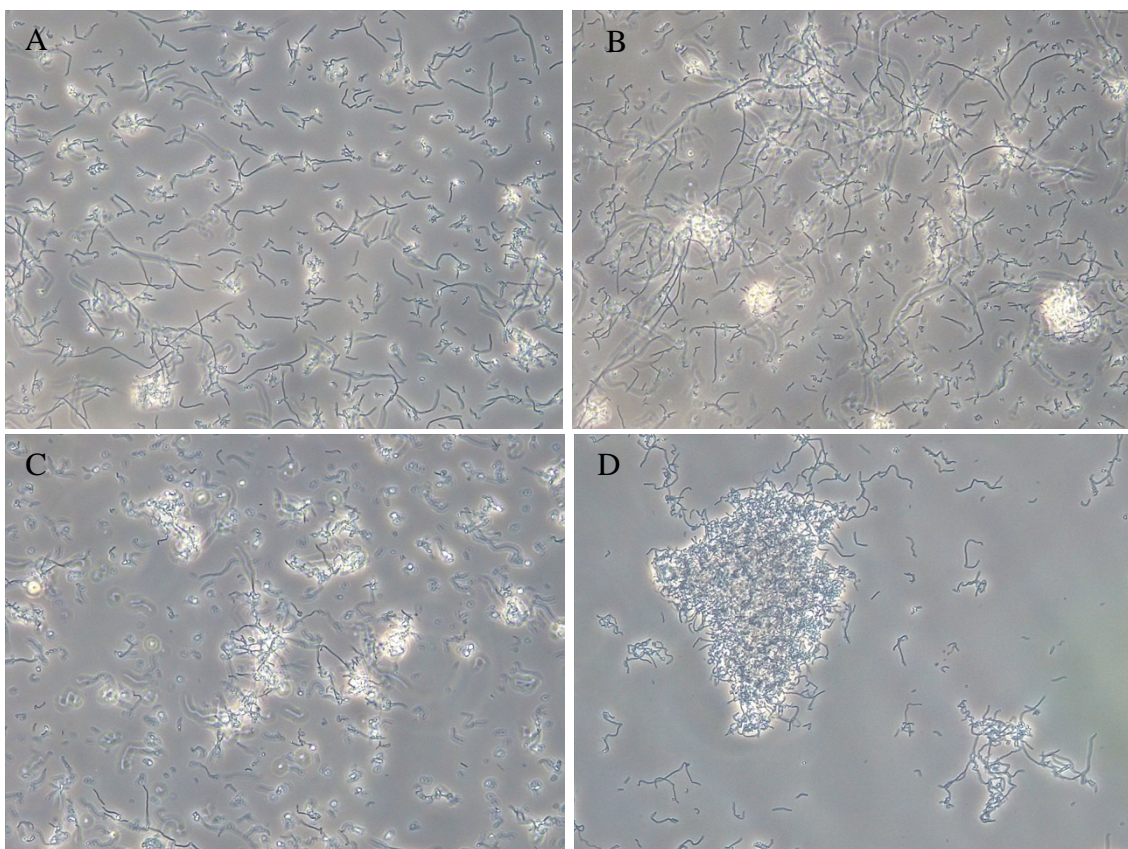


Figure 6. Microscopy images at 40X magnification of the continuous fermentation at different mixtures of air during 288 h. (A) Cells at 0 h and 0% of air, (B) 120 h and 33% of air, (C) 216 h and 66% of air, and (D) 288 h and 100% of air

Enzymatic Assays

Enzymatic assays were carried out to establish the presence and level of activity of NAD(P)H oxidase, NAD(P)H peroxidase and glutathione peroxidase in the strain. The results of the measurements showed that all the activity of the 5 enzymes were significantly the same during the first 96 hours; 0% and 33% of air. After 120 hours of fermentation, the results of the activity assays clearly show

a significant and proportional increase of activity of all the enzymes. The aforementioned indicates that under the condition of the experiment, the strain has a threshold near 33% of air at a rate of 0.5 L/L/min, under which it is able to tolerate oxygen without any further expressing of its oxidative stress protective enzyme system (Figure 7 and 8). Furthermore, once the system achieves a 100% aeration (after 216 h), the activity of the peroxidases almost equalise the ones of the oxidases. This finding suggests that this condition; 100% air at 0.5 L/L/min, is close to the limit of the amount of oxygen that the strain can process without producing a significant amount of H₂O₂ and others ROS.

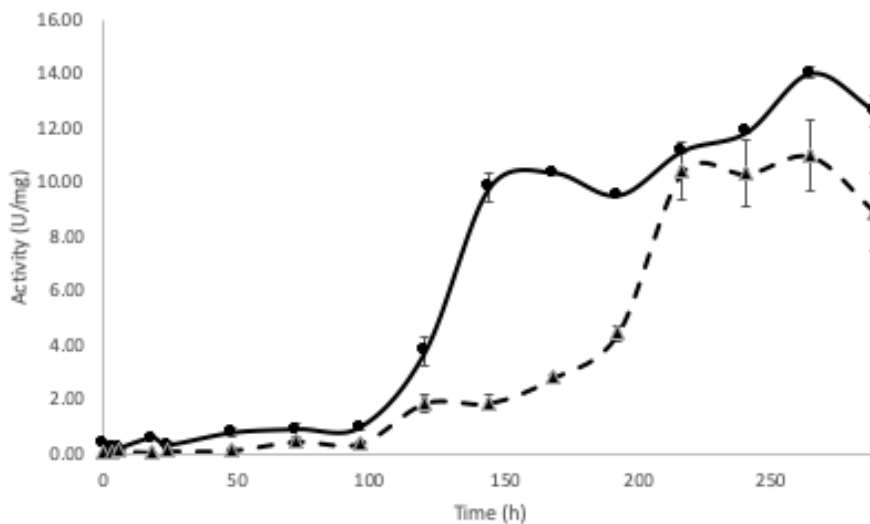


Figure 7 Enzyme activity of the NADH oxidase (●) and the NADH peroxidase (▲) of *L. reuteri* DSM 17938 over time when aerated with 500 mL/min of 4 different mixtures of air (0% at 0h, 33% from 0 to 120 h, 66% from 120 to 216 h and 100% from 120 to 216 h) together with stirring speed of 200 rpm.

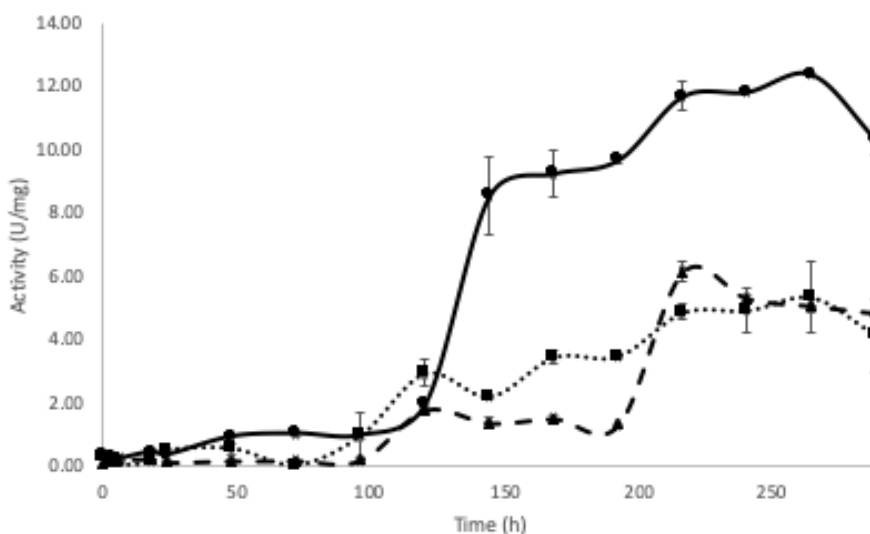


Figure 7 Enzyme activity of the NADPH oxidase (●), NADPH peroxidase (■), and glutathione peroxidase (▲) of *L. reuteri* DSM 17938 over time when aerated with 500 mL/min of 4 different mixtures of air (0% at 0h, 33% from 0 to 120 h, 66% from 120 to 216 h and 100% from 120 to 216 h) together with stirring speed of 200 rpm.

Metabolite Analysis

Supernatants of all the samples were analysed with HPLC at the end of the fermentation. The results showed that lactate is the most produced metabolite during the fermentation. However, its concentration constantly decreased with an increase in the level of oxygen; and oxidative stress. The same pattern was observed with the ethanol while the evolution of the amount of acetate increased proportionally with the amount of oxygen supplied to the system. The carbon balance on these 3 metabolites shows that under aerobic conditions, the strain tends to divert the carbon flow to the production of acetate than to the production of lactate and ethanol as the regeneration of cofactors is no longer done through these ways due to the use of oxygen as electron acceptor. Also, the high oxidative stress conditions during the fermentation produced elevated internal concentrations of H_2O_2 which chemically reacts with the intracellular pyruvate producing acetate and H_2O without ATP production as it was reported by van Niel et al 2002.

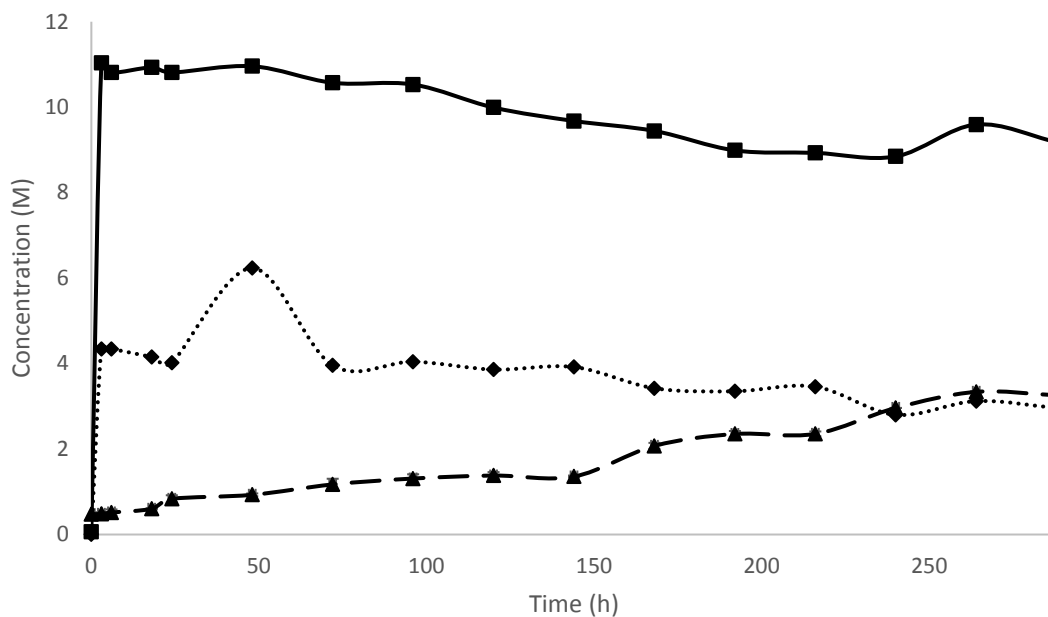


Figure 9. Metabolite profile through the continuous fermentation of *L. reuteri* DSM 17938 over time when aerated with 500 mL/min of 4 different mixtures of air (0% at 0h, 33% from 0 to 120 h, 66% from 120 to 216 h and 100% from 120 to 216 h) together with stirring speed of 200 rpm. (▲) Acetate, (◆) ethanol, and (■) lactate

Discussion

The goal of this project was to find out whether the oxidative stress resistance of *L. reuteri* DSM 17938 is associated with increased expression of the NAD(P)H (per)oxidases and/or glutathione peroxidase as a function of the aeration rate and the evaluation of its effects on the metabolism and survivability of the cells. Firstly, enzymatic assays were performed to quantify the activity of the enzymes which presumably conferred high resistance to oxidative stress to the evaluated strain. Secondly, the extracellular metabolites of the fermentation were quantified to establish the effect of the aerobic conditions on the metabolism. Lastly, pictures of the cells throughout the fermentation were taken to determine physical changes produced by the oxidative stress.

The enzymatic assays presented evidence of the use and increased expression of NAD(P)H oxidase, glutathione peroxidase, and NAD(P)H peroxidase as the enzymatic mechanisms of defense against the oxidative stress by *L. reuteri* DSM 17938. Activities of all the enzymes analyzed are present, especially that of glutathione peroxidase, which have a similar level of activity as the NADPH oxidase at all mixes of air evaluated. Furthermore, the activity profiles of all the enzymes were particularly the same during the fermentation, with increased levels of activity after 120 h and 216 h; 24 hours after the aeration mix was changed to 66% and 100% of air, respectively (Figure 7 and 8). This finding is particularly important since throughout the fermentation there was a reduced production of H₂O₂ (less than 10 μM) despite that aerobic growth of LAB is accompanied by the formation of ROS. Therefore, the increased level of activity of the enzymes after each increment of the amount aeration indicates that the strain possesses a finetuned enzymatic system of protection against ROS in which the oxidases are highly expressed under high oxygen fluxes in the culture; in a proportional way, to reduce all the oxygen in the system while the glutathione peroxidase and the NADH(P) peroxidases diminish the H₂O₂ produced to non-harmful concentrations.

The similar level of expression of the glutathione peroxidase compared to the NAD(P)H peroxidases indicates that the mechanism of defense against ROS in *L. reuteri* DSM 17938 is based on both enzymes but especially on glutathione peroxidase at high oxygen concentrations. This finding is particularly important as glutathione in association with the glutathione peroxidase is a major non-enzymatic antioxidant associated with the reduction of H₂O₂ and another ROS like hydroxyl radical and lipid peroxides while the physiological glutathione redox ratio (GSH/GSSG) is maintained within the cell (Kullisaar et al., 2010). In a previous investigation, Artsanchorn (2018) reported that this strain is able to produce H₂O₂ and thrive at a concentration as high as 450 μM (double of aeration) for some hours until the radical is almost eliminated of the medium. The

aforementioned evidences that the strain has a demonstrated high ROS tolerance at concentrations oxygen that could hamper other microorganisms, even of the same species, which suggest that the strain has a complete glutathione system (glutathione peroxidase/reductase) to maintain the glutathione redox balance. However, it remains pending to establish if *L. reuteri* DSM 17938 has a complete functional glutathione system to maintain the internal glutathione redox balance as *L. fermentum* ME-3 (Kullisaar et al., 2010), considering that the activity of the glutathione peroxidase has been demonstrated in this study and bioinformatic analysis shows that *L. reuteri* possesses more than 10 potential genes that could codify an homologous protein (~35% homology) to the *Lactobacillus acidophilus* glutathione reductase (GenBank AAV42951.1).

Quantification of the extracellular metabolites revealed that during the fermentation there was simultaneous production of acetate, lactate and ethanol as has been described in previous reports (Arsköld et al., 2008; Ianniello et al., 2015). The trends of the metabolites analyzed show that in aerobic conditions there is a shift towards the increase of the production of acetate with a reduction of the lactate and ethanol produced as a consequence. The shift could be explained by the fact that the use of oxygen as electron acceptor eliminates the need to regenerate the NADH through the production of ethanol; while the decline in the production of lactate is explained by the spontaneous reaction of pyruvate with H₂O₂ not driven by the ATP production (van Niel, Hofvendahl, & Hahn-Hägerdal, 2002). Moreover, the increase in the production of acetate; proportional with the supply of oxygen, allows the obtention of additional energy supply through extra ATP generation (via acetyl phosphate to acetate and ATP) (Figure 3) which could be used to overexpress and maintain the oxidative stress protective enzymes; oxidases and peroxidases. The aforementioned confirms previous findings in heterofermentative LAB, which showed that oxygen shift ethanol production to acetate as main product of acetyl phosphate (AcP) and also redirects the metabolism from pyruvate to acetate at the expense of lactate (Gänzle, Vermeulen, & Vogel, 2007; Kang et al., 2013; Lorquet et al., 2004). However, the pathway of the acetate formation from pyruvate in *L. reuteri* is still unclear; therefore, it should be necessary to examine if the strain possesses an active pyruvate dehydrogenase (Jensen, Melchiorsen, Jokumsen, & Villadsen, 2001), a pyruvate oxidase (Goffin et al., 2006) or any other enzyme to completely elucidate the aerobic metabolic pathway of the strain.

Finally, the microscopic images show that the strain exhibits a strong autoaggregation phenotype which have an apparent positive correlation with the oxygen in the medium. This ability has been found in some LAB to escape and mitigate the effect of the oxygen stress (Yan et al., 2016), possibly due to the significant reduction of oxygen diffusivity (Xu, Xie, Feng, & Su, 1998) produced by the aggregates which could generate micro-anaerobic environments at the center of the clumps.

Conclusion

In conclusion, three techniques have been applied to answer the question on how *L. reuteri* DSM 17938 resist oxidative stress. The assays of different enzymes have shown that the strain is able to use oxygen as an electron acceptor using and adjust the expression levels of the enzymes NAD(P)H oxidases/peroxidases and glutathione peroxidase depending on the amount of oxygen in the system. The quantification of the metabolites also indicated that the strain is using oxygen as electron acceptor by diverting the metabolism of acetyl phosphate and pyruvate to produce more acetate, at the expense of ethanol and lactate, as the use of oxygen as electron acceptor eliminates the need to regenerate the NADH via catabolic metabolites (pyruvate and AcP) and allows the generation of extra ATP. Finally, the cells have been visualized using microscopy pictures, displaying that the strain exhibit a strong autoaggregation phenotype with an apparent positive correlation with the amount of the oxygen in the medium, which mechanism has to be further investigated. Combining the results from the enzymatic assays and the pictures obtained from the microscopes, the results suggest that the great resistance to oxidative stress of *L. reuteri* DSM 17938 is a combination of the physical protection provided by the autoaggregation ability of the strain and its highly active NAD(P)H oxidases/peroxidases and glutathione peroxidases.

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Appendix

SD4 Medium

The recipe for 1 liter of SD4 medium for *L. reuteri* is the following:

- Sugar solution

25 g of glucose dissolved in 200 ml of H₂O sterilized in the autoclave.

- Salt solution

K₂HPO₄ (2.5 g), KH₂PO₄ (2.5 g), MgSO₄ • 7H₂O (0.25 g), Sodium acetate (0.5 g), and MnCl₂ • 2H₂O (0.013 g) dissolved in 200 ml of H₂O. The pH of the solution is adjusted to 5.5 with 5M H₂SO₄ and sterilized in the autoclave.

- Tween 80

10 g of glucose dissolved in 90 g of H₂O sterilized in the autoclave.

- Casamino acid solution

5g of glucose dissolved in 50 ml of H₂O sterilized by filtration.

- Amino acids solution

Reduced glutathione (0.01 g), L-Alanine (0.06 g), L-Serine (0.2 g) L-Tryptophan (0.03 g), L-Asparagine (0.2 g), L-Cysteine (0.17 g), and L-Glutamine (0.2 g) dissolved in 50 ml of H₂O sterilized by filtration.

- UAG solution

Uracil (0.4 g), adenine (0.2 g), guanine (0.2 g) and NaOH (50%) (0.24 g) in 30 ml of H₂O sterilized by filtration.

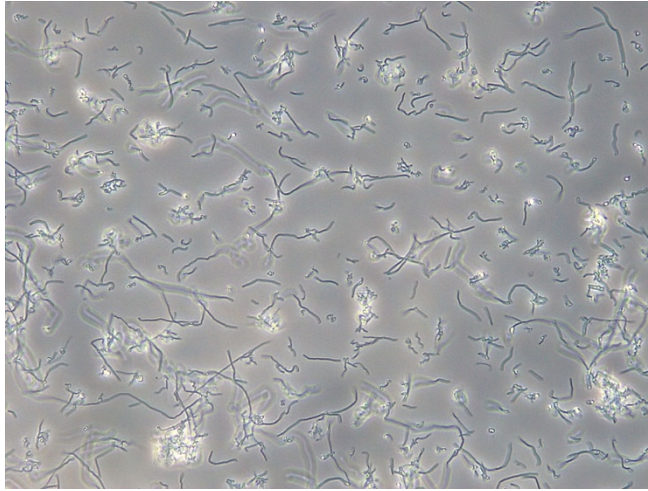
- Vitamin solution

10 ml of the solution composed by biotine (10 mg/L), pyridoxal – HCl (206 mg/L), folic acid (10 mg/L), riboflavin (98 mg/L), nicotinic acid (98 mg/L), thiamine – HCl (101 mg/L) Ca-Pantothenate (95 mg/L), and P-Aminobenzoic acid (10 mg/L) dissolved in H₂O

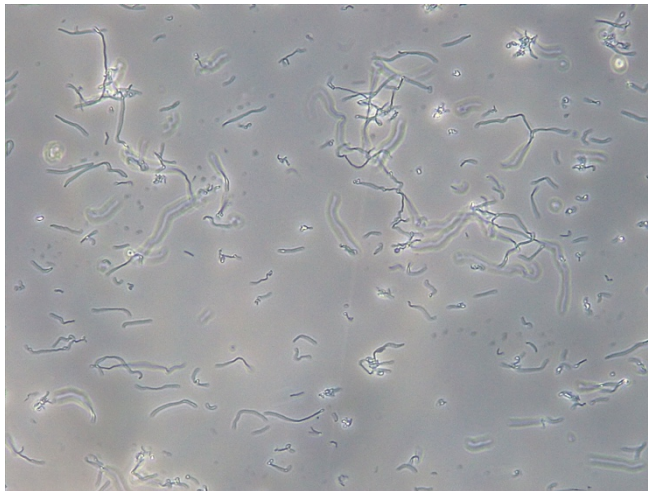
- Trace element solution

1 ml of a 100 ml solution prepared dissolving EDTA (1.5 g) and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.45 g) in 50 ml of H_2O , adjusting the pH to 6.0. Then $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1 g), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.03 g), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.03 g), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.04 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.45 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.3 g), H_3BO_3 (0.1 g), and KI (0.01 g) are added and the pH is adjusted to 6.0 after each addition. The solution is sterilized by filtration.

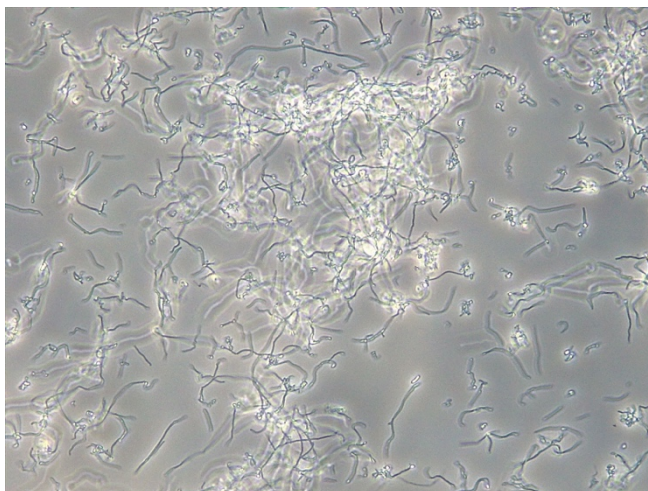
Microscopy images



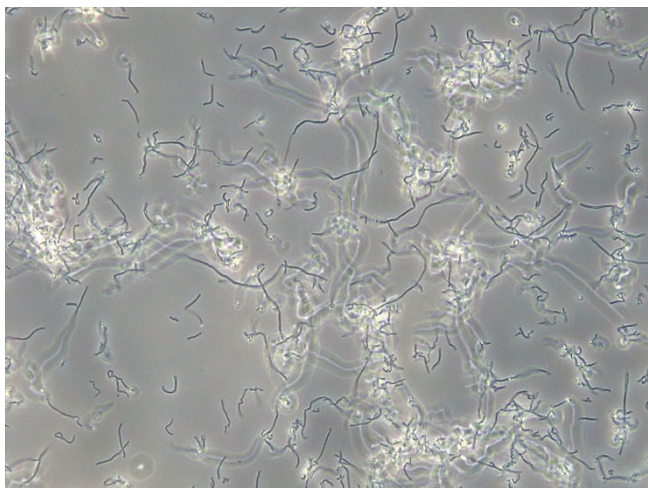
Microscopy image at 40X magnification of the continuous fermentation at 0 h and 0% of air (100% N₂) with an air rate of 0.5 L/V/min.



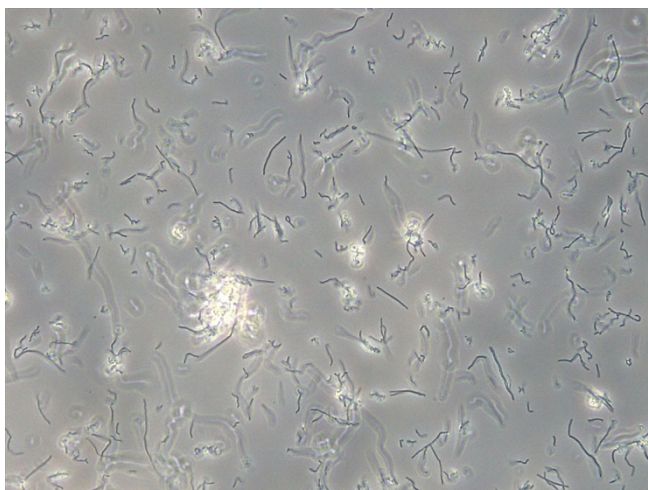
Microscopy image at 40X magnification of the continuous fermentation at 3 h and 33.3% of air (66.6% N₂) with an air rate of 0.5 L/V/min.



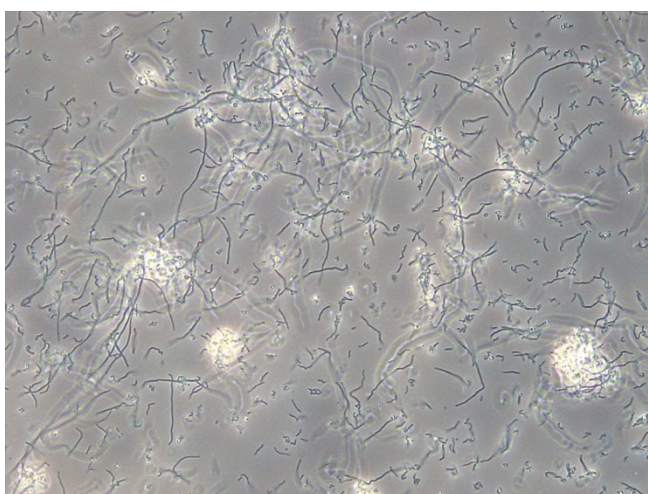
Microscopy image at 40X magnification of the continuous fermentation at 18 h and 33.3% of air (66.6% N₂) with an air rate of 0.5 L/V/min.



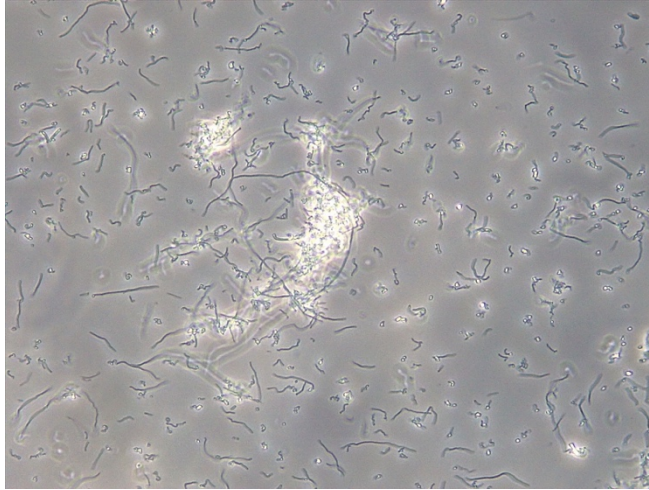
Microscopy image at 40X magnification of the continuous fermentation at 24 h and 33.3% of air (66.6% N₂) with an air rate of 0.5 L/V/min.



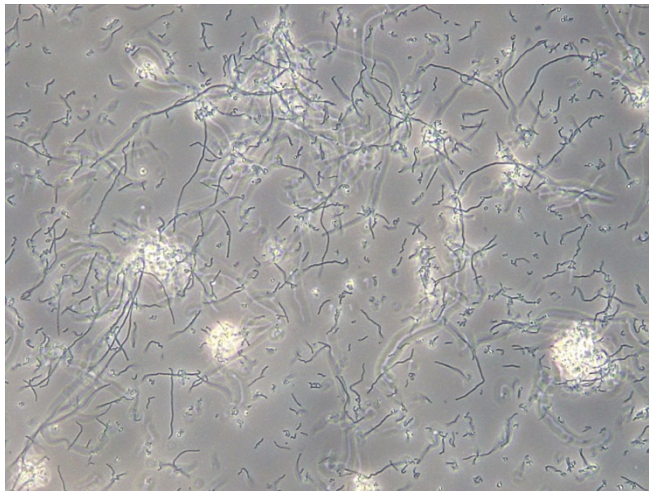
Microscopy image at 40X magnification of the continuous fermentation at 48 h and 33.3% of air (66.6% N₂) with an air rate of 0.5 L/V/min.



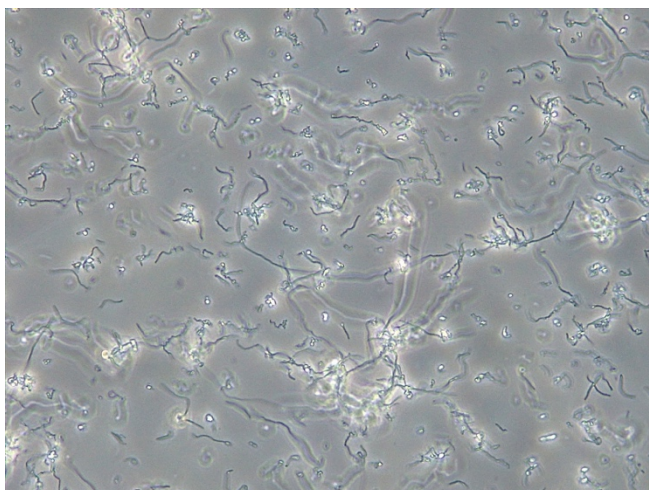
Microscopy image at 40X magnification of the continuous fermentation at 72 h and 33.3% of air (66.6% N₂) with an air rate of 0.5 L/V/min.



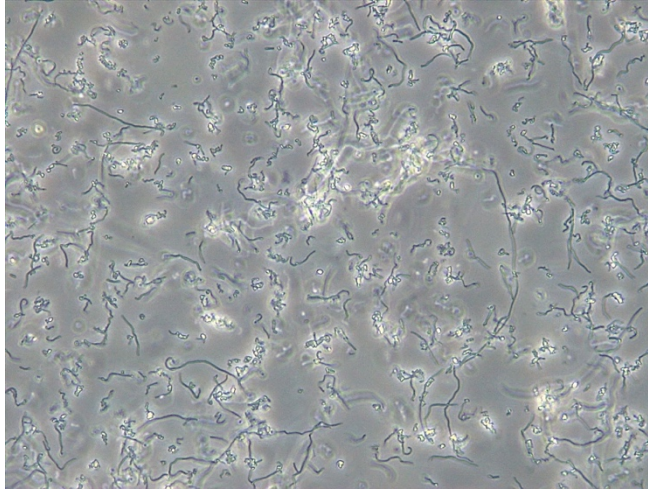
Microscopy image at 40X magnification of the continuous fermentation at 96 h and 33.3% of air (66.6% N₂) with an air rate of 0.5 L/V/min.



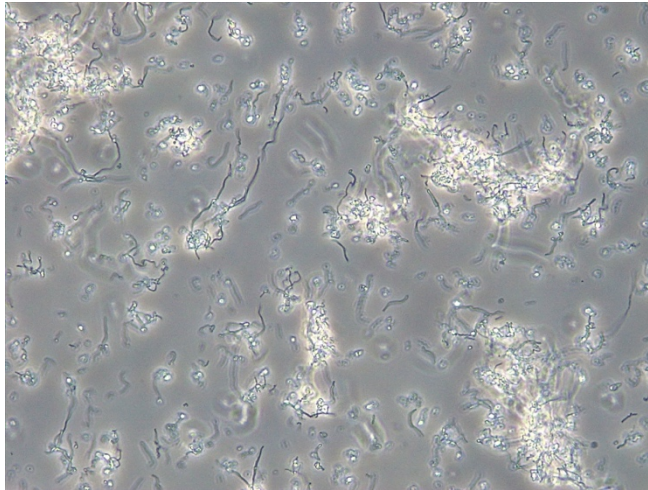
Microscopy image at 40X magnification of the continuous fermentation at 120 h and 33.3% of air (66.6% N₂) with an air rate of 0.5 L/V/min.



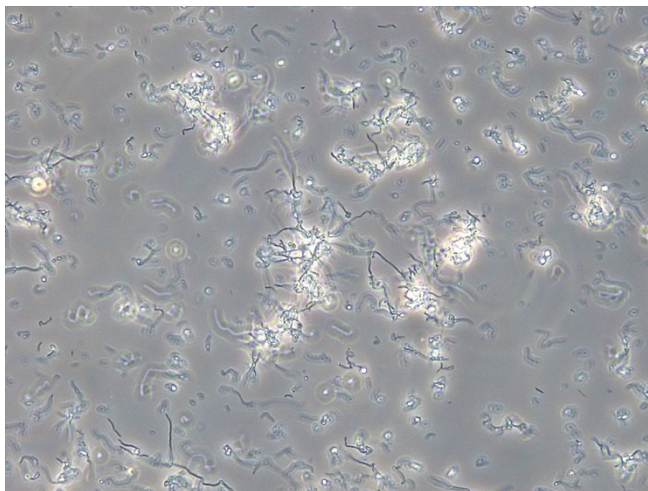
Microscopy image at 40X magnification of the continuous fermentation at 144 h and 66.6% of air (33.3% N₂) with an air rate of 0.5 L/V/min.



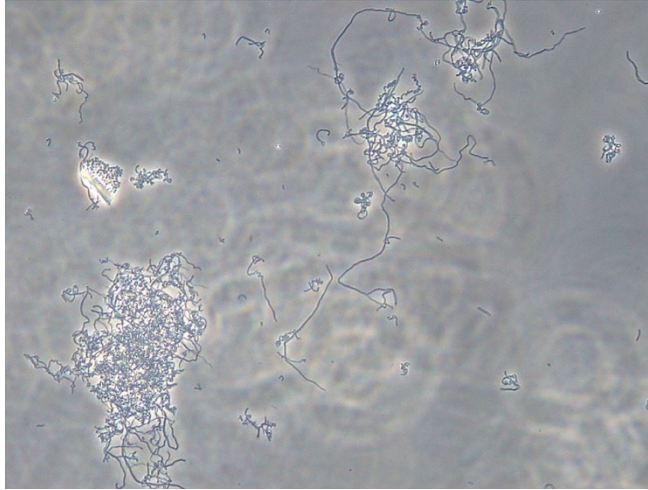
Microscopy image at 40X magnification of the continuous fermentation at 168 h and 66.6% of air (33.3% N₂) with an air rate of 0.5 L/V/min.



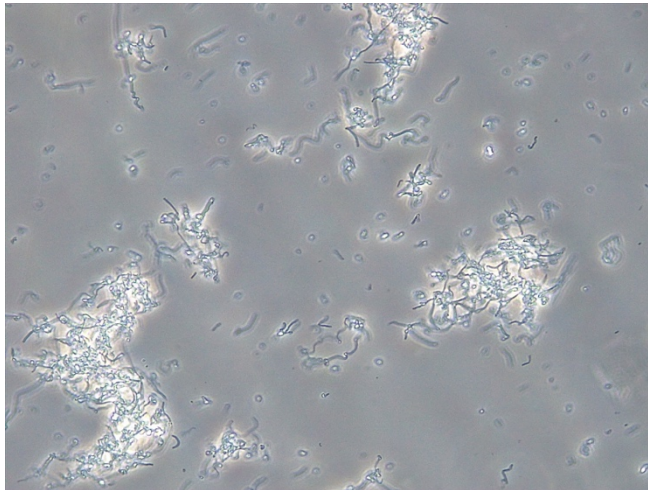
Microscopy image at 40X magnification of the continuous fermentation at 192 h and 66.6% of air (33.3% N₂) with an air rate of 0.5 L/V/min.



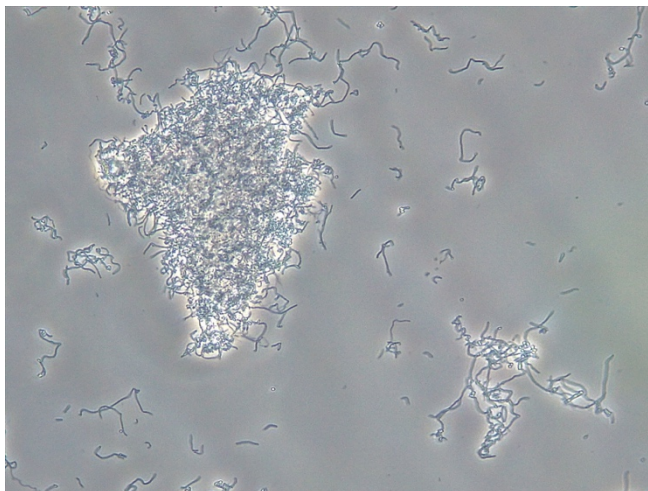
Microscopy image at 40X magnification of the continuous fermentation at 216 h and 66.6% of air (33.3% N₂) with an air rate of 0.5 L/V/min.



Microscopy image at 40X magnification of the continuous fermentation at 240 h and 100% of air (0% N₂) with an air rate of 0.5 L/V/min.



Microscopy image at 40X magnification of the continuous fermentation at 264 h and 100% of air (0% N₂) with an air rate of 0.5 L/V/min.



Microscopy image at 40X magnification of the continuous fermentation at 280 h and 100% of air (0% N₂) with an air rate of 0.5 L/V/min.

Data of the enzymatic assays and the continuous fermentation

Time (h)	DCW (mg/ml)	H ₂ O ₂ (uM)	Protein (mg/ml)	NADH Oxidase (U/mg)	NADH Peroxidase (U/mg)	NADPH Oxidase (U/mg)	NADPH Peroxidase (U/mg)	Glutathione Peroxidase (U/mg)	Lactate (M)	Acetate (M)	Ethanol (M)
0	1.39 ± 0.04	1.6 ± 0.4	0.28 ± 0.01	0.41 ± 0.06	0.05 ± 0.06	0.38 ± 0.01	0.27 ± 0.07	0.05 ± 0.04	0.06	0.48	0.00
3	1.48 ± 0.03	2.0 ± 0.4	0.28 ± 0.00	0.18 ± 0.15	0.04 ± 0.04	0.31 ± 0.01	0.16 ± 0.04	0.18 ± 0.00	11.04	0.48	4.34
6	1.38 ± 0.07	0.0 ± 0.4	0.24 ± 0.00	0.21 ± 0.01	0.10 ± 0.07	0.24 ± 0.00	0.04 ± 0.01	0.21 ± 0.03	10.81	0.52	4.34
18	1.29 ± 0.01	5.6 ± 0.5	0.19 ± 0.00	0.57 ± 0.05	0.05 ± 0.01	0.45 ± 0.12	0.13 ± 0.07	0.22 ± 0.02	10.93	0.60	4.15
24	1.56 ± 0.08	6.4 ± 0.1	0.25 ± 0.01	0.32 ± 0.12	0.10 ± 0.04	0.36 ± 0.13	0.50 ± 0.09	0.12 ± 0.03	10.81	0.83	4.02
48	1.17 ± 0.03	1.6 ± 0.6	0.23 ± 0.00	0.76 ± 0.15	0.12 ± 0.10	0.93 ± 0.02	0.55 ± 0.18	0.17 ± 0.03	10.96	0.93	6.24
72	1.35 ± 0.13	5.0 ± 0.6	0.32 ± 0.01	0.89 ± 0.25	0.45 ± 0.09	1.02 ± 0.11	0.03 ± 0.02	0.17 ± 0.07	10.57	1.17	3.96
96	1.49 ± 0.10	7.5 ± 0.6	0.34 ± 0.01	0.99 ± 0.09	0.38 ± 0.10	0.97 ± 0.12	0.97 ± 0.72	0.19 ± 0.08	10.53	1.31	4.04
120	1.37 ± 0.08	5.9 ± 0.7	0.28 ± 0.01	3.78 ± 0.52	1.87 ± 0.32	1.95 ± 0.02	2.93 ± 0.42	1.77 ± 0.09	9.99	1.38	3.86
144	1.31 ± 0.02	4.0 ± 0.6	0.32 ± 0.02	9.82 ± 0.55	1.87 ± 0.29	8.53 ± 1.25	2.19 ± 0.07	1.36 ± 0.16	9.68	1.36	3.92
168	1.39 ± 0.07	0.6 ± 0.7	0.32 ± 0.01	10.36 ± 0.05	2.79 ± 0.15	9.23 ± 0.76	3.44 ± 0.19	1.50 ± 0.08	9.44	2.07	3.42
192	1.26 ± 0.07	3.1 ± 0.3	0.36 ± 0.01	9.53 ± 0.05	4.45 ± 0.29	9.67 ± 0.14	3.47 ± 0.09	1.31 ± 0.04	8.99	2.35	3.35
216	1.31 ± 0.06	6.3 ± 0.7	0.50 ± 0.03	11.16 ± 0.08	10.38 ± 1.06	11.68 ± 0.44	4.86 ± 0.26	6.13 ± 0.32	8.93	2.36	3.46
240	1.55 ± 0.00	8.2 ± 0.4	0.60 ± 0.07	11.86 ± 0.01	10.31 ± 1.24	11.82 ± 0.01	4.92 ± 0.68	5.29 ± 0.10	8.85	2.96	2.80
264	1.52 ± 0.01	8.2 ± 0.4	0.59 ± 0.04	14.04 ± 0.23	10.96 ± 1.32	12.37 ± 0.08	5.33 ± 1.10	5.06 ± 0.19	9.59	3.34	3.12
288	1.56 ± 0.01	8.4 ± 0.5	0.62 ± 0.05	12.62 ± 0.55	8.90 ± 1.40	10.29 ± 0.47	4.12 ± 1.17	4.82 ± 0.09	9.18	3.26	2.96

Statistics

Statistical analysis of the NADH oxidase

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Air 0%	2	0.82326206	0.41163103	0.00345474
Air 33&	8	5.9093379	0.73866724	0.09084769
Air 66%	8	66.9551145	8.36938931	8.22451583
Air 100%	8	99.3624289	12.4203036	1.35152489

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	647.406096	3	215.802032	70.1570457	2.0156E-11	3.04912499
Within Groups	67.6716736	22	3.07598517			
Total	715.077769	25				

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 33%</i>
Mean	0.411631028	0.738667238
Variance	0.00345474	0.090847691
Observations	2	8
Pooled Variance	0.079923572	
Hypothesized Mean Difference	0	
df	8	
t Stat	-1.46324952	
P(T<=t) one-tail	0.090774602	
t Critical one-tail	1.859548038	
P(T<=t) two-tail	0.181549203	
t Critical two-tail	2.306004135	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 66%</i>
Mean	0.41163103	8.36938931
Variance	0.00345474	8.22451583
Observations	2	8
Pooled Variance	7.19688319	
Hypothesized Mean Difference	0	
df	8	
t Stat	-3.7521354	
P(T<=t) one-tail	0.00280353	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	0.00560707	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 100%</i>
Mean	0.41163103	12.4203036
Variance	0.00345474	1.35152489
Observations	2	8
Pooled Variance	1.18301612	
Hypothesized Mean Difference	0	
df	8	
t Stat	-13.965602	
P(T<=t) one-tail	3.3484E-07	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	6.6968E-07	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 66%</i>
Mean	0.738667238	8.369389311
Variance	0.090847691	8.224515831
Observations	8	8
Pooled Variance	4.157681761	
Hypothesized Mean Difference	0	
df	14	
t Stat	-7.48462437	
P(T<=t) one-tail	1.47343E-06	
t Critical one-tail	1.761310136	
P(T<=t) two-tail	2.94686E-06	
t Critical two-tail	2.144786688	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 100%</i>
Mean	0.73866724	12.4203036
Variance	0.09084769	1.35152489
Observations	8	8
Pooled Variance	0.72118629	
Hypothesized Mean Difference	0	
df	14	
t Stat	-27.511226	
P(T<=t) one-tail	6.8804E-14	
t Critical one-tail	1.76131014	
P(T<=t) two-tail	1.3761E-13	
t Critical two-tail	2.14478669	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 66%</i>	<i>Air 100%</i>
Mean	8.36938931	12.4203036
Variance	8.22451583	1.35152489
Observations	8	8
Pooled Variance	4.78802036	
Hypothesized Mean Difference	0	
df	14	
t Stat	-3.7025852	
P(T<=t) one-tail	0.00118259	
t Critical one-tail	1.76131014	
P(T<=t) two-tail	0.00236518	
t Critical two-tail	2.14478669	

Statistical analysis of the NADH peroxidase

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Air 0%	2	0.09803992	0.04901996	0.00380885
Air 33&	14	2.47183586	0.1765597	0.02873574
Air 66%	8	21.969531	2.74619138	1.31193266
Air 100%	8	81.1165851	10.1395731	1.56620318

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	534.411671	3	178.137224	243.021021	3.8161E-20	2.94668527
Within Groups	20.5243243	28	0.73301158			
Total	554.935996	31				

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 33%</i>
Mean	0.049019961	0.176559704
Variance	0.003808851	0.028735744
Observations	2	14
Pooled Variance	0.026955252	
Hypothesized Mean Difference	0	
df	14	
t Stat	-1.02764442	
P(T<=t) one-tail	0.160772898	
t Critical one-tail	1.761310136	
P(T<=t) two-tail	0.321545796	
t Critical two-tail	2.144786688	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 66%</i>
Mean	0.04901996	2.74619138
Variance	0.00380885	1.31193266
Observations	2	8
Pooled Variance	1.14841718	
Hypothesized Mean Difference	0	
df	8	
t Stat	-3.1836015	
P(T<=t) one-tail	0.00646361	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	0.01292722	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 100%</i>
Mean	0.04901996	10.1395731
Variance	0.00380885	1.56620318
Observations	2	8
Pooled Variance	1.37090389	
Hypothesized Mean Difference	0	
df	8	
t Stat	-10.901129	
P(T<=t) one-tail	2.2207E-06	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	4.4415E-06	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 66%</i>
Mean	0.176559704	2.74619138
Variance	0.028735744	1.311932655
Observations	14	8
Pooled Variance	0.477854663	
Hypothesized Mean Difference	0	
df	20	
t Stat	-8.38727019	
P(T<=t) one-tail	2.78639E-08	
t Critical one-tail	1.724718243	
P(T<=t) two-tail	5.57278E-08	
t Critical two-tail	2.085963447	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 100%</i>
Mean	0.1765597	10.1395731
Variance	0.02873574	1.56620318
Observations	14	8
Pooled Variance	0.56684935	
Hypothesized Mean Difference	0	
df	20	
t Stat	-29.857577	
P(T<=t) one-tail	2.3025E-18	
t Critical one-tail	1.72471824	
P(T<=t) two-tail	4.605E-18	
t Critical two-tail	2.08596345	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 66%</i>	<i>Air 100%</i>
Mean	2.74619138	10.1395731
Variance	1.31193266	1.56620318
Observations	8	8
Pooled Variance	1.43906792	
Hypothesized Mean Difference	0	
df	14	
t Stat	-12.326293	
P(T<=t) one-tail	3.3117E-09	
t Critical one-tail	1.76131014	
P(T<=t) two-tail	6.6234E-09	
t Critical two-tail	2.14478669	

Statistical analysis of the NADPH oxidase

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Air 0%	2	0.75152553	0.37576276	0.00021005
Air 33%	14	8.59637427	0.61402673	0.11462858
Air 66%	8	58.7647553	7.34559442	11.5828767
Air 100%	8	92.3226218	11.5403277	0.73249459

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	698.29748	3	232.765827	74.3169125	1.90096E-13	2.94668527
Within Groups	87.6979805	28	3.13207073			
Total	785.99546	31				

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 33%</i>
Mean	0.375762764	0.614026734
Variance	0.000210046	0.114628577
Observations	2	14
Pooled Variance	0.106455825	
Hypothesized Mean Difference	0	
df	14	
t Stat	0.966034602	
P(T<=t) one-tail	0.17520955	
t Critical one-tail	1.761310136	
P(T<=t) two-tail	0.3504191	
t Critical two-tail	2.144786688	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 66%</i>
Mean	0.37576276	7.34559442
Variance	0.00021005	11.5828767
Observations	2	8
Pooled Variance	10.1350434	
Hypothesized Mean Difference	0	
df	8	
t Stat	-2.7692966	
P(T<=t) one-tail	0.01216154	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	0.02432308	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 100%</i>
Mean	0.37576276	11.5403277
Variance	0.00021005	0.73249459
Observations	2	8
Pooled Variance	0.64095902	
Hypothesized Mean Difference	0	
df	8	
t Stat	-17.639516	
P(T<=t) one-tail	5.4525E-08	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	1.0905E-07	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 66%</i>
Mean	0.614026734	7.345594416
Variance	0.114628577	11.58287669
Observations	14	8
Pooled Variance	4.128515417	
Hypothesized Mean Difference	0	
df	20	
t Stat	7.475098716	
P(T<=t) one-tail	1.63598E-07	
t Critical one-tail	1.724718243	
P(T<=t) two-tail	3.27195E-07	
t Critical two-tail	2.085963447	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 100%</i>
Mean	0.61402673	11.5403277
Variance	0.11462858	0.73249459
Observations	14	8
Pooled Variance	0.33088168	
Hypothesized Mean Difference	0	
df	20	
t Stat	-42.858254	
P(T<=t) one-tail	1.8607E-21	
t Critical one-tail	1.72471824	
P(T<=t) two-tail	3.7215E-21	
t Critical two-tail	2.08596345	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 66%</i>	<i>Air 100%</i>
Mean	7.34559442	11.5403277
Variance	11.5828767	0.73249459
Observations	8	8
Pooled Variance	6.15768564	
Hypothesized Mean Difference	0	
df	14	
t Stat	-3.3808476	
P(T<=t) one-tail	0.00224019	
t Critical one-tail	1.76131014	
P(T<=t) two-tail	0.00448038	
t Critical two-tail	2.14478669	

Statistical analysis of the NADPH peroxidase

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Air 0%	2	0.544002	0.272001	0.00540987
Air 33%	14	4.76395014	0.34028215	0.15548439
Air 66%	8	24.0624072	3.0078009	0.34036947
Air 100%	8	38.4585122	4.80731402	0.66326418

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	115.252512	3	38.417504	118.832653	4.93506E-16	2.946685266
Within Groups	9.0521425	28	0.3232908			
Total	124.304655	31				

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 33%</i>
Mean	0.272001001	0.340282153
Variance	0.005409869	0.155484392
Observations	2	14
Pooled Variance	0.144764783	
Hypothesized Mean Difference	0	
df	14	
t Stat	0.237404201	
P(T<=t) one-tail	0.407891097	
t Critical one-tail	1.761310136	
P(T<=t) two-tail	0.815782194	
t Critical two-tail	2.144786688	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 66%</i>
Mean	0.272001	3.0078009
Variance	0.00540987	0.34036947
Observations	2	8
Pooled Variance	0.29849952	
Hypothesized Mean Difference	0	
df	8	
t Stat	-6.3339189	
P(T<=t) one-tail	0.00011222	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	0.00022445	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 100%</i>
Mean	0.272001	4.80731402
Variance	0.00540987	0.66326418
Observations	2	8
Pooled Variance	0.58103239	
Hypothesized Mean Difference	0	
df	8	
t Stat	-7.5260498	
P(T<=t) one-tail	3.3796E-05	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	6.7592E-05	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 66%</i>
Mean	0.340282153	3.007800902
Variance	0.155484392	0.340369473
Observations	14	8
Pooled Variance	0.22019417	
Hypothesized Mean Difference	0	
df	20	
t Stat	12.82632509	
P(T<=t) one-tail	2.07732E-11	
t Critical one-tail	1.724718243	
P(T<=t) two-tail	4.15464E-11	
t Critical two-tail	2.085963447	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 100%</i>
Mean	0.34028215	4.80731402
Variance	0.15548439	0.66326418
Observations	14	8
Pooled Variance	0.33320732	
Hypothesized Mean Difference	0	
df	20	
t Stat	-17.460612	
P(T<=t) one-tail	7.09E-14	
t Critical one-tail	1.72471824	
P(T<=t) two-tail	1.418E-13	
t Critical two-tail	2.08596345	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 66%</i>	<i>Air 100%</i>
Mean	3.0078009	4.80731402
Variance	0.34036947	0.66326418
Observations	8	8
Pooled Variance	0.50181682	
Hypothesized Mean Difference	0	
df	14	
t Stat	-5.0805696	
P(T<=t) one-tail	8.3794E-05	
t Critical one-tail	1.76131014	
P(T<=t) two-tail	0.00016759	
t Critical two-tail	2.14478669	

Statistical analysis of the glutathione peroxidase

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Air 0%	2	0.10446205	0.05223102	0.001755
Air 33&	14	2.5219092	0.18013637	0.00194887
Air 66%	8	11.8876098	1.48595123	0.04334306
Air 100%	8	42.5890703	5.32363379	0.30145897

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	142.954063	3	47.6513545	546.660976	6.06713E-25	2.946685266
Within Groups	2.44070454	28	0.08716802			
Total	145.394768	31				

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 33%</i>
Mean	0.052231024	0.180136371
Variance	0.001755001	0.00194887
Observations	2	14
Pooled Variance	0.001935022	
Hypothesized Mean Difference	0	
df	14	
	-	
t Stat	3.846491654	
P(T<=t) one-tail	0.000889908	
t Critical one-tail	1.761310136	
P(T<=t) two-tail	0.001779815	
t Critical two-tail	2.144786688	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 66%</i>
Mean	0.05223102	1.48595123
Variance	0.001755	0.04334306
Observations	2	8
Pooled Variance	0.03814455	
Hypothesized Mean Difference	0	
df	8	
t Stat	-9.2855604	
P(T<=t) one-tail	7.3603E-06	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	1.4721E-05	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 0%</i>	<i>Air 100%</i>
Mean	0.05223102	5.32363379
Variance	0.001755	0.30145897
Observations	2	8
Pooled Variance	0.26399597	
Hypothesized Mean Difference	0	
df	8	
t Stat	-12.977395	
P(T<=t) one-tail	5.8898E-07	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	1.178E-06	
t Critical two-tail	2.30600414	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 66%</i>
Mean	0.180136371	1.485951229
Variance	0.00194887	0.043343063
Observations	14	8
Pooled Variance	0.016436837	
Hypothesized Mean Difference	0	
df	20	
t Stat	22.98106497	
P(T<=t) one-tail	3.7477E-16	
t Critical one-tail	1.724718243	
P(T<=t) two-tail	7.4954E-16	
t Critical two-tail	2.085963447	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 33%</i>	<i>Air 100%</i>
Mean	0.18013637	5.32363379
Variance	0.00194887	0.30145897
Observations	14	8
Pooled Variance	0.1067774	
Hypothesized Mean Difference	0	
df	20	
t Stat	-35.515384	
P(T<=t) one-tail	7.6159E-20	
t Critical one-tail	1.72471824	
P(T<=t) two-tail	1.5232E-19	
t Critical two-tail	2.08596345	

t-Test: Two-Sample Assuming Equal Variances

	<i>Air 66%</i>	<i>Air 100%</i>
Mean	1.48595123	5.32363379
Variance	0.04334306	0.30145897
Observations	8	8
Pooled Variance	0.17240102	
Hypothesized Mean Difference	0	
df	14	
t Stat	-18.485412	
P(T<=t) one-tail	1.5593E-11	
t Critical one-tail	1.76131014	
P(T<=t) two-tail	3.1186E-11	
t Critical two-tail	2.14478669	