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Bacteriophages as antimicrobial agents against bacterial contaminants in yeast fermentation processes

by

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ABSTRACT

Bacterial contaminants are ubiquitous in yeast fermentation processes for biofuel production. In general, they compete with yeasts for nutrients, reducing overall production yield. The present project investigates the application of bacteriophages (phages), viruses infecting and killing prokaryotes, as potential antibacterial agents in yeast fermentation processes. A phage cocktail was applied to control contamination while preventing the development of bacterial resistance. The study identifies and quantifies the effects of parameters influencing infection/contamination. The data obtained were ultimately used to develop a mathematical model detailing the dynamics of the populations involved (yeasts, bacteria and phages). The results showed that the addition of phages cocktail at relatively low initial multiplicity of infection was sufficient to reduce contamination and allowed the yields of yeast and ethanol to reach values equivalent to those of axenic yeast cultures. Moreover, the model showed good fit to the experimental results.

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LIST OF SYMBOLS, NOMENCLATURE, OR ABBREVIATIONS

µL - microlitre

^{o}C - Celsius degrees

ATCC - American Type Culture Collection

ATP - adenosine triphosphate

BC - before Christ

bp - base pairs

BV - bacterial vaginosis

cfu - colony forming units

DNA - deoxyribonucleic acid

dsDNA - double-stranded DNA

g - gram

GC - guanine and cytosine nitrogenous bases

kbp - kilo base pairs

L - litre

LAB - lactic acid bacteria

M - molar concentration

M9 – a type of minimal salts culture medium

Mb - mega base pairs

min - minute

mL - millilitre

MOI - multiplicity of infection

MRS - lactobacilli culture medium designed by de Man, Rogosa and Sharpie

N - normal concentration

nm - nanometer

OD_{600} - optical density at 600nm wavelength

ORF - open reading frame

pfu - plaque forming units

pH - potential hydrogen

RID - refractive index detector

rpm - revolutions per minute

tRNA - transfer ribonucleic acid

v/v - volume/volume

1. INTRODUCTION

Yeasts have been utilized for their beneficial attributes for millennia. The ancient use of yeasts for the production of breads and beverages makes them the oldest 'domesticated' organisms (Walker, 1998). In fact, archaeologists found evidences of intentional production of alcoholic beverages fermented by yeasts from the Neolithic times (8500-4000 BC) (Johnson and Echavarri-Erasun, 2011; McGovern *et al*., 1997). *Saccharomyces cerevisiae* is undoubtedly the most important yeast for mankind. Its growth and fermentation is fundamental to the manufacture of an immense variety of useful products.

Yeast fermentation for the production of biofuels, especially ethanol, is a consolidated technology that poses as an alternative renewable source of energy (Lynd, 1991). The advent of this industry has translated in significant research efforts to improve production and quality and to implement the use of non-food sources of sugars as raw materials (Wyman, 1994).

However, little attention has been paid for an issue common for virtually all yeast fermentation processes, the contamination by undesirable microorganisms (Muthaiyan and Ricke, 2010). Lactic acid bacteria (LAB) are the most abundant and common bacterial contaminants in bioethanol processes (Skinner and Leathers, 2004). These contaminants compete with yeasts for nutrients in the mash and may release substances that can impede yeast growth (Bayrock and Ingledew, 2004). Currently, addition of antibiotics and pH reduction are the main measures taken by the industry to control contamination (Bischoff *et al*., 2007). However, some concerns arise from these actions: the use of antibiotics contributes to the emergence of resistant bacterial strains (Silbergeld *et al*., 2008), and reduction of pH, while lowering the growth rate of

many bacterial contaminants, also leads to lower ethanol yields (Narendranath and Brey, 2009). These consequences contribute to the search for alternative antimicrobials.

The present study proposes the application of bacteriophages (phages) to reduce contamination in yeast fermentation processes. Bacteriophages are viruses that infect prokaryotes. They are ubiquitous biological entities, representing the most numerous organisms in the world (Brüssow and Kutter, 2005). Phages are being investigated for applications in several different fields that range from health sciences (Manoutcharian, 2011) to sensing devices (Rontó *et al*., 1994). However, for industries in which bacteria are used to ferment substrates into useful products, phages are troublesome and unwanted. The most common cases of phage contamination are found in the dairy industry, where lactic acid bacteria are added to dairy raw materials in order to produce fermented products, such as cheese and yoghurt (Moineau and Lévesque, 2005).

The present study proposes to take advantage of the spread of phage infection in lactic acid bacteria fermentation processes to reduce contamination and improve biofuel yield in yeast fermentation processes.

2. LITERATURE REVIEW

2.1 Ethanol

2.1.1 General characteristics

Ethyl alcohol is a substance of fundamental importance throughout the world. Its molecular formula is C_2H_6O and it is best known for its CAS name, ethanol. It is a clear and colorless chemical with melting point of - 114.1^{o}C and boiling point of 78.15^{o}C (O'Neil *et al*., 2006). It is widely used as solvent, fuel, octane enhancer, chemical reagent and in beverages.

2.1.2 Ethanol as fuel

Much attention has been paid in the last decades to the use of ethanol as fuel for internal combustion engines. This application has an environmental appeal and poses as an alternative solution for the worldwide dependence on petroleum for energy purposes. In the past, several misconceptions regarding using ethanol as fuel led to a limited growth of its production. Such wrong ideas about alcohol included claiming it a 'boutique fuel' and even a harmful chemical to car engines (Pilgrim, 2009). After long period of successful fuel ethanol utilization, these claims are now extinct. Nowadays, automotive technology allows some vehicles to consume pure ethanol, pure gasoline or any blend of both fuels. This technology, named flex fuel, was widely embraced in Brazil, one of the largest producers of ethanol. According to a report issued by the Brazilian government in September of 2012, it is estimated that flex fuel vehicles compose half of the total consumer fleet in the country (Ministério de Minas e Energia, 2012).

Recently, the production of ethanol has been experiencing a dramatic increase, not only in the leading producing countries, such as the US and Brazil, but also in other parts of the world, with emphasis to Europe and Asia (Pilgrim, 2009). While sugar cane and corn are the main raw materials in Brazil and United States, respectively, Europe is adopting small grains such as wheat and barley as feedstocks. In 2007, the production of bioethanol in the US reached 6.5 billion gallons, an increase of more than 32% in relation to the previous year. After 30 years of overall steady revenue reduction, even the market for distilled alcoholic beverages faced slight increase in the last decade (Collicutt, 2009).

The application of ethanol as fuel has also been a matter of legislations. The United States approved, in 1990, the Clean Air Act Amendments that required blending oxygenates into gasoline. Methyl tertiary-butyl ether (MTBE) was initially the main source of such oxygenates, acting also as octane enhancer. However, due to environmental concerns caused by the presence of MTBE in ground water, many states banned its usage. Ethanol is now the best substitute for this compound (Pilgrim, 2009). In addition, a program named Renewable Fuel Standard (RFS), created under the Energy Policy Act (EPAct) of 2005, required mixing 7.5 billion gallons of renewable fuel with gasoline by 2012. In 2007 the program was expanded and one new requirement was to increase this objective to 36 billion gallons of renewables by 2022 (Environmental Protection Agency, 2012).

Considering these factors, a certain level of optimism can be perceived in the industry, and according to Pilgrim (2009), “the potential for ethanol has never been better.”

2.1.3 Production of bioethanol

Several different processes can be applied for the production of bioethanol. What mainly determines the differences are the agricultural products used as raw materials. Currently, starch- and sugar-based crops compose the most common feedstocks used. The processing methods and technologies can be divided, as a general rule, as a function of these two crops categories.

Production from sugar-based plants requires major upstream extraction work (Monceaux, 2009). It involves washing, breaking, milling/extraction, straining, clarification and evaporation. After the steps of clarification/evaporation, the sugar juice is ready to be fermented by yeasts, followed by distillation/dehydration of the ethanol.

Starch-based cereals processes can be further divided in two sub-categories: dry-grind and wet-milling processes. The former consists of grain milling, mashing and cooking, liquefaction, saccharification/fermentation, distillation, centrifugation, evaporation and drying; and the latter consists of cooking, liquefaction, fermentation, distillation/dehydration and evaporation (Monceaux, 2009).

The differences in processing are mostly based on the initial treatment and mash formation (upstream operations). After the ethanol is produced, the downstream operations of distillation and dehydration tend to be the same for all processes. More importantly, fermentation is the common unit operation for all cases, as it is responsible for the conversion of sugars to ethanol.

The modes of operation of fermenters mainly applied in industries are batch, continuous and fed-batch (Zhang, 2009). The first is a process in which the substrate is added at once, before the inoculation of the microorganism. After the completion of

fermentation, the contents are removed for further processing. The primary disadvantage of this process is the loss of productivity caused by downtime (cleaning, sanitizing and filling) between runs (Bayrock and Ingledew, 2001a). Continuous fermentation is characterized by the constant addition of substrate into the equipment along with the constant removal of the fermentation contents. While this process is attractive, it is the most susceptible to long- term contamination (Bayrock and Ingledew, 2001a; 2001b). Fed-batch is a process in which substrate is continuously added to the system, while the final contents are removed once the maximum fermenter volume is reached.

Bioethanol can be produced from fermentation by bacteria (Ingram *et al*., 1987; Wills *et al*., 1981) or yeasts (Williamson *et al*., 1980). Currently yeasts are, by far, the most used organisms for this purpose. In fact, the yeast *Saccharomyces cerevisiae* is still perceived as the most economical way to produce ethanol (Bayrock and Ingledew, 2001a).

2.1.4 *Saccharomyces cerevisiae*

S. cerevisiae, also referred to as baker's or brewer's yeast, is an eukaryotic unicellular microorganism that belongs to the kingdom Fungi, division Ascomycota (Walker, 2009). This yeast is predominantly diploid and reproduces asexually by budding (Oda and Ouchi, 2000). They are capable of growing under aerobic or anaerobic conditions. The chromosomes inside its nucleus vary in number and in size from 0.2 to 6Mb (Van Zandycke, 2009).

The production of ethanol by *S. cerevisiae* starts with the rapid uptake and conversion of sugars to pyruvate through 12 enzymes (Ingledew, 2009a). Acetaldehyde

and carbon dioxide (CO_2) are then produced from pyruvate. CO_2 is released by diffusion and acetaldehyde transforms into ethanol through an alcohol dehydrogenase enzyme. These glycolysis reactions generate ATP for the yeasts. Interestingly, more than 90% of the sugars consumed anaerobically are generally converted into ethanol. In addition to alcohols, glycerol, organic acids, esters and aldehydes are also substances produced by *S. cerevisiae*.

Most commonly, ethanol production is performed under anaerobic conditions. The reduced amount of oxygen in the medium results from the high height to volume ratio of the fermenter and the absence of aeration. This makes the diffusion of the oxygen present in the head space of the equipment possible only in the upper levels of mash. Besides, CO_2 released by considerable amount of yeasts replaces the air in the head space of the equipment. Nevertheless, small quantities of oxygen must be present in the medium to allow the yeast to synthesize components of the cell membrane (Ingledew, 2009a).

2.1.5 Contaminants in ethanol fermentation

Contaminants in yeast fermentation processes for biofuel production can be considered ubiquitous and permanent. Suppression of these contaminants is currently a major challenge for this industry (Muthaiyan and Ricke, 2010). Because the presence of unwanted organism is unavoidable, some authors consider chronic infections expected and tolerated (Leja and Broda, 2009; Skinner and Leathers, 2004).

Bacteria and wild yeasts are the main contaminants present in biofuel processes (Ingledew *et al*., 2009). Several studies conducted at ethanol plants in different locations throughout the world identified lactic acid bacteria (LAB) as the

major group of bacterial contaminants (Bischoff *et al*., 2007; de Carvalho-Netto *et al*., 2008; Leja and Broda, 2009; Skinner and Leathers, 2004; Sossa Urrego *et al*., 2009). LAB, or 'the Lactics', are Gram-positive, aerobic to facultative anaerobic, and generally non-sporulating rod or coccus shaped bacteria (Azhari Ali, 2010; Carr *et al*., 2002; Martin Patrick, 2012). They come from the following genera: *Carnobacterium, Enterococcus, Lactoccoccus, Lactobacillus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Paralactobacillus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella.* These bacteria are famous as starters for dairy products, however they also have importance in the production of other foods (meat products, beverages and vegetables), and have impact in human health. LAB are divided into two groups based on the end products of glucose metabolism, the homofermenters and the heterofermenters. The first group transforms glucose solely into lactic acid while the second produces additional substances (carbon dioxide, acetic acid and ethanol). In general, LAB are relatively heat-resistant and tolerant to low pH and high concentrations of ethanol (Chin and Ingledew, 1994; Thomas *et al*., 2001).

Since LAB are naturally encountered in plants and soil (Chen *et al*., 2005), they are commonly found in agricultural raw materials serving as feedstocks for biofuel production. However, sources of contamination in manipulated yeasts (Sossa Urrego *et al*., 2009) and within the processing facilities (Narendranath and Brey, 2009; Skinner and Leathers, 2004) were also identified.

The main issue arising from the presence of contaminants in yeast fermentations is the decreased final yield of ethanol (Narendranath *et al*., 1997). The bacteria in the medium compete with *S. cerevisiae* for nutrients and produce substances, such as organic acids, that may directly inhibit the growth of the yeast

(Narendranath *et al*., 2001); and alcohol production is directly related to yeast development (Ingledew, 2009a). In fact, acute contamination can lead to 'stuck' fermentation that requires process shut down and full cleaning, causing expensive loss of productivity. Moreover, Thomas *et al*. (2001) stated that *S. cerevisiae* cells produce more glycerol when growing with bacteria. A plant is considered under control if losses due to contaminants do not exceed 4% (Ingledew, 2009a), however higher losses are encountered in industry. In a study performed in fermentation tanks of an ethanol plant in Colombia, Sossa Urrego *et al*. (2009) determined that the presence of contaminants reduced efficiency by almost 8% in comparison to the theoretical value.

2.1.6 Current solutions for combating the contaminants in ethanol fermentation

One measure taken to reduce contamination in industry is heat treatment. This physical treatment can be performed indirectly, through steps that require heat for a purpose other than exclusively eliminating contaminants (for example the cooking of starch - Kelsall and Piggot, 2009) or through equipment dedicated to decontamination (e.g. pasteurizers). Both methods are not substantially effective in reducing contamination to levels that do not affect yield. In fact, Skinner and Leathers (2004) even observed an increase in contamination of steep water after pasteurization.

Cleaning and sanitation are important steps to be considered in fermentation plants. Not limited to reducing contamination levels and, therefore, increasing ethanol yield, these steps also enable cost reduction through improvements in energy efficiency and maintenance costs (Richards, 2009). Still, these procedures only lower the amount of unwanted microorganisms. A complete removal of contaminants would require complex and expensive processes of disinfection or sterilization. Although they

don’t provide a complete solution for contamination issues, cleaning and sanitation are good practices that plants must follow.

Lowering pH of the mash is a practice highly adopted by industries. pH level between 4.0 to 4.5 is commonly applied, but lower values can be often found, especially in continuous fermentation processes (Graves *et al*., 2006). The goal is to inhibit contaminants as most lactic acid bacteria have reduced growth rate at these pH values (Narendranath and Power, 2005). However, pH values lower than the optimal for *S. cerevisiae* (5.0 to 5.5) also affect yeasts yield and ethanol production (Narendranath and Brey, 2009). Besides, it was observed that the organic acids produced by LAB are present at increased undissociated form in a medium with lower pH, enhancing their inhibitory potential towards yeasts (Graves *et al*., 2006).

The addition of chemical substances with antibiotic effect is currently the solution mostly applied in industries. These substances are able to selectively kill bacteria without affecting yeasts. Virginiamycin, penicillin, tetracycline, erythromycin, tylosin, and monensin are the most common antibiotics used for this purpose (Bischoff *et al*., 2009; Hynes *et al*., 1997; Leja and Broda, 2009; Paulus Compart *et al*., 2013). These antibiotics can be applied alone or in combination. They can be added directly to the mash prior to fermentation or in different steps of the process (e.g. saccharification or yeast propagation tanks - Bischoff *et al*., 2007; Skinner and Leathers, 2004). The amount and frequency of dosage applied is also highly variable among plants.

Although antibiotics, in general, offer a sensible solution for the reduction of contamination, their application for non-therapeutic purposes poses major concerns on the emergence of resistance (Silbergeld *et al*., 2008). In the case of ethanol production, this concern extends not only to the appearance of resistant strains inside

the process, turning the application of certain antibiotics no longer effective to control contamination (Bischoff *et al*., 2007), but also to the presence of residual amounts in by-products used as animal feed (mostly distillers dried grains with solubles - DDGS) (Ensley, 2011). In fact, regulations, mainly in Europe, requiring antibiotic-free feed are contributing to phase out these antimicrobials (Narendranath and Brey, 2009). However, this theme has been controversial: a recent study on the presence of antibiotics in co-products made the authors conclude that the risk of resistance development threatening human health is minimal (Paulus Compart *et al*., 2013). Besides this debate, there are increased efforts into the investigation of alternative antimicrobials to be applied for the reduction of contaminants in biofuel production.

Several different alternatives, including chemical treatment, natural compounds and plant extracts, have been proposed (see Muthaiyan *et al*., 2011 for a review). An example of chemical treatment is the addition of sulfite (Chang *et al*., 1997). In this study, the authors concluded that although sulfite could be seen as a potential antimicrobial, its bactericidal activity was effective only in the presence of molecular oxygen. A study by Limayem *et al.* (2011) investigated the efficiency of four different natural antimicrobials, nisin, ε-polylysine, chitosan and lysozyme, and concluded that nisin, a bacteriocin, had the best potential. The efficiency of nisin was also proved in another study (Peng *et al*., 2012). Hop extract is a natural plant-derived product known to have antimicrobial effects. A recent study proved its efficiency (Leite *et al*., 2013), however, according to Muthaiyan *et al*. (2011), the application of hop compounds alone is not ideal. Another alternative recently investigated was the application of phage endolysins, lytic enzymes produced during phage replication cycle

that degrade peptidoglycan and contribute to cell lysis (Roach *et al*., 2013). These enzymes can be added to the mash or be expressed on the surface of yeasts.

The concept of applying bacteriophages (phages) for the control of bacterial contaminants in yeast fermentation processes for the production of biofuels was mentioned in literature (Narendranath and Brey, 2009) and has been the subject of two patent applications (US 2009/0104157 A1, US 2010/0330041 A1). The work of Narendranath and Brey (2009) and the patent US 2010/0330041 A1 proposed the concept but didn't present experimental support. Patent US 2009/0104157 A1 showed preliminary results of phages infecting LAB in MRS medium and corn mash. According to these experiments, phages presented higher efficiency in MRS medium in comparison to corn mash under the conditions tested. Yet, despite these preliminary results, no systematic studies to establish comprehensive knowledge have been shown, and this alternative is currently not applied in industry.

2.2 Bacteriophages

2.2.1 General characteristics

Bacteriophages, or phages, are viruses whose hosts are bacteria, eubacteria and archaea (Ackermann, 2007). They are ubiquitous on Earth and are the most abundant biological entities – with a population estimated at 10^{30} to 10^{32} individuals (Brüssow and Kutter, 2005). As viruses, phages do not present any metabolic activity and are absolute parasites. They were discovered independently by two researchers: Frederick William Twort in 1915 and Félix Hubert d'Hérelle in 1917 (Ackermann, 2006). Since then, they have been extensively studied and used in a variety of applications.

In spite of all efforts made, there is still a lot to learn about phages. Their abundance in nature makes bacteriophage research an immense field of study. It is estimated a rate of 100 new phages discovered per year (Ackermann, 2007). Phages are classified mainly based on nucleic acid type and overall morphology (Ackermann, 2005). This mode of classification was first introduced by Bradley (1967) after several unsuccessful classification proposals.

Phages can have various different morphotypes. The largest group of known phages falls within the classification of tailed phages (order *Caudovirales*). These are further divided into three families: *Myoviridae*, phages with long and contractile tails composed by a sheath and a central tube (e.g. coliphage T4); *Siphoviridae*, with long and non-contractile tails (e.g. coliphage λ); and *Podoviridae*, with short and non-contractile tails (e.g. coliphage T7) (Ackermann, 2005). Some other well-known phages are classified in different families: ϕX174 – *Microviridae*, PM2 – *Corticoviridae*, M13 – *Inoviridae*, ϕ6 – *Cystoviridae*, and PRD1 – *Tectiviridae*.

2.2.2 Replication

The first step involved in phage infection is the adsorption of phage organelles on bacterial receptors. Phages are capable of adsorbing to receptors located on the cell walls of both Gram-positive and Gram-negative bacteria, on the host's capsule or slime layers, or on appendages (i.e. pili and flagella). In general, adsorption involves three sub-steps: initial contact, reversible binding and irreversible binding (Duckworth, 1987). Initial contact involves random collisions between phage and host due to the flow of both entities in a medium. The second sub-step, reversible binding, is an initial attachment of the parts in which electrostatic forces are involved but are not strong

enough to allow permanent adsorption. This sub-step was identified based on experimental observations in which the number of attached phages reduced after elution (Garen and Puck, 1951). In the irreversible sub-step, a specific connection occurs, sometimes with enzymatic cleavage, between bacterial receptor and phage anti-receptor. When this last sub-step is achieved chemical bonds are strong enough to avoid desorption and trigger arrangements in other phage molecules that allow the advent of the second step of phage infection, insertion of the genetic material inside the host.

The delivery of phage's nucleic acid is conducted, in general, through enzymatic penetration of the cell membrane followed by the insertion of the genetic material. Upon entering the host, the nucleic acid takes over the bacterial reproduction machinery and reprograms the cell for the synthesis of phage progeny (Guttman *et al*., 2005). Once new phages are assembled, endolysin and holin enzymes are produced to cleave the peptidoglycan layer in the host cell wall to cause the release of the progeny followed by the death of the cell (Young and Wang, 2006). This phenomenon is called cell lysis. In the case of filamentous phages (e.g. M13, f1, fd) the host secretes the progeny through extrusion without killing the cell (Russel and Mode, 2006).

In the case of temperate phages, the phage can undergo the lytic cycle (describe above) or the lysogenic cycle. In the latter case, the initial steps of adsorption and genome insertion are the same as in the lytic cycle; however, the inserted genetic material is then integrated into the genome of the host (Campbell, 2006), rather than being expressed. When the host containing the temperate phage genome (termed prophage) divides, the prophage is also replicated along with the cell. Upon changes in

chemical or physical properties of the environment, the prophage may be activated, triggering the lytic cycle and producing progeny (Paul, 2008).

Another phenomenon, pseudolysogeny, may be observed in some phages (Łoś and Węgrzyn, 2012; Ripp and Miller, 1997). This phenomenon is characterized by an unstable state, in which the phage genome resides within the cell, but neither triggers the production of progeny by the host, nor is integrated in the host's genome. Unfavorable cell growth conditions (such as starvation) were determined to induce pseudolysogeny. Once growth conditions improve, this state is terminated and the phage's genome initiates either the lytic or the lysogenic cycle.

2.2.3 Parameters linked to phages replication

The beginning of the infection, determined by the adsorption and nucleic acid insertion in the host, initiate the eclipse and latent periods (Abedon, 2006). The period between genome insertion and the formation of the first mature phage within the cell is called the eclipse period. The latent period is defined as the time between genome insertion and the initial release of newly formed progeny. These two periods demonstrate that there is a time gap between the formation of mature phages and cell lysis. Rise or lysis period starts with the appearance of the first released phages and ends with the complete lysis of the cell. The average amount of phages produced per infected cell in a population is termed burst size. These parameters of phage replication can be determined from the one-step growth experiment (Ellis and Delbruck, 1939). Other terms used in virology applied in this thesis are multiplicity of infection (MOI), which corresponds to the ratio of phages to hosts; and plaques, the area of lysis formed on a lawn of cells (Mahy, 2009).

2.2.4 General applications

Phages have been considered to an enormous variety of applications. One use is in the medical field to control bacterial infections. This application, termed phage therapy, has been extensively studied to prevent the problematic emergence of antibiotic resistant bacterial strains (Schoolnik *et al*., 2004). This technology has already been applied for a while in some Eastern European countries (Brüssow, 2012; Housby and Mann, 2009).

A major breakthrough for the technological application of phages was the advent of phage display (Smith, 1985; Smith and Petrenko, 1997). This method conveys the expression of random peptides on the coat of phage vectors that are then applied in the interaction with several different substances.

Several works have been conducted using phages in sensing technology (Balasubramanian *et al.*, 2007; Gervais *et al*., 2007; Ignatov *et al.*, 2005; Nanduri *et al.*, 2007; Shen *et al*., 2009; Souza *et al*., 2006; Tolba *et al.*, 2010). In such cases, they are generally used as recognition probes that bind to an entity of interest present in a sample. After binding occurs, a physical transducer recognizes a resulting change in a variable and activates a quantifiable signal that expresses the recognition of the entity.

In the materials field, Willis *et al*., 2008 reported organic polymer built with phages. The material was polymerized through the reaction between phage M13 and acrylamide monomers. The final macroscopic result was an opaque gel-like resilient structure with a good 'shape memory'. The formation of materials combining phages and inorganic chemicals was also investigated (Cao and Mao, 2011). Genetically modified M13 and inorganic chemicals formed ordered flexible films that, when assembled on the anode of a battery, improved its capacity by several orders of

magnitude (Nam *et al.*, 2006). The combination of phages with noble metals, transition metals and semiconductor materials have also been investigated (Yoo *et al.*, 2006). Due to the high surface area of phages, they have also promising applications in the catalysis field. One example involved unmodified M13 filamentous phages coated with Rh, Pd, and Ru used in hydrogenation reactions (Avery *et al*., 2009).

2.2.5 Phages against lactic acid bacteria

LAB fermentation for the production of desirable products (e.g. food - Adams and Moss, 2008) faces a frequent problem with bacteriophage contamination. Unwanted phages attack LAB and lead to faulty fermentations. Although this type of contamination is a problem in the manufacture of all products derived from bacterial fermentation, such as butanol, antibiotics and gluconic acid, the dairy industry is the one that mostly exposed the issues related to phages (Moineau and Lévesque, 2005). Due to the rapid replication of phages, controlling their proliferation, once the contamination has started, is an arduous task. In fact, Watanabe *et al*. (1970) declared that controlling phage contamination is harder than controlling bacterial contamination.

Villion and Moineau (2009) conducted a review study on bacteriophages of *Lactobacillus*, one of the main genera of LAB. Their searches identified 231 phages of which a significant portion was isolated from contaminated food. Only nine phages had their complete genome sequenced and made available for studies. All of the phages belong to the *Caudovirales* order, of which almost 60% are of the *Siphoviridae* family. According to the authors, the problematic of phage contamination is likely to increase in the future with the higher production of probiotics.

2.2.6 *Lactobacillus plantarum* phages

Several *L. plantarum* phages were isolated in the recent years. The sources varied considerably, but most of them were found during LAB fermentation for the production of foods and feeds. All the phages belong to the *Caudovirales* order.

Chibani-Chennoufi *et al*. (2004) screened several *L. plantarum* phages from fermented food/feed sources. 15 phages were isolated from coffee (CC1 to CC15), phage LP45 from silage of a farm, LP76 from maize fermentation, and LP65 from salchichon salami. Phages LP65, CC12, CC13 have *Myoviridae* morphology and the others are *Siphoviridae*. Another isolate from meat products was phage fri (Trevors *et al*., 1983). This *L. plantarum* myophage has a latent period of 75min, a lysis period of 75min and a sizeable burst size of approximately 200pfu/cell.

Fermented feed silage is a common source of phages. Doi *et al*. (2003) isolated 10 virulent phages from silage in Japan. They were named ϕPY1-ϕPY10. Seven of them are members of the *Siphoviridae* family and three are *Myoviridae*. Several LAB were screened for sensitivity towards the phages and eight phages were able to infect the *L. plantarum* species tested.

De Antoni *et al*. (2010) isolated two *L. plantarum* phages from dairy processing. The fermented milk kefir was the source of phages FAGK1 and FAGK2. The two isolated siphophages have latent period of approximately 30min, lysis period of 80min and burst size of 11pfu/cell.

Several studies with phages isolated from fermenting vegetables have been conducted. Lu *et al*. (2003) identified phage ϕJL-1 in commercial cucumber fermentation. The phage is also a member of the *Siphoviridae* family and has a latent period of 35min, a lysis period of 40min, and a burst size of 22pfu/cell. Its genome has

36.7kbp and consists of linear dsDNA. In a study conducted in fermentation of table olives, Lanza *et al*. (2011) was able to isolate phages against *L. plantarum*. The natural fermentation of olives (Greek-style) occurs in a brine of 6-8% sodium chloride. Nine different brines were investigated and all of them had phage activity. The study isolated nine phages, ϕS1-ϕS9. The estimated genome sizes of the phages were approximately 36kbp for eight phages and 47kbp for one. Yoon *et al*. (2001; 2002) isolated two *L. plantarum* phages from kimchi, a Korean fermented food from cabbage. Phage SC921 is a member of the *Siphoviridae* family, with an isometric head of 60nm in diameter and a tail 260nm long and 10nm wide; and phage Y1 belongs to the *Podoviridae* family, with a short non-contractile tail.

Three phages were isolated from ruminal fluid samples and further characterized (Nemcová *et al*., 1993). The phages L1, L2 and L20 have similar characteristics: they all belong to the *Myoviridae* family and have hexagonal heads of 69-78nm in diameter and tails of 106-108nm in length.

L. plantarum phages were also identified in vaginal samples (Pavlova *et al*., 1997). The authors were investigating if phages against indigenous vaginal lactobacilli, bacteria important for vaginal health, were facilitating the replacement of the predominant vaginal flora with anaerobic bacteria causative of bacterial vaginosis (BV).

Caso *et al*. (1995) isolated several lactobacilli phages from samples of cheese, cheese whey, com silage, soil, river water and sewage. Temperate phages were also induced from strains of *L. plantarum*, *L. casei*, *L. brevis* and *L. hilgardii*. The authors further characterized the lytic *L. plantarum* phages ϕLP1 -A, ϕLP1-B and ϕLP2. All of them belong to the *Siphoviridae* family.

Sewage has also been an important source of phages infecting *L. plantarum*. Kelly *et al*. (2011) isolated four phages against a strain of *L. paraplantarum* (a species related to *L. plantarum* - Curk *et al*., 1996) from a wastewater treatment plant and a farm slurry pit in Ireland. The phages, named cIL1, cIL2, mmL1 and mmL2, all belong to the *Siphoviridae* family. In this and in other study (Kelly *et al*., 2012) the authors suggested the application of these isolated phages for the control of spoilage bacteria in the brewing industry.

The increased identification and characterization of, not only *L. plantarum* phages, but LAB phages in general is extremely important to provide fundamental knowledge and to allow a better understanding on the potential application of phages. Moreover, they provide sources for the composition of phage cocktails. A mixture of phages is generally more effective in killing the target bacteria and helps avoiding the appearance of resistant cells (Chan *et al*., 2013; Sulakvelidze and Kutter, 2005; Tanji *et al*., 2005). In addition, polyphage composition can have synergistic effect (Gu *et al*., 2012).

2.2.7 Phages *Lactobacillus plantarum* ATCC® 8014-B1™ and ATCC® 8014-B2™

The virulent phages ATCC® 8014-B1™ (phage B1) and ATCC® 8014-B2™ (phage B2) are well studied organisms and can be considered model phages against *Lactobacillus plantarum*. They have a relatively narrow host range, especially phage B1 (Briggiler Marcó *et al*., 2012; De Antoni *et al*., 2010), and have demonstrated significant resistance to thermal and chemical treatments and to pH inactivation (Briggiler Marcó *et al*., 2009; 2010).

Phage B1 was isolated from corn silage (Douglas and Wolin, 1971) and is a member of the *Siphoviridae* family, with an icosahedral capsid of around 54nm in diameter and tail of 157nm in length and 8nm in width (Briggiler Marcó *et al*., 2012). It has a linear dsDNA genome of *pac*-type with 38kbp, GC content of 47.6%, 60 open reading frames (ORFs), no tRNA, and 13 structural proteins (Briggiler Marcó *et al*., 2012). In addition, it has latent period of 30min, lysis time of 75min and burst size of 60pfu/infective centre at 37°C in MRS broth (de Man *et al*., 1960; Briggiler Marcó *et al*., 2010). Finally, galactose was shown to be the likely polysaccharide component involved in phage adsorption onto the host wall (Douglas and Wolin, 1971).

Phage B2 was isolated from anaerobic sewage sludge (Douglas and Wolin, 1971) and is also a member of the *Siphoviridae* family (Briggiler Marcó *et al*., 2012; Nes *et al*., 1988). Briggiler Marcó *et al*. (2012) reported an icosahedral capsid with a diameter of 74nm and a tail of 240nm in length and 10nm in width, whereas Nes *et al*. (1988) determined that the phage particle has isometric head of 110nm and a flexible tail of 500nm. This phage has linear dsDNA genome of *cos*-type with 80.5kbp, GC content of 37%, 127 ORFs, six tRNAs, and 9 structural proteins (Briggiler Marcó *et al*., 2012). Nes *et al*. (1988) found the same GC content but their genome size, estimated through restriction enzyme analysis, diverged (73kbp). According to Briggiler Marcó *et al*. (2010), the phage has latent period of 30min, lysis time of 75min and burst size of 83pfu/infective centre at 37°C in MRS broth. Nes *et al*., (1988) found latent period of 75min, lysis time of 90min and burst size of 12-14pfu/infective centre in an experiment performed in MRS at 30^{o}C. The glucose substituents in the teichoic acid were suggested to be the receptors responsible for phage adsorption (Douglas and Wolin, 1971).

3. HYPOTHESIS AND OBJECTIVES

It is hypothesised that virulent phages are capable of reducing bacterial contamination in yeast fermentation processes to levels that prevent reduction in ethanol yield.

Three main objectives were developed according to this hypothesis. The first objective to fulfill was to evaluate and quantify the reduction in the yields of yeast and ethanol due to the presence of *Lactobacillus plantarum* in conditions similar to those commonly found in industries. The next objective was to evaluate the effectiveness of phages in reducing contamination and their impact on yeast behaviour and ethanol production. The third objective was to develop a mathematical model to simulate the population dynamics and to help understand the phenomena involved in the growth of the three types of organisms present in the system (yeast, bacteria and phages).

4. MATERIALS AND METHODS

4.1 Microorganisms and culture media

The yeast used for this work was *Saccharomyces cerevisiae* Superstart™. This commercial yeast was kindly provided by Lallemand Inc. (Montreal, QC). The bacterium and phages used were *Lactobacillus plantarum* ATCC® 8014™ and bacteriophages *Lactobacillus plantarum* ATCC® 8014-B1™ (phage B1) and *Lactobacillus plantarum* ATCC® 8014-B2™ (phage B2).

The media MRS and M9 were utilized in this work. MRS (BD Difco™ lactobacilli broth, Sparks, MD) is a medium designed by de Man *et al*. (1960), appropriate for growth of lactobacilli. The broth was prepared following instructions of the manufacturer, dissolving 55g of the powder per 1L of deionized water. M9 minimal salts medium was prepared in three different stages. First, a solution of 5 × M9 was prepared by adding 33.92g of sodium phosphate monobasic anhydrous 99% (Fisher Scientific, Fair Lawn, NJ), 15g of potassium phosphate monobasic 99% (Fisher Scientific, Fair Lawn, NJ), 5g of ammonium chloride (Fisher Scientific, Fair Lawn, NJ) and 2.5g of sodium chloride 100.1% (Fisher Scientific, Fair Lawn, NJ) into 1L of deionized water. Then, 1.1 × M9 solution was prepared by adding 200mL of the 5 × M9 solution into 700mL of deionized water with 0.4914g of magnesium sulfate heptahydrate 101.9% (Fisher Scientific, Fair Lawn, NJ) and 0.0147g of calcium chloride dehydrate (Fisher Scientific, Fair Lawn, NJ). Finally, 1g of yeast extract (BD Bacto™, Sparks, MD) was added to the 900mL of 1.1 × M9 solution and autoclaved. This solution was then mixed with 100mL of sterile 20g/L glucose (Fisher Scientific, Fair Lawn, NJ) solution. Both media had their pH adjusted with 1N HCl and 1M NaOH solutions, as required. A pH meter (Fisher Scientific Accumet® AR20) was used for measurements.

Hard and soft agars were prepared by the addition of 1.5% and 0.75% of agar technical (BD Difco™, Sparks, MD) in MRS broth, respectively. Hard agar was immediately poured in petri dishes while soft agar was kept in a water bath at 60°C prior to cell addition and pouring.

4.2 Cells growth measurements

Cells growth was monitored either by measuring the optical density of a sample at a wavelength of 600nm (OD_{600}) using a spectrophotometer (Ultrospec 50, Biochrom, Cambridge, UK), by counting the number of cells in a counting chamber (Hausser Scientific, improved Neubauer brightline, Horsham, PA) with a microscope (Leica Mycrosystems, DMRXA2, Heerbrugg, Switzerland) equipped with a digital camera (QImaging, Retiga EX, Surrey, BC), or by serial dilutions method with colony forming units counted on petri dishes with hard agar after overnight incubation at 30°C.

4.3 Aerobic and anaerobic conditions

All aerobic experiments were conducted in shake flasks capped with foam stoppers incubated in a rotary incubator shaker (Infors HT, Ecotron, Bottmingen, Switzerland). The anaerobic conditions were carried out in sealed culture bottles with medium height to vessel diameter ratio greater than 60%. The experiments were not conducted under strictly anaerobic conditions because some oxygen was initially present in the system. However, anaerobic conditions were expected to be achieved once the oxygen dissolved in the medium was consumed by the organisms. The low

head space of the containers and lack of exchange with the surroundings represented a good simulation of the fermentation conditions encountered in industry.

The anaerobic experiments with agitation were conducted on the tray of the rotary incubator shaker and those without agitation were in the same equipment, but in a section without rotation.

4.4 Phage titer

Titer measurements were performed using the agar layering technique, similar to the one described in Storms *et al*. (2010).

10µL of a host's overnight culture (2µL of host culture were used for phage B2) were added to 3mL of MRS soft agar kept at 60^{o}C. The mixture was then poured on a layer of hard agar and let to dry and solidify for 5min. 10-µL drops of dilutions of phage solution were placed on the soft agar. Once the drops were dried, the plate was incubated overnight at 30^{o}C (37^{o}C in the case of phage B2). The titer in pfu/mL was calculated by counting plaques of phages after overnight incubation.

4.5 Inoculation loads

Unless otherwise specified, late exponential growing cultures were diluted in the following manner before addition into the recipients: in experiments involving axenic yeast cultures, *S. cerevisiae* culture was diluted to 10^{6}cells/mL. The growth involving yeasts and bacteria at low inoculation level had the dilution of the cultures to $2x10^{6}$cells/mL of yeasts and $2x10^{7}$cells/mL of bacteria. For the experiments with yeasts, bacteria at low inoculation level and phages, $3x10^{6}$cells/mL of yeasts were mixed with $3x10^{7}$cells/mL of bacteria, $1.5x10^{6}$pfu/mL of phage B1 and $1.5x10^{6}$pfu/mL of phage B2

in a tube (microcentrifuge or bottom round). The mixture was vortexed and let adsorbing for 5min before being added into the recipient. Likewise, for the growth of yeasts and bacteria at high inoculation level, yeasts were diluted to $2x10^6$cells/mL and bacteria to $2x10^9$cells/mL; and for the cases when phages were added, $3x10^6$cells/mL of yeasts were mixed with $3x10^9$cells/mL of bacteria, $1.5x10^8$pfu/mL of phage B1 and $1.5x10^8$pfu/mL of phage B2 in a tube, vortexed and let adsorbing for 5min before being added into the recipients.

In all cases the cultures were added into the recipients in an amount that allowed 10^4cells/mL of yeasts, $10^5/10^7$cells/mL of bacteria and phages at a multiplicity of infection (MOI) of 0.1 to be present at the beginning of the experiment. The inoculation load of yeasts (10^4cells/mL) corresponded to the recommendation of the yeast manufacturer. The lowest bacterial inoculation level (10^5cells/mL) simulated the contamination concentration mostly found in industries (Leja and Broda, 2009; Skinner and Leathers, 2004; Sossa Urrego *et al*., 2009), whereas the highest (10^7cells/mL) exemplified a more critical contamination level.

4.6 Impact of pH on aerobic cultures

All experiments to evaluate pH effects on organisms were performed aerobically at 30^{o}C and 150rpm. Optical density measurements were taken periodically.

For experiments with yeast, shake flasks with 25mL of M9 medium at pH3, 3.5, 4, 4.5, 5 and 6 were used. The flasks were inoculated with 100µL of an exponentially growing culture. Two replicates were performed for this experiment and the results were reported as the average of both.

For the bacterium, M9 at pH3.5, 4, 4.5, 5, 6 and 7 was used. 20mL of medium were added to shake flasks and were inoculated with 200µL of exponentially growing cultures diluted to 10^7cfu/mL. Three replicates were performed in this experiment.

Tests involving phages B1 and B2 were carried out with each individual phage and with both phages in combination. Experiments were conducted in shake flasks with 25mL of M9 at pH3.5, 4, 4.5, 5, 6 and 7. For the tests with individual phages, exponentially growing bacteria diluted to 10^7cfu/mL were mixed in a microcentrifuge tube with phages at a concentration of 10^6pfu/mL (MOI of 0.1), followed by adsorption (resting) for 5min before 250µL of the mixture were added to the flask. When testing the phage cocktail, bacteria at 10^7cfu/mL were mixed with phages B1 and B2 at $5x10^5$pfu/mL each in a microcentrifuge tube (MOI of 0.1). The mixture was let to allow phage adsorption for 5min before 250µL were added to the flasks.

4.7 Population dynamics of yeasts, bacteria and phages B1 and B2

The experiments for population dynamics studies were all performed at 30^{o}C and 150rpm (in the case of experiments with agitation). Cells concentrations (yeasts and bacteria) were determined using microscopy and counting chamber.

4.7.1 Experiments in M9 medium

Aerobic experiments with several different bacterial inoculation levels (from 10^5cells/mL to 10^8cells/mL) were performed, but only the one with 10^5cells/mL was reported. This experiment was conducted in shake flasks containing 25mL of M9 media at pH4 and 6. For axenic yeast cultures, 250µL of medium containing 10^6cells/mL were added to the flasks. For the cases where yeasts grew along with bacteria, 250µL of a

mixture containing 10^6cells/mL of yeasts and 10^7cells/mL of bacteria were added to the flasks. For the experiments involving phages, 10^6cells/mL of yeasts were mixed with 10^7cells/mL of bacteria, $5x10^5$pfu/mL of phage B1 and $5x10^5$pfu/mL of phage B2 in a microcentrifuge tube. The mixture was vortexed and let to adsorb for 5min before 250µL were added to the flasks.

Anaerobic experiments in M9 medium at pH4 or 6 were also carried out. Capped bottles with capacity of 130mL were filled with 100mL of medium. The inoculation load of the cultures followed the procedures explained in **Section 4.5**. Cells concentrations were determined by microscopy after 41h.

4.7.2 Experiments in MRS medium

Population dynamics studies were also performed in MRS under aerobic and anaerobic conditions. In all cases cultures were inoculated according to **Section 4.5**. The aerobic experiments were divided in two, one with a single measurement after 20h of fermentation and another with periodic measurements throughout 60 hours of fermentation. Both were conducted in shake flasks containing 25mL of MRS medium at pH6, but in the former case bacteria at high and low inoculation levels were applied and the experiments were conducted in triplicates. The latter evaluated only bacteria at high inoculation level and had one replicate. For the anaerobic experiments, bottles with a capacity of 130mL were filled with 120mL of MRS medium at pH6. The experiments were carried out without agitation and in triplicates.

4.8 Ethanol production

Ethanol production was measured for cultures growing aerobically and anaerobically in MRS medium at pH6. The procedures for the preparation of cultures grown under aerobic condition were the same as described for evaluation of the kinetics throughout 60 hours of fermentation. In the case of anaerobic conditions, procedures were the same as the ones for anaerobic experiments carried out without agitation (both in **Section 4.7.2**). Triplicate experiments were performed for both cases.

Procedures for the measurement of ethanol were the same in both cases: for experiments without phages, a 1-mL sample was transferred into a microcentrifuge tube and centrifuged (Eppendorf 5424R, rotor Eppendorf FA-45-24-11, Hamburg, Germany) for 1min at 14,000×g and 25^{o}C. When phages were present in the samples, different conditions were used for centrifugation (90min at 20,000×g and 25^{o}C). This was done to remove the phages from the supernatant and avoid them to pass through the high performance liquid chromatography (HPLC) column. Samples were then filtered (Fischer Scientific, 0.2µm, Windsor, ON) and transferred into chromatography vials. The ethanol content was measured by HPLC (Agilent Technologies 1200 series, SupelcoGel Pb carbohydrate column with 7.8mm internal diameter and 30cm length, with guard column and refractive index detector – RID). Sample injection volume for measurement was 10µL and elution flow-rate was 0.5 mL/min with deionized sterile water (MilliQ, MilliPore).

The measurements taken in the HPLC were transformed to ethanol concentration through the calibration curve presented in **Figure 4-1**.

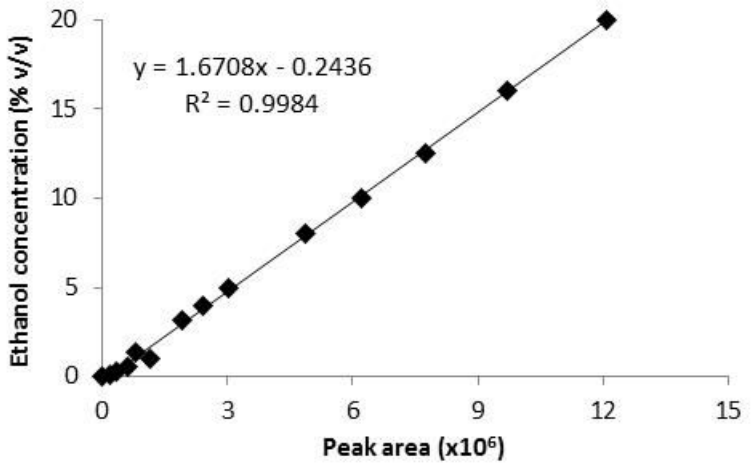


Figure 4-1: Calibration curve for ethanol concentration measured in HPLC. The insert expresses the linear equation and the coefficient of determination.

4.9 Determination of parameters for the mathematical model

Several experiments were conducted to either determine the parameters to be applied in the model or test the fit of the model with experimental data. Basically, parameters were determined from experiments with each individual organism and the model was tested from population dynamics experiments. For the case of yeast growth, the model was tested with data taken from the experiments conducted for axenic yeast cultures under aerobic conditions in MRS medium at pH6 sampled throughout 60h of fermentation (**Section 4.7.2**).

4.9.1 Bacteria-related parameters

Experiments were carried out with 25mL of MRS at pH6 in shake flasks under aerobic conditions at 30^{o}C and 150rpm. 250µL of a late exponential growth culture diluted to 10^{7}cells/mL were added to the flasks. OD_{600} measurements were taken

periodically and cells concentrations were obtained with a calibration curve (data not shown).

4.9.2 One step growth curves

One step growth experiments were performed for each phage in MRS medium at pH6, 30^{o}C and 150rpm. For both cases, late exponential phase bacterial cultures were diluted to 2x10^{8}cells/mL. 500µL of the culture were mixed in a microcentrifuge tube with 500µL of phage solution to a MOI of 0.1 (the low MOI is applied to avoid that more than one phage infects a cell). The mixture was vortexed and let to rest. Time started once vortexing was done. At time zero, 10µL of the mixture were diluted in 1mL of fresh medium and the titer was measured following the procedures found in **Section 4.4**. At 5min, 20µL of the mixture were transferred into a flask with 20mL of fresh medium and titer was measured periodically. At 100min, 2mL of the diluted mixture were transferred into another flask containing 20mL of fresh medium and more titer measurements were taken. These dilution steps were conducted to avoid secondary infection of cells. The burst size (b) was calculated by dividing the released phages – calculated as the average titer after lysis time ($titer_{LT}$) minus the unadsorbed phages based on adsorption efficiency measurements ($Ɛ_{ads}$ - Storms *et al*., 2010; 2012) – by the infected cells – calculated as the average titer in latent period ($titer_{LP}$) minus the unadsorbed phages (based on adsorption efficiency). This is expressed in **Equation 4-1**:

$$b = \frac{release\ phages}{infected\ cells} = \frac{(titer_{LT}) - (Ɛ_{ads}\ .\ titer_{LP})}{(titer_{LP}) - (Ɛ_{ads}\ .\ titer_{LP})} \qquad \textbf{(Eq. 4-1)}$$

Adsorption efficiencies for both phages were determined in adsorption experiments.

4.9.3 Adsorption experiments

Similar procedures were used for adsorption experiments performed with both phages. The experiments were conducted in MRS medium at pH6 and at room temperature. 500µL of a culture of *L. plantarum* at a concentration of $2x10^8$cells/mL were mixed with 500µL of a phage solution containing $2x10^7$pfu/mL (MOI of 0.1) in a microcentrifuge tube. The mixture was vortexed before the chronometer started. At different times, 10-µL samples were transferred into a microcentrifuge tube with 1mL of fresh medium to avoid further adsorption. The diluted samples were then centrifuged for 1min at 14,000×g and 25°C, to allow only unadsorbed phages to remain in the supernatant. After centrifugation, the titer measurements were performed on the diluted samples. These titers corresponded to the amount of unadsorbed phages remaining at that time. These values were then subtracted from the initial concentration of phages in the phage stock.

4.9.4 Infection of bacterial population (non-susceptible/susceptible)

This experiment was conducted aerobically, at 30°C, 150rpm, and in shake flasks. *L. plantarum* cultures in late exponential phase were diluted to a concentration of $3x10^9$cells/mL. Phage B1 stock was diluted to reach a concentration of approximately $4x10^8$pfu/mL, and phage B2 was diluted to reach $4x10^7$pfu/mL. 2mL of the diluted bacteria were mixed with 1mL of phage B1 and 1mL of phage B2 diluted stocks in a 5mL bottom round tube, vortexed and let to adsorb for 5min. 2mL of this mixture were then added to a shake flask containing 18mL of MRS at pH6. This high inoculation load (10%) allowed a more accurate evaluation of the behaviour of the host. Periodically, the growth of the host was monitored by optical density and the

corresponding cell concentrations were calculated from a calibration curve (data not shown).

4.9.5 Phage amplification

These experiments were conducted at either of two MOI, 0.1 or 100. 25mL of MRS at pH6, 30°C and 150rpm under aerobic conditions were inoculated with bacteria and phage. Bacterial cultures in late exponential phase were diluted to reach $2x10^7$cells/mL. Phage B1 stock was diluted to reach $2x10^6$pfu/mL (for a corresponding MOI of 0.1) or $2x10^9$pfu/mL (for a MOI of 100). Phages were mixed with bacteria, vortexed, and let to adsorb for 5min. 250-µL of this mixture were then added to a shake flask containing 25mL of medium. OD_{600} was measured and cell concentration values were obtained from a calibration curve (data not shown). The titers of free phages in the solution were evaluated on centrifuged samples (1min, at 14,000×g and 25°C).

5. MATHEMATICAL MODEL

A mathematical model was developed to simulate and help understand the dynamics of the different populations involved in the system studied (yeast (Y), bacteria (B), phages (P)).

5.1 Yeast population

Monod kinetics was used to describe a simplified model of the growth of yeast (Shuler, 2002; Monod, 1949). In this form, it is based on the following reaction:

$$Y + S \xrightarrow{\mu_{yeast}} 2\,Y \qquad \textbf{(Eq. 5-1)}$$

where Y represents yeast cells and S the substrate.

According to this model, the growth rate and rate of substrate consumption are given by **Equations 5-2** and **5-3**, respectively.

$$\frac{dY}{dt}|_{growth} = Y\left(\frac{\mu_{\max - SC} \cdot S}{K_{Monod-SC}+S}\right) \qquad \textbf{(Eq. 5-2)}$$

$$\frac{dS}{dt}|_{growth} = -\left(\frac{\mu_{\max - SC} \cdot S}{K_{Monod-SC}+S}\right)\left(\frac{Y}{Yi_{SC}}\right) \qquad \textbf{(Eq. 5-3)}$$

where the subscript $_{SC}$ stands for parameters for the yeast *Saccharomyces cerevisiae* Superstart™.

This model determines the growth rate (μ) of the organism through the correlation between its maximum specific growth rate (μ_{max}), affinity constant towards the limiting substrate (Monod constant – K_{Monod}), and the yield of the organism to substrate (Yi). μ_{max} is the maximum growth rate that can be achieved by the organism regardless of the concentration of the limiting substrate (in the present case, glucose). μ_{max} can be obtained experimentally through the measurement of the growth rate at different concentrations of the limiting nutrient. Monod constant (K_{Monod}) is the half

velocity constant and corresponds to the concentration of substrate required to achieve half μ_{max}. Yield (Yi) is the amount of biomass or, in the case of the present study, the quantity of cells produced per gram of the limiting nutrient.

5.2 Bacterial population growth

Monod kinetics was also used to describe bacterial growth, according to the reaction:

$$B + S \xrightarrow{\mu_{bacteria}} 2\ B \qquad \textbf{(Eq. 5-4)}$$

where B represents bacterial cells.

Similarly to the growth of yeast cells, the growth rate and substrate consumption can be described by **Equations 5-5** and **5-6**:

$$\frac{dB}{dt}|_{growth} = B\left(\frac{\mu_{max-LP}\cdot S}{K_{Monod-LP}+S}\right) \qquad \textbf{(Eq. 5-5)}$$

$$\frac{dS}{dt}|_{growth} = -\left(\frac{\mu_{max-LP}\cdot S}{K_{Monod-LP}+S}\right)\left(\frac{B}{Yi_{LP}}\right) \qquad \textbf{(Eq. 5-6)}$$

where the subscript $_{LP}$ stands for parameters for the lactic acid bacterium *Lactobacillus plantarum* ATCC® 8014™.

The parameters μ_{max}, K_{Monod} and Yi are determined in a similar fashion as for the yeast.

5.3 Susceptible and non-susceptible bacterial populations

To account for the introduction of phages into the system, the bacterial population was divided into two sub-groups: 1) bacteria that are susceptible to phage infection (SB) and 2) those that are non-susceptible to infection (NSB). It is important to note that non-susceptible cells are different than resistant cells. The former, unlike

resistance, is a transient state; non-susceptible cells can eventually revert back to a susceptible state and vice-versa. This idea was derived from epidemiological studies of certain types of viral infections in humans, which define that groups within a population can be less susceptible to infections at different stages in their development (Palese, 2004). A major type of group differentiation is age. It is recognized, for instance, that children and elderly are more susceptible to certain types of viral diseases (Neuzil *et al*., 2000). The exact causes of host non-susceptibility in phage-bacteria systems are not well defined and may differ from system to system; however phenotype, metabolism and cell age are suggested as potential factors involved. To our knowledge, this is the first study that considers the concept of non-susceptibility of bacterial hosts.

In the present study both groups of bacteria were assumed to grow at the same rate and, therefore, have the same parameters for Monod kinetics. In addition, the combined populations of these two bacterial groups must equal the overall bacterial population. **Equations 5-7** and **5-8** present the reactions of formation of susceptible and non-susceptible bacteria. Note that the kinetics is the same for both.

$$SB + S \xrightarrow{\mu_{bacteria}} 2\ SB \qquad \textbf{(Eq. 5-7)}$$

$$NSB + S \xrightarrow{\mu_{bacteria}} 2\ NSB \qquad \textbf{(Eq. 5-8)}$$

where SB is susceptible bacterium and NSB is non-susceptible bacterium.

The growth rate and substrate consumption for both bacterial groups are described below:

$$\frac{dSB}{dt}|_{growth} = SB\left(\frac{\mu_{max-LP} \cdot S}{K_{Monod-LP}+S}\right) \qquad \textbf{(Eq. 5-9)}$$

$$\frac{dNSB}{dt}|_{growth} = NSB\left(\frac{\mu_{max-LP} \cdot S}{K_{Monod-LP}+S}\right) \qquad \textbf{(Eq. 5-10)}$$

$$\frac{dS}{dt}|_{growth} = -\left(\frac{\mu_{max-LP} \cdot S}{K_{Monod-LP}+S}\right)\left(\frac{SB}{Yi_{LP}}\right) - \left(\frac{\mu_{max-LP} \cdot S}{K_{Monod-LP}+S}\right)\left(\frac{NSB}{Yi_{LP}}\right) \quad \textbf{(Eq. 5-11)}$$

As previously explained, susceptible bacteria may become non-susceptible and vice-versa. **Equation 5-12** shows the interrelation between these groups of bacteria.

$$NSB \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SB \quad \textbf{(Eq. 5-12)}$$

This reaction has a forward and reverse rate constant, k_1 and k_2 respectively. At equilibrium, the forward and reverse reaction rates are equal (**Equation 5-13**), and the equilibrium rate constant (K_{eq}) is equal to the ratio of the rate constants, k_1 and k_2. K_{eq} is also equal to the ratio of the concentrations of susceptible and non-susceptible bacteria at equilibrium (**Equation 5-14**).

$$k_1.NSB|_{eq} = k_2.SB|_{eq} \quad \textbf{(Eq. 5-13)}$$

$$K_{eq} = \frac{k_1}{k_2} = \frac{[SB]_{eq}}{[NSB]_{eq}} \quad \textbf{(Eq. 5-14)}$$

The rates of bacterial cell transformation are given by **Equations 5-15** and **5-16**:

$$\frac{dSB}{dt}|_{transformation} = k_1.NSB - k_2.SB \quad \textbf{(Eq. 5-15)}$$

$$\frac{dNSB}{dt}|_{transformation} = k_2.SB - k_1.NSB \quad \textbf{(Eq. 5-16)}$$

Susceptible and non-susceptible bacterial groups are assumed to be existent in all populations, independently of the presence or absence of phages. Hence, equilibrium between these groups can be considered to exist in a normal growing population in the absence of phages. Should a bacterial culture be initiated at groups ratio different from the equilibrium ratio, after a certain period of time, the population would reach equilibrium within a few generations. In fact, simulations using this model showed that, indeed, equilibrium was achieved rapidly in the system without phages (data not shown). Therefore, it can be assumed that all experiments conducted in this study were initiated with bacterial populations at equilibrium between the different

groups and that the initial ratio of the bacterial groups was equal to the equilibrium constant K_{eq}, as expressed in **Equation 5-17**:

$$K_{eq} = \frac{[SB]_{initial}}{[NSB]_{initial}} \quad \textbf{(Eq. 5-17)}$$

5.4 Phage infection

The mathematical model for phage infection applied in this system considers multiple parameters involved in phage amplification. These parameters are adsorption rate constant (for phage adsorption onto bacterial receptors - k_{ads}), adsorption efficiency (proportion of phages that effectively bind to a host cell - $\mathcal{E}_{ads}$), latent period (U), lysis time (Lt) and burst size (b). All these parameters were obtained experimentally.

The model was built on the assumption that only one phage attaches to a bacterium to form an infected cell. The phage then overtakes the infected host replication machinery to create and release new progeny. The process of phage infection and development considered in this model is represented in **Equation 5-18**:

$$SB + P \xrightarrow{k_{ads}} IC \xrightarrow{k_{Lt}} bP \quad \textbf{(Eq. 5-18)}$$

where P is phage, IC is infected cell and b is burst size. Note that only susceptible bacteria (SB) are infected by phages

The reaction leading to the formation of progeny was assumed to be first order, as this is an adequate generalization for this mechanism. From the rates of the reactions present in **Equation 5-18**, the following equations are obtained:

Equation for susceptible bacteria infection:

$$\frac{dSB}{dt}|_{infection} = -k_{ads}.\mathcal{E}_{ads}.P.SB \quad \textbf{(Eq. 5-19)}$$

The parameter $\mathcal{E}_{ads}$, or adsorption efficiency, was introduced by Storms *et al*. (2010; 2012) to account for the unadsorbing fraction of phages in tailed phage populations.

Equation for phage production:

$$\frac{dP}{dt} = k_{Lt} \,.\, b.IC - k_{ads} \,.\, \mathcal{E}_{ads} \,.\, P.SB \qquad \textbf{(Eq. 5-20)}$$

Equation for the rate of change of infected cells:

$$\frac{dIC}{dt} = k_{ads} \,.\, \mathcal{E}_{ads} \,.\, P.SB - k_{Lt} \,.\, IC \qquad \textbf{(Eq. 5-21)}$$

In a system in which only the infected bacteria are evaluated, as is the purpose of the one step growth experiment, SB is equal to zero right after initiation and the first term of the right side of **Equation 5-21** becomes zero. The differential equation can then be solved as:

$$IC = IC_0 . e^{-k_{Lt}.Lt} \qquad \textbf{(Eq. 5-22)}$$

Rearranging **Equation 5-22** and assuming lysis of 99% of the infective cells (a common assumption in one-step experiments, $IC/IC_0 = 1/100$), the rate constant of the lysis reaction is given by the following equation:

$$k_{Lt} = \frac{-1}{Lt} \ln\left(\frac{1}{100}\right) \qquad \textbf{(Eq. 5-23)}$$

The parameters linked to infection were determined through one-step experiments and had to be evaluated for each phage (B1 and B2) individually.

5.5 Combined model

The combination of all terms described yields the final model for the system; the general scheme of which is presented in **Figure 5-1**:

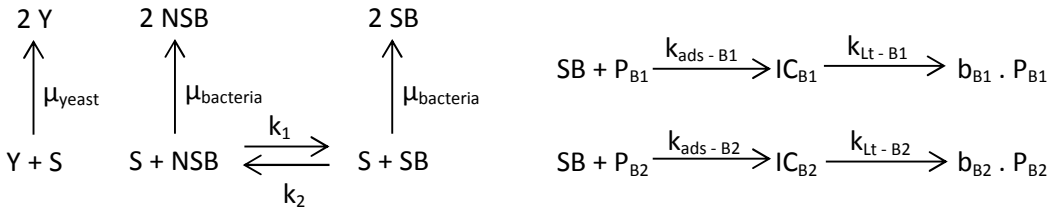


Figure 5-1: General scheme of the final mathematical model configuration used for the populations dynamics studied. Subscripts $_{B1}$ and $_{B2}$ stand for parameters for phages B1 and B2, respectively.

Rearranging the formulas according to the system presented in **Figure 5-1**, the following equations for the complete mathematical model were obtained:

Equation for yeast growth:

$$\frac{dY}{dt} = Y\left(\frac{\mu_{\max - SC} \cdot S}{K_{Monod-SC}+S}\right) \quad \textbf{(Eq. 5-24)}$$

Equation for susceptible bacteria:

$$\frac{dSB}{dt} = SB\left(\frac{\mu_{\max - LP} \cdot S}{K_{Monod-LP}+S}\right) + k_1 . NSB - k_2 . SB - k_{ads-B1} . \mathcal{E}_{ads-B1} . P_{B1} . SB - k_{ads-B2} . \mathcal{E}_{ads-B2} . P_{B2} . SB \quad \textbf{(Eq. 5-25)}$$

where the first term of the right side of the equation corresponds to susceptible bacteria growth, the second corresponds to the transformation of non-susceptible cells into susceptible cells, the third to the transformation of susceptible cells into non-susceptible cells, the fourth to phage B1 infection and the fifth to phage B2 infection.

Equation for non-susceptible bacteria:

$$\frac{dNSB}{dt} = NSB\left(\frac{\mu_{\max - LP} \cdot S}{K_{Monod-LP}+S}\right) + k_2 . SB - k_1 . NSB \quad \textbf{(Eq. 5-26)}$$

where the first term of the right side of the equation corresponds to non-susceptible bacteria growth, the second to the transformation of susceptible cells into non-

susceptible cells and the third to the transformation of non-susceptible cells into susceptible cells.

Equation for overall bacterial population (susceptible plus non-susceptible - B):

$$\frac{dB}{dt} = \frac{dSB}{dt} + \frac{dNSB}{dt} \quad \textbf{(Eq. 5-27)}$$

Equation for cells infected by phage B1:

$$\frac{dIC_{B1}}{dt} = k_{ads-B1} \,.\, \mathcal{E}_{ads-B1} . P_{B1} \,.\, SB - k_{Lt-B1} \,.\, IC_{B1} \quad \textbf{(Eq. 5-28)}$$

where the first term of the right side of the equation corresponds to infected cell formation and the second to cell lysis.

Equation for phage B1 production:

$$\frac{dP_{B1}}{dt} = k_{Lt-B1} \,.\, b_{B1} \,.\, IC_{B1} - k_{ads-B1} \,.\, \mathcal{E}_{ads-B1} . P_{B1} \,.\, SB \quad \textbf{(Eq. 5-29)}$$

where the first term of the right side of the equation corresponds to phage production and the second to infected cell formation.

Equation for cells infected by phage B2:

$$\frac{dIC_{B2}}{dt} = k_{ads-B2} \,.\, \mathcal{E}_{ads-B2} . P_{B2} \,.\, SB - k_{Lt-B2} \,.\, IC_{B2} \quad \textbf{(Eq. 5-30)}$$

where the first term of the right side of the equation corresponds to infected cell formation and the second to cell lysis.

Equation for phage B2 production:

$$\frac{dP_{B2}}{dt} = k_{Lt-B2} \,.\, b_{B2} \,.\, IC_{B2} - k_{ads-B2} \,.\, \mathcal{E}_{ads-B2}\, P_{B2} \,.\, SB \quad \textbf{(Eq. 5-31)}$$

where the first term of the right side of the equation corresponds to phage production and the second to infected cell formation.

Equation for overall phage production (phages B1 and B2 free in solution - P):

$$\frac{dP}{dt} = \frac{dP_{B1}}{dt} + \frac{dP_{B2}}{dt} \quad \textbf{(Eq. 5-32)}$$

Equation for substrate consumption:

$$\frac{dS}{dt} = -\left(\frac{\mu_{max-SC} \cdot S}{K_{Monod-SC}+S}\right)\left(\frac{Y}{Yi_{SC}}\right) - \left(\frac{\mu_{max-LP} \cdot S}{K_{Monod-LP}+S}\right)\left(\frac{SB}{Yi_{LP}}\right) - \left(\frac{\mu_{max-LP} \cdot S}{K_{Monod-LP}+S}\right)\left(\frac{NSB}{Yi_{LP}}\right)$$

(**Eq. 5-33**)

where the first term of the right side of the equation corresponds to substrate consumption by yeast, the second to substrate consumption by susceptible bacteria, and the third to substrate consumption by non-susceptible bacteria.

The latent period (U) parameters were added to the model as conditional terms that prevent the second term of **Equations 5-28** and **5-30** and the first term of **Equations 5-29** and **5-31** to be calculated for an inital period corresponding to the latent period of each corresponding phage (Jain *et al*., 2006).

The main assumptions for this model are: 1) all susceptible cells are sensitive to phage infection; 2) no more than one phage infects a bacterium; 3) there are no resistant bacteria (Cairns *et al*., 2009; Jain *et al*., 2006); 4) substrate consumption by bacteria infected by phages is negligible; 5) the conversion from one bacterial group to the other does not require substrate consumption; 6) the death of bacterial cells is caused only by phage infection (natural cell death is negligible); 7) the rate of yeast death is negligible; 8) yeast buds are not differentiated from yeast cells (both entities compound yeast development); and 9) phages are not inactivated. Additionally, secondary characteristics of phage infections such as pseudolysogeny and abortive infection (Chopin *et al*., 2005) were not taken into account.

6. RESULTS

The presence of multiple organisms in a system (*e.g.* yeasts, bacteria, phages) may lead to differences in behaviour of the individual entities when compared to axenic cultures. These differences can involve simple phenomena – such as competition for nutrients – or more complex situations – such as the release of inhibitory substances by competing organisms. Differences in environmental or processing conditions, such as pH, aeration and medium composition, also impact the behaviour of strains. This combination of organisms and environmental conditions make up the ecology of a system or process, which may greatly impact productivity. In this study, several sets of conditions were investigated to understand the ecological parameters interfering in the dynamics of the populations involved.

6.1 Impact of pH on aerobic cultures

In order to better understand the impact of pH on the behaviour of the cultures involved in this study, the yeast *Saccharomyces cerevisiae* Superstart™ and the lactic acid bacterium *Lactobacillus plantarum* ATCC® 8014™ were grown in M9 minimal media at pH ranging from 3 to 7. In addition, infections of the bacterial strain by bacteriophages ATCC® 8014-B1™ (phage B1) and ATCC® 8014-B2™ (phage B2) were also carried out at a similar pH range.

6.1.1 *Saccharomyces cerevisiae* Superstart™

The aerobic growth of yeast in M9 medium with different pH values is presented in **Figure 6-1**. The trends did not show clear differences in cell growth for the pH range investigated. At all pH tested the yeast reached an optical density of

approximately 2.5, which corresponds to a cell concentration of $4x10^7$cells/mL, at stationary phase after approximately 28 hours of fermentation.

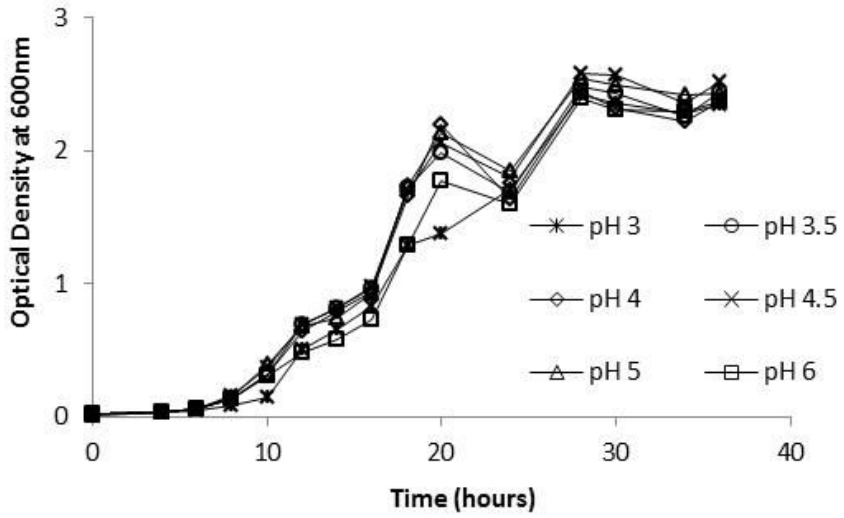


Figure 6-1: Aerobic growth of *S. cerevisiae* in M9 minimal medium at pH values ranging from pH3 to pH6.

6.1.2 *Lactobacillus plantarum* ATCC® 8014™

Figure 6-2 shows the aerobic growth of *L. plantarum* in M9 minimal medium at pH ranging from 3.5 to 7. Reducing the pH directly inhibited the yield of the bacterial culture. The lowest cell concentration ($4x10^7$cfu/mL) was observed at the lowest pH.

The longer lag phase observed at pH 7 can be explained by suboptimal growth at this pH and/or salts agglomeration occurred during medium sterilization by autoclave. This precipitation may have reduced the availability of some of the salts and hampered their uptake by the microorganisms. In spite of the longer lag phase, it is important to note that the final cells yield was still greatest at pH 7.

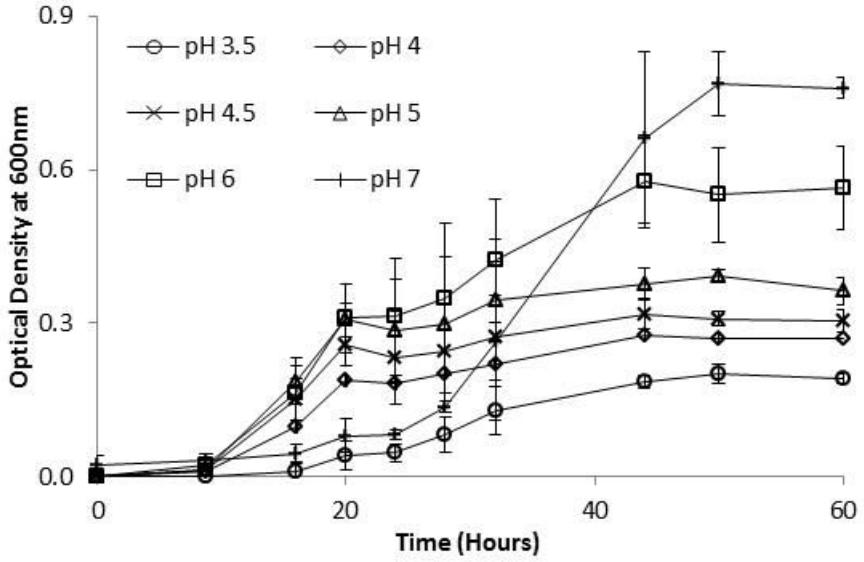


Figure 6-2: Aerobic growth of *L. plantarum* in M9 minimal medium at pH ranging from pH3.5 to pH7. M9 medium at pH3.61 was used as reference for OD measurements. Error bars indicate the standard deviation of three experiments.

6.1.3 *Lactobacillus plantarum* bacteriophages ATCC® 8014-B1™ and ATCC® 8014-B2™

The results of *L. plantarum* cultures infected by phage B1 in media at different pH can be found in **Figure 6-3**. According to this figure, the yield of the bacteria infected by phage was greater at higher pH, except at pH 6 which had the lowest growth. This indicates that phage B1 has a greater infection potential (is mostly active) at pH 6. As in **Figure 6-2** the host had a longer lag phase at pH 7, again probably caused by suboptimal growth and/or salt availability.

Figure 6-4 shows the results for the growth of bacterial host infected by phage B2 in M9 media at different pH. These results indicate that phage B2 infection was most effective in the pH range from 4 to 6.

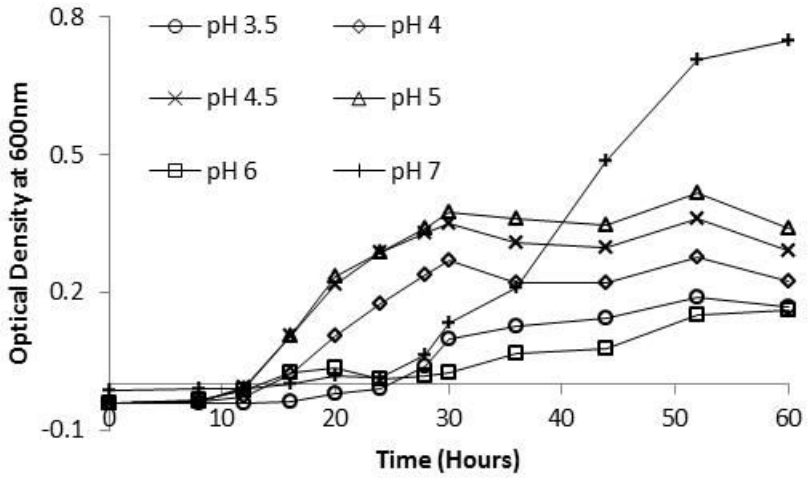


Figure 6-3: Aerobic growth of *L. plantarum* infected by phage B1, at an initial MOI of 0.1, in M9 minimal medium at pH ranging from pH3.5 to pH7. M9 medium at pH3.61 was used as reference for OD measurements.

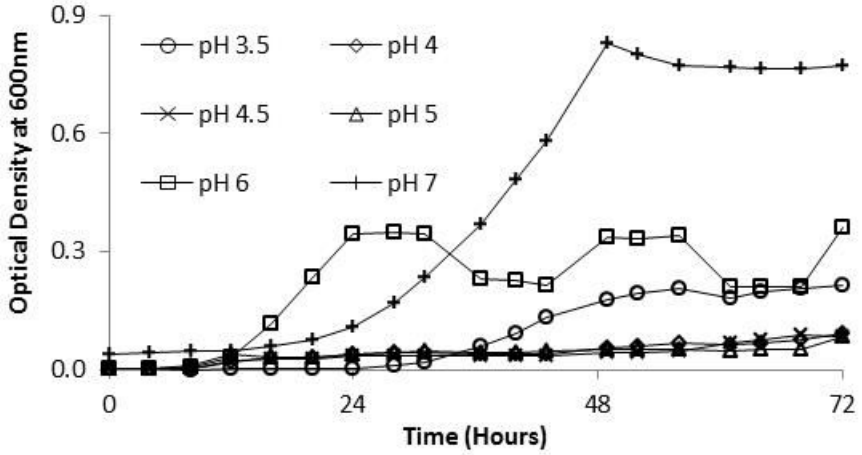


Figure 6-4: Aerobic growth of *L. plantarum* infected by phage B2, at an initial MOI of 0.1, in M9 minimal medium at pH ranging from pH3.5 to pH7. M9 medium at pH3.61 was used as reference for OD measurements.

Combined infection by both phages B1 and B2 on the host growing in M9 medium at different pH is shown in **Figure 6-5**. It can be seen that both phages present in the medium clearly supressed bacterial growth at pH ranging from pH 4 to pH 6.

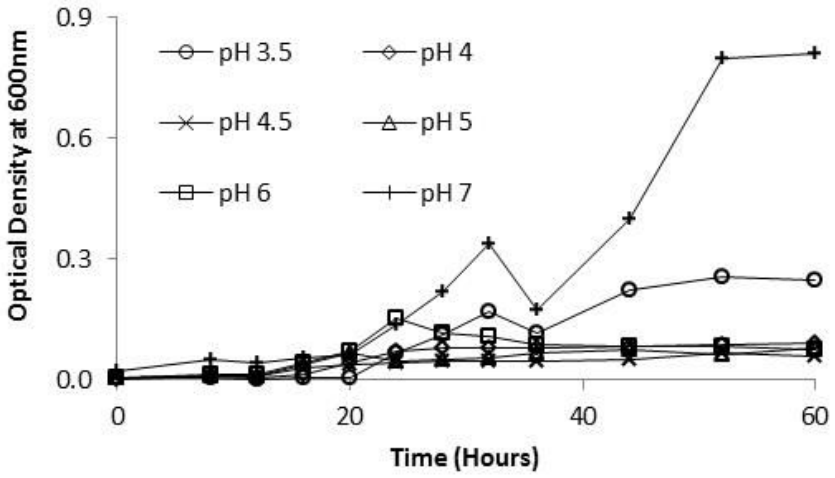


Figure 6-5: Aerobic growth of *L. plantarum* infected by phages B1 and B2, at an initial MOI of 0.1, in M9 minimal medium at pH ranging from pH3.5 to pH7. M9 medium at pH3.61 was used as reference for OD measurements.

To better understand the effect of phages on their bacterial host under different pH conditions, the data obtained in **Figure 6-2** to **Figure 6-5** were compared to quantify the infectivity potential of the phages. For that, the integral of the curves in the figures was calculated – with units of OD·time. A larger value of integrated optical density indicates that more cells have proliferated over the length of the infection. When subtracting the values obtained in **Figure 6-3** from those obtained in **Figure 6-2** at the corresponding pH, it is possible to quantify the reduction in the overall growth caused by phage B1. Likewise, the host cell death caused by phage B2 and by phages B1 and B2 together can be obtained at each pH by subtracting the values obtained in

Figure 6-4 and **Figure 6-5** from those obtained in **Figure 6-2**. These measurements were reported as percentage of cells killed by phages (phage infectivity).

Phage infectivity for phage B1, B2 and phages B1 and B2 together is presented in **Figure 6-6**. It can be observed, in **Figure 6-6a,** that phage B1 was highly effective at killing its host at pH6 and, to a lower extent, at pH7. Phage B2 was able to kill its host at all pH tested (**Figure 6-6b**), however its infectivity was higher at pH4 to pH5. The infectivity of both phages combined was high and similar for pH4 to pH6. Infectivity in **Figure 6-6a** at pH4.5 and **Figure 6-6b** at pH3.5 were slightly negative (-8.9% and -3.6%, respectively). As those values were low, it is unlikely that phages at these specific pH conditions are improving bacterial growth. Probably experimental error caused this.

6.2 Population dynamics of yeasts, bacteria and phages B1 and B2

The results from the study of the pH effect on culture development and infectivity (**Section 6.1**) served as basis for the experiments of population dynamics. The infectivity pattern observed in **Figure 6-6c** revealed that a phage cocktail containing phages B1 and B2 was most efficient between pH4 and pH6. For this reason, the population dynamics experiments were conducted at the outermost values of this range, namely pH4 and pH6.

6.2.1 Aerobic conditions in M9 minimal medium

As explained in **Section 4.7.1**, population dynamics experiments in M9 medium under aerobic conditions were tested with bacterial inoculation levels ranging from 10^5 to 10^8cfu/mL. Because the results were similar for all tests, it was reported only the results for the inoculation of 10^5cfu/mL. The results for the growth of yeast in the

absence or presence of bacteria and/or phages B1 and B2 in M9 minimal medium at pH 4 are found in **Figure 6-7.** While yeast and bud concentrations did not differ greatly between experiments (**Figure 6-7a)** and **b)**), bacterial level remained a full order of magnitude lower when phages were added to the system (**Figure 6-7c)**).

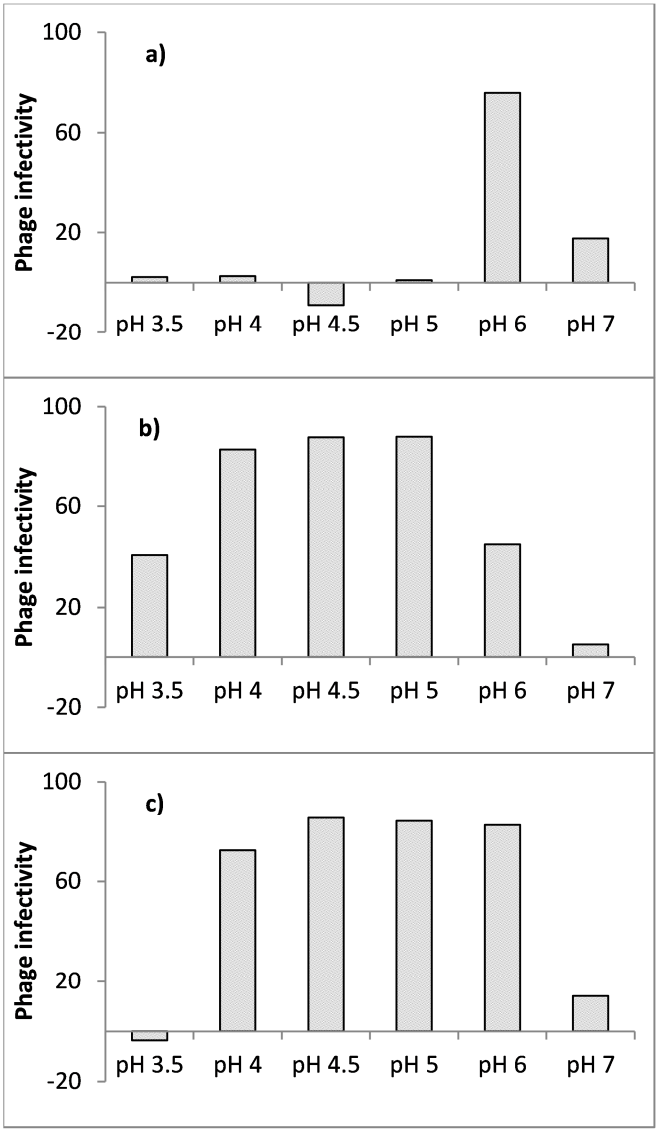


Figure 6-6: Quantification of phage infectivity (percentage of cells killed) at different pH for a) phage B1, b) phage B2, and c) phages B1 and B2 combined**.**

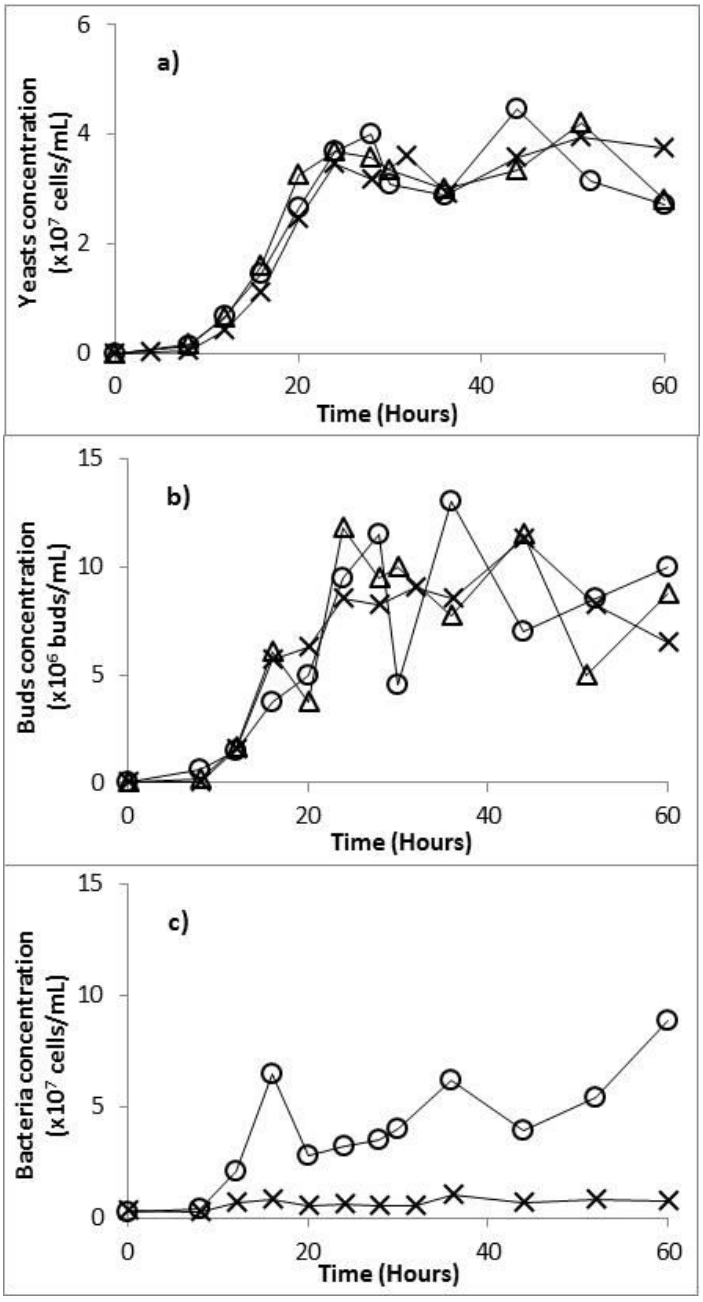


Figure 6-7: Aerobic growth of yeast, bacteria, phage B1 and phage B2 in M9 minimal medium at pH4. Concentrations of a) yeast cells, b) yeast buds and c) bacterial cells are presented. Data is reported for cases where yeasts were grown alone (Δ); in the presence of bacteria (O); and in the presence of bacteria and phages B1 and B2 (×).

The same experiment was conducted in M9 medium at pH 6. Results are presented in **Figure 6-8.** Again, the most clear difference was found in **Figure 6-8c)**, where, the bacterial level was one order of magnitude lower in the presence of phages.

6.2.2 Anaerobic conditions in M9 minimal medium

As explained in materials and methods (**Section 4**), anaerobic conditions were achieved by conducting the experiments in sealed culture bottles. It should be noted that the agitation of the bottles, provided by the trays of the rotary incubator shakers, wasn't sufficient to spread evenly the yeasts as cells settling was observed. This flaw in agitation could have slightly affected the results.

Figure 6-9 shows final concentrations for anaerobic growth experiments in M9 minimal medium at pH4. It can be seen in this figure that yeasts concentration was directly reduced by the presence of bacteria. The addition of phages allowed some recovery by the yeasts.

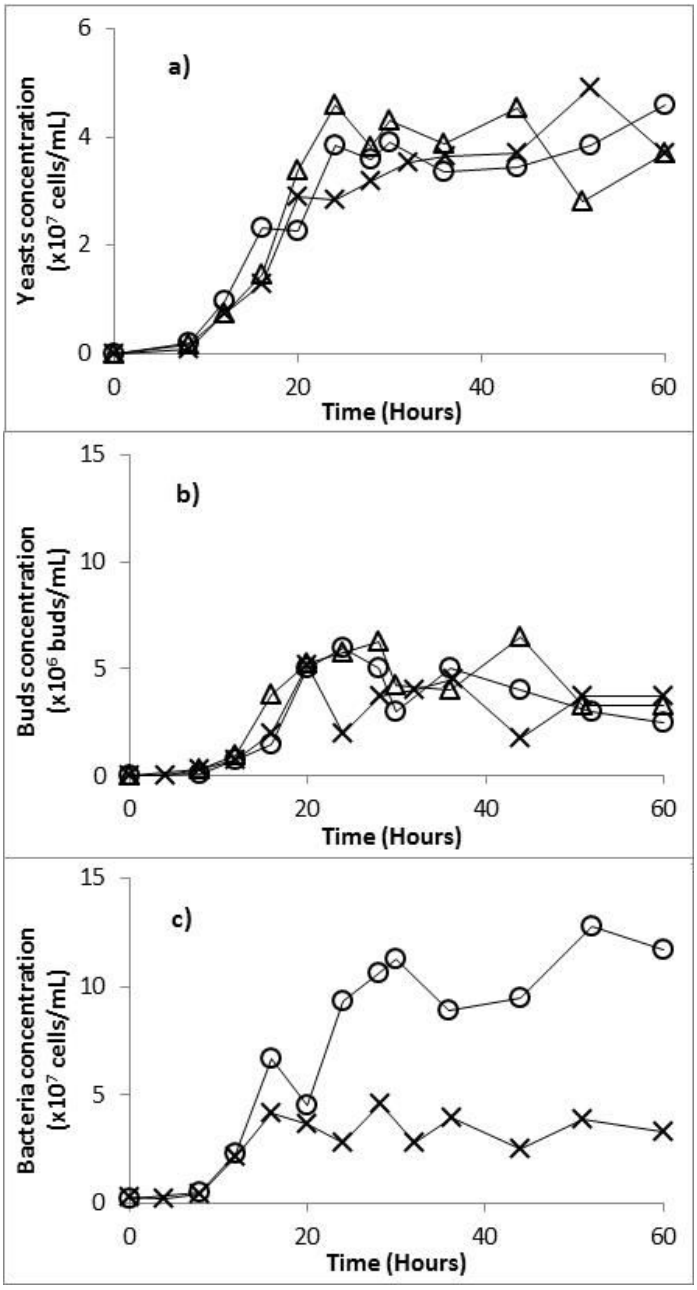
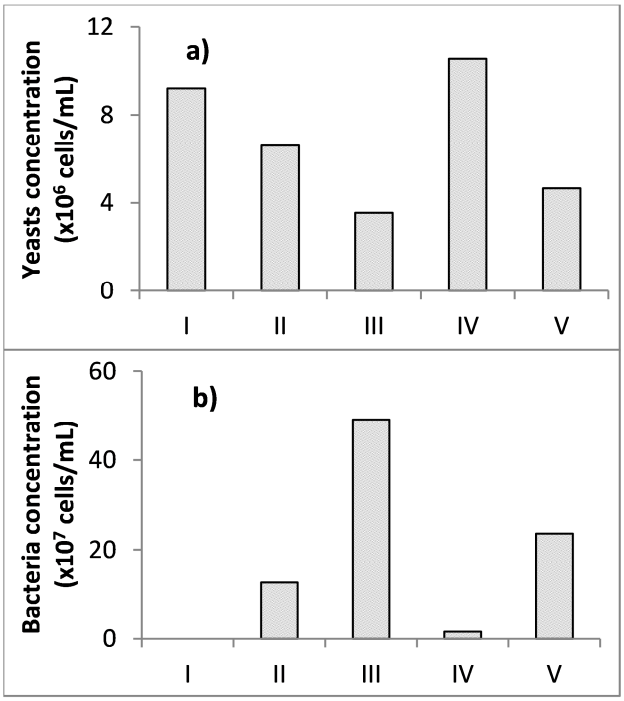


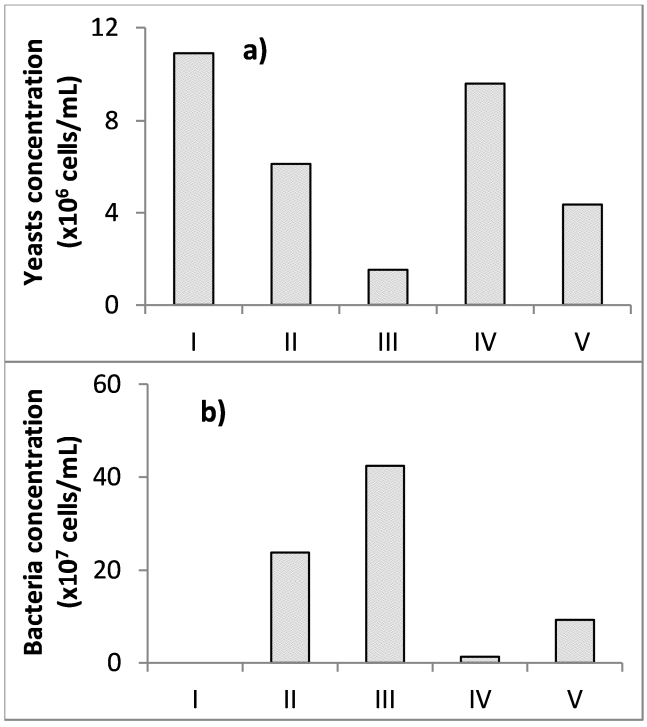
Figure 6-8: Aerobic growth of yeast, bacteria, phage B1 and phage B2 in M9 minimal medium at pH6. Concentrations of a) yeast cells, b) yeast buds and c) bacterial cells are presented. Data is reported for cases where yeasts were grown alone (Δ); in the presence of bacteria (O); and in the presence of bacteria and phages B1 and B2 (×).



Inoculation load	I	II	III	IV	V
Yeasts (cells/mL)	10^4	10^4	10^4	10^4	10^4
Bacteria (cells/mL)	-	10^5	10^7	10^5	10^7
Phages (pfu/mL)	-	-	-	10^4	10^6

Figure 6-9: Anaerobic growth of yeast, bacteria, phage B1 and phage B2 in M9 minimal medium at pH4. Concentrations of a) yeast cells and b) bacterial cells are presented. Data is reported for cases where yeasts were grown alone (I); in the presence of bacteria at low inoculation level (II); in the presence of bacteria at high inoculation level (III); in the presence of bacteria at low inoculation level and phages B1 and B2 (IV); and in the presence of bacteria at high inoculation level and phages B1 and B2 (V). The table presents the inoculation loads for the organisms. Measurements were taken after 41h of fermentation.

Figure 6-10 shows the final concentrations for anaerobic experiments at pH6. Similar trends to those seen at pH4 (**Figure 6-9**) were observed at pH6 (**Figure 6-10**).



Inoculation load	I	II	III	IV	V
Yeasts (cells/mL)	10^4	10^4	10^4	10^4	10^4
Bacteria (cells/mL)	-	10^5	10^7	10^5	10^7
Phages (pfu/mL)	-	-	-	10^4	10^6

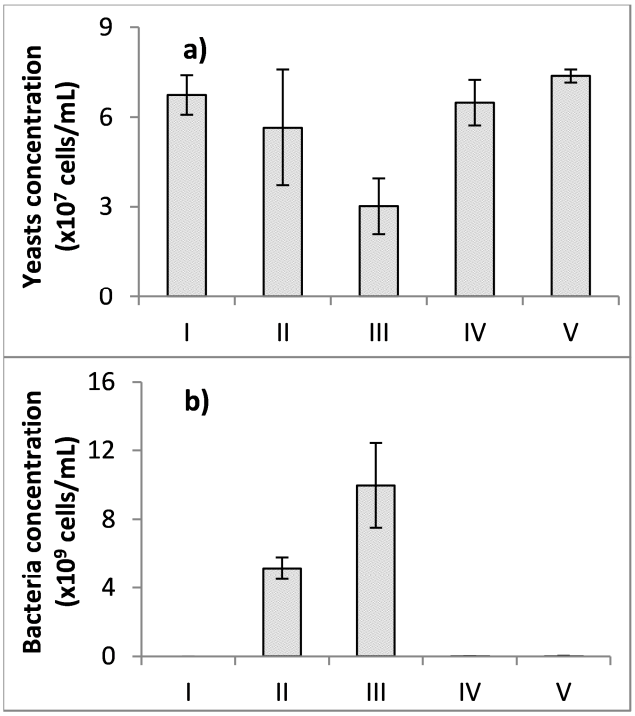
Figure 6-10: Anaerobic growth of yeast, bacteria, phage B1 and phage B2 in M9 minimal medium at pH6. Concentrations of a) yeast cells and b) bacterial cells are presented. Data is reported for cases where yeasts were grown alone (I); in the presence of bacteria at low inoculation level (II); in the presence of bacteria at high inoculation level (III); in the presence of bacteria at low inoculation level and phages B1 and B2 (IV); and in the presence of bacteria at high inoculation level and phages B1 and B2 (V). The table presents the inoculation loads for the organisms. Measurements were taken after 41h of fermentation.

6.2.3 Aerobic conditions in MRS medium

MRS medium is a suitable medium for growth of lactobacilli. Interestingly, growth experiments with *S. cerevisiae* in MRS showed that this organism displays faster kinetics and reaches higher yields in comparison to M9. Therefore, since MRS is a rich and complex medium – much like mash used in industrial fermentations – and is suitable for both, *L. plantarum* and *S. cerevisiae*, it was also used for population dynamics experiments. The aim of these experiments was to evaluate the efficiency of phages when bacterial cultures were growing under highly favourable conditions, thus creating a case of high contamination by lactic acid bacteria.

Experiments were conducted at pH 6. Medium at pH 4 presented particles sedimentation after sterilization. It is not entirely known if the precipitated particles were only salts, because MRS is a medium with a considerable variety of components. Since this medium discrepancy affected the reliability of the results (data not shown), experiments at pH 4 were not considered in the analysis.

The results of cells yields measured after 20h of fermentation under aerobic conditions are shown in **Figure 6-11**. It can be seen, in **Figure 6-11a)**, that the presence of bacteria at high inoculation load led to a significant decrease in yield of yeast cells. Moreover, when phages were added and killed most of the bacterial host, yeast concentrations reached the same levels as yeast growing alone. Bacterial concentrations in experiments IV and V of **Figure 6-11b)** were found to be $2.6x10^6$cells/mL and $1.7x10^7$cells/mL, respectively.



Inoculation load	I	II	III	IV	V
Yeasts (cells/mL)	10^4	10^4	10^4	10^4	10^4
Bacteria (cells/mL)	-	10^5	10^7	10^5	10^7
Phages (pfu/mL)	-	-	-	10^4	10^6

Figure 6-11: Aerobic growth of yeast, bacteria, phage B1 and phage B2 in MRS medium at pH6. Concentrations of a) yeast cells and b) bacterial cells are presented. Data is reported for cases where yeasts were grown alone (I); in the presence of bacteria at low inoculation level (II); in the presence of bacteria at high inoculation level (III); in the presence of bacteria at low inoculation level and phages B1 and B2 (IV); and in the presence of bacteria at high inoculation level and phages B1 and B2 (V). The table presents the inoculation loads for the organisms. Measurements were taken after 20h of fermentation. Error bars indicate the standard deviation of three experiments.

An experiment evaluating the overall growth (kinetics) throughout 60 hours of fermentation in MRS at pH 6 was made. These results, shown in **Figure 6-12**, present a pattern similar to the ones found in **Figure 6-11** in terms of reduction of yeast cell concentration when bacteria are present and massive killing of bacteria when phages were present.

In order to better visualize the differences observed in yeast populations in the absence or presence of bacteria and/or phages, sample pictures of the final concentrations of the cultures growing in MRS at pH 6 are presented in **Figure 6-13**. The samples were placed on the counting chamber and the pictures were taken with the digital camera of the microscope. This figure shows a clear reduction in yeast concentration in the presence of bacteria (**Figure 6-13b)**); and when phages were added, the concentration of *L. plantarum* was greatly reduced and yeast concentration returned to its original value (**Figure 6-13c**)). Note that phages are too small to be seen by visible microscopy.

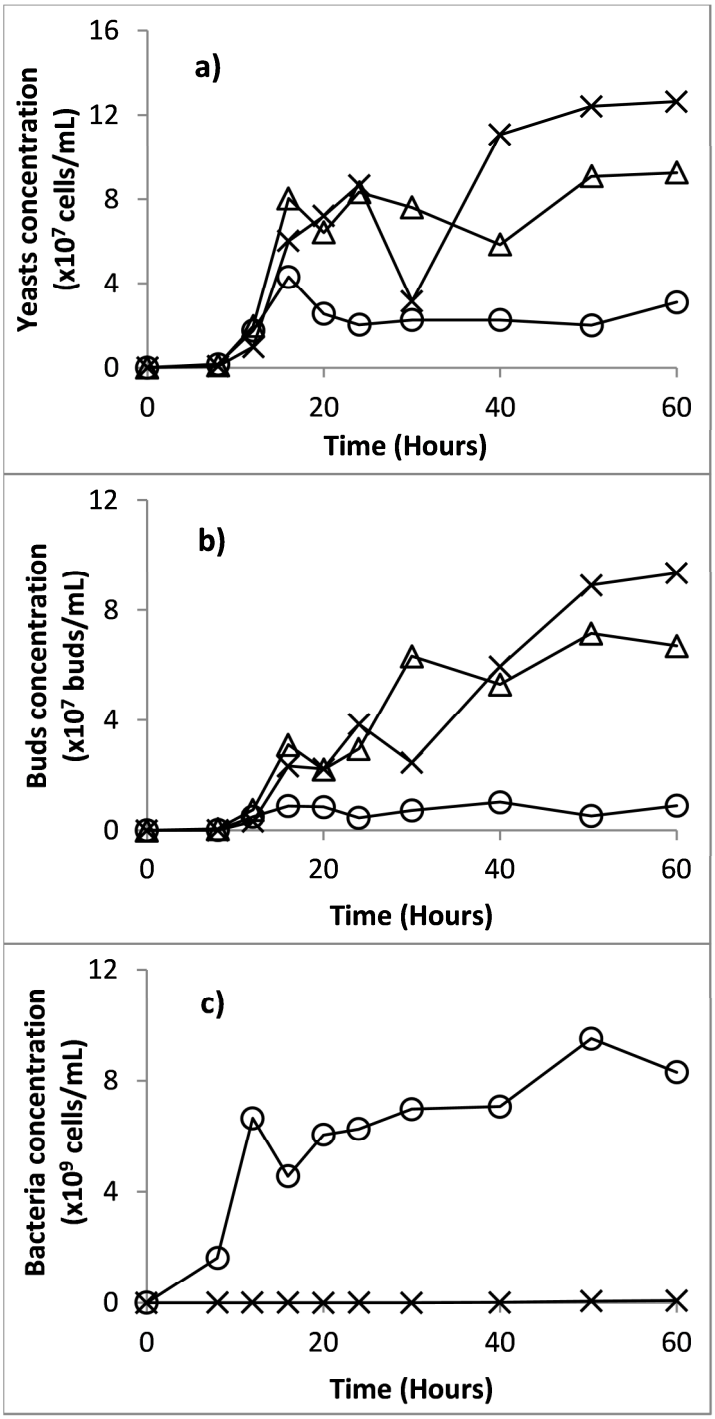


Figure 6-12: Aerobic growth of yeast, bacteria, phage B1 and phage B2 in MRS medium at pH6. Concentrations of a) yeast cells, b) yeast buds and c) bacterial cells are presented. Data is reported for cases where yeasts were grown alone (Δ); in the presence of bacteria (O); and in the presence of bacteria and phages B1 and B2 (×).

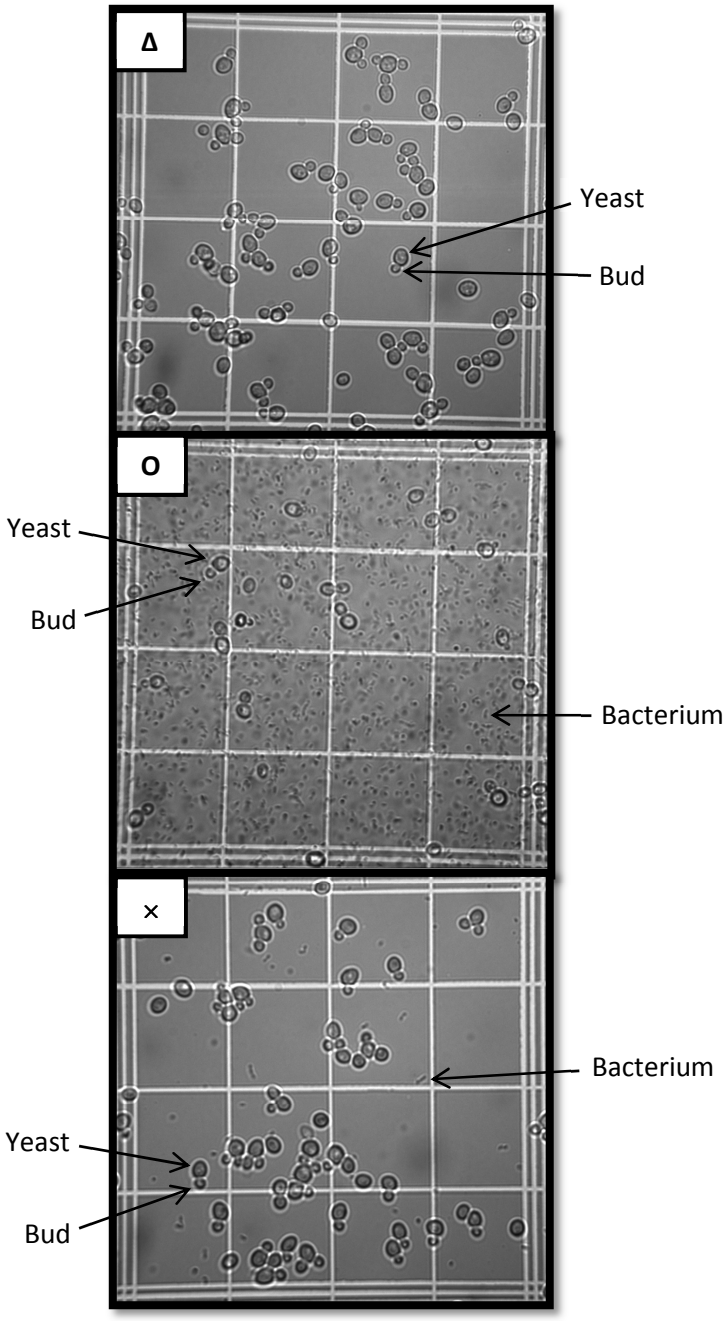


Figure 6-13: Sample pictures for the population dynamics experiment in MRS medium at pH6. Data is reported for cases where yeasts were grown alone (Δ); in the presence of bacteria (O); in the presence of bacteria and phages B1 and B2 (×). Examples of cells of yeasts, buds and bacteria are pointed in the figure. All samples were diluted 5 times.

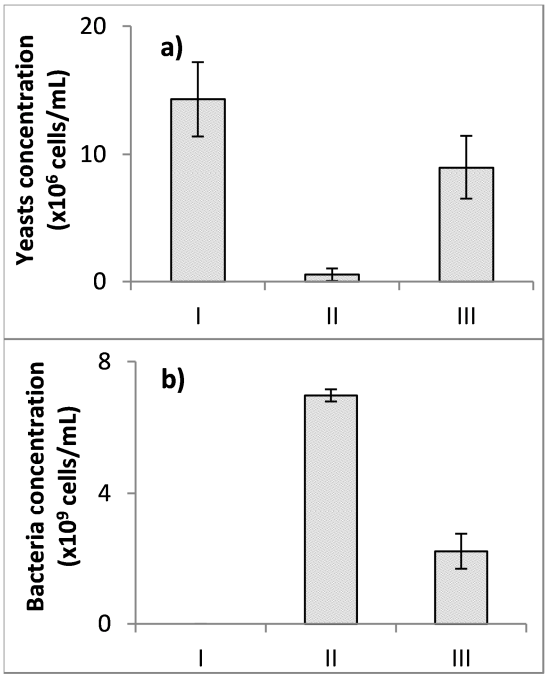
6.2.4 Anaerobic conditions in MRS medium

As opposed to anaerobic experiments in M9 medium, anaerobic conditions in MRS were performed without agitation. **Figure 6-14** shows results from anaerobic population experiments in MRS medium at pH6. The trends observed in **Figure 6-14** resemble those for aerobic growth in the same medium (**Figure 6-11**) . This indicates that competition between yeast and bacteria, and infection by phages had similar consequences in aerobic and anaerobic environments.

6.3 Ethanol production

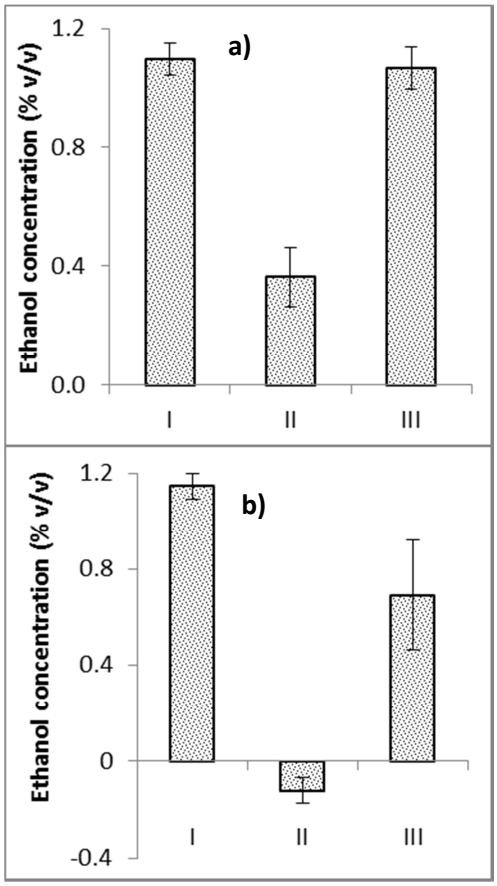
Although ethanol production is generally considered to correlate with yeast growth (Ingledew, 2009a), it is important to evaluate if and how it is affected by the presence of bacteria and phages.

Ethanol concentration was measured in experiments performed in MRS medium at pH6 under aerobic and anaerobic conditions. In a preliminar experiment it was identified that the highest production of ethanol under aerobic conditions occured at 24h, whereas the highest production under anaerobic conditions happened at 65h (data not shown). Thus, **Figure 6-15** shows the results for ethanol production (% v/v) under aerobic and anaerobic conditions sampled at 24h and 65h, respectively. It can be seen that ethanol production was significantly reduced when bacterial contaminants were present. The addition of phages in the system caused recovery of alcohol production. The negative ethanol concentration seen in **Figure 6-15b)** when yeasts and bacteria were present was due to extremely low measurements, outside of the range of the calibration curve. They can thus be considered virtually zero.



Inoculation load	I	II	III
Yeasts (cells/mL)	10^4	10^4	10^4
Bacteria (cells/mL)	-	10^7	10^7
Phages (pfu/mL)	-	-	10^6

Figure 6-14: Anaerobic growth of yeast, bacteria, phage B1 and phage B2 in MRS medium at pH6. Concentrations of a) yeast cells and b) bacterial cells are presented. Data is reported for cases where yeasts were grown alone (I); in the presence of bacteria at high inoculation level (II); and in the presence of bacteria at high inoculation level and phages B1 and B2 (III). The table presents the inoculation loads for the organisms. Measurements were taken after 65h of fermentation. Error bars indicate the standard deviation of three experiments.



Inoculation load	I	II	III
Yeasts (cells/mL)	10^4	10^4	10^4
Bacteria (cells/mL)	-	10^7	10^7
Phages (pfu/mL)	-	-	10^6

Figure 6-15: Ethanol production in MRS medium at pH6 under aerobic a) and anaerobic b) conditions. Data is reported for cases where yeasts were grown alone (I); in the presence of bacteria at high inoculation level (II); and in the presence of bacteria at high inoculation level and phages B1 and B2 (III). The table presents the inoculation loads for the organisms. Measurements for aerobic condition were taken at 24 hours and for anaerobic condition at 65 hours of fermentation. Error bars indicate the standard deviation of three experiments.

6.4 Determination of parameters for the mathematical model

The parameters used for the mathematical model developed to describe the population dynamics were obtained experimentally, from literature or through best-fit analysis. All experiments and assumptions were made considering MRS medium at pH6. Below are experimental results used to determine the model parameters.

6.4.1 Yeast-related parameters

For yeast growth, the values for $\mu_{max - SC} = 0.65\ h^{-1}$ and $K_{Monod - SC} = 0.108$ g/L applied in the model were obtained from the literature (Leuenberg, 1972; Papagianni *et al*., 2007). Yield (Yi_{SC}) was obtained experimentally through the cultivation of the organism in 50mL of MRS medium with pH6 (50mL of medium has 1g of glucose (de Man *et al*., 1960)). Once the culture reached stationary phase, the number of yeasts and buds were counted in the microscope (with a counting chamber). Yield was found to be $5.9x10^{9}$cells/g of glucose.

Figure 6-16 shows the fit of the Monod kinetics model for yeast growth. The experimental data was obtained from the yeast population grown under aerobic conditions in MRS medium at pH 6 (**Figure 6-12**). Because the model doesn't differentiate yeast cells and buds, the experimental data points correspond to the sum of the (Δ) measurements in **Figure 6-12a)** and **Figure 6-12b)**.

6.4.2 Bacteria-related parameters

Monod parameters for bacterial growth were obtained from the literature (Helanto *et al*., 2007). The values are $\mu_{max - LP} = 0.823\ h^{-1}$ and $K_{Monod - LP} = 7.33$ g/L. The yield (Yi_{LP}) of $4.8x10^{11}$cells/g of glucose was determined from a best-fit evaluation

through least squares and residuals analysis. The fit of the Monod kinetics model for bacteria growing alone in MRS medium at pH 6 is represented in **Figure 6-17**.

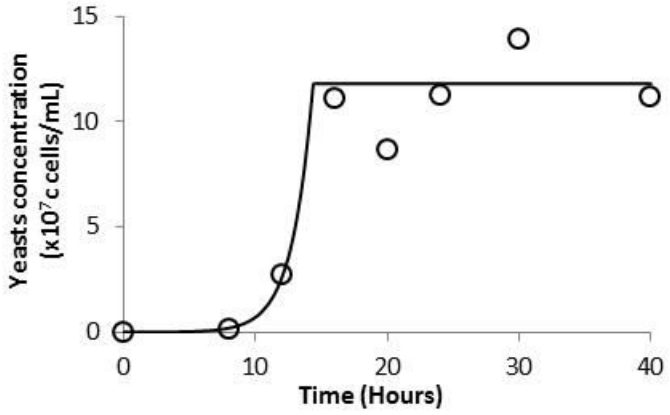


Figure 6-16: Comparison of experimental yeast concentration (O) with the Monod kinetics model.

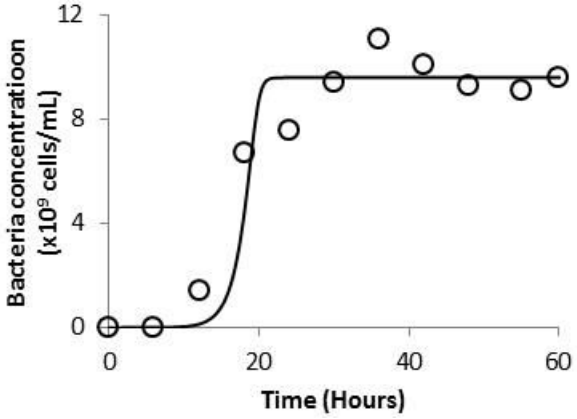


Figure 6-17: Comparison of experimental bacteria concentration (O) with the Monod kinetics model.

6.4.3 Phages-related parameters

Three of the parameters necessary for modelling phage development (burst size, latent period and lysis time) can be obtained from the one step growth experiment. These experiments were done for both phages B1 and B2 individually. **Figure 6-18** contains the results for the experiments.

From the results found in **Figure 6-18**, the latent period for phage B1 was found to be 1.33h (or 80min), the lysis time was approximately 2.67h (or 160min) and the burst size was 154 phages per infected cell. Likewise, the parameters determined for phage B2 were latent period of 1.33h (or 80min), lysis time of 0.67h (or 40min), and burst size of 21 phages per infected cell. Using these parameters, phage infection models were plotted in **Figure 6-18**. It should be noted that the model was built considering the development of a single phage infecting only one bacterium.

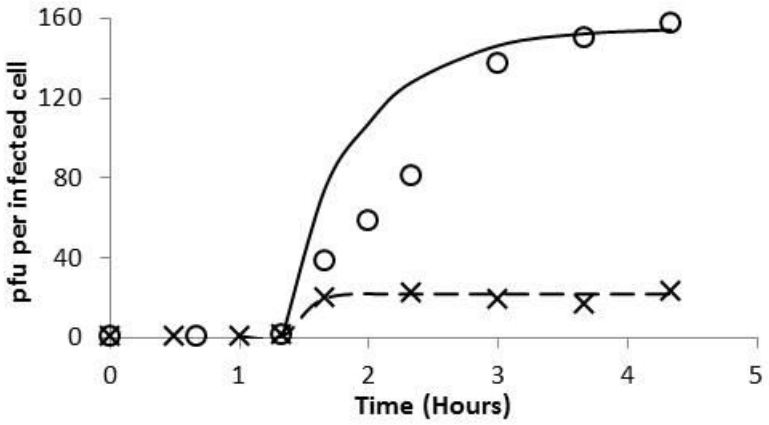


Figure 6-18: One step growth curves for phages B1 (O) and B2 (X). The full line represents the model for phage B1 and the dashed line represents the model for phage B2.

Adsorption rate constants and adsorption efficiencies for each phage were found from adsorption experiments in which the binding of phages to their host is monitored. The parameters were determined from least squares methods from a model for adsorption kinetics. The model adopted was the first-order adsorption efficiency (Storms *et al*., 2010; 2012). **Figure 6-19** shows results of adsorption experiments and the corresponding models for phages B1 and B2.

As can be seen, the adsorption of either phage to its host was not highly efficient under the conditions tested. Adsorption rate constants and efficiencies obtained from the model were $k_{ads\text{ - }B1}$=2.76x10^{-10}mL/min.cell and ε_{B1}=0.57; and $k_{ads\text{ - }B2}$=9.99x10^{-8}mL/min.cell and ε_{B2}=0.35.

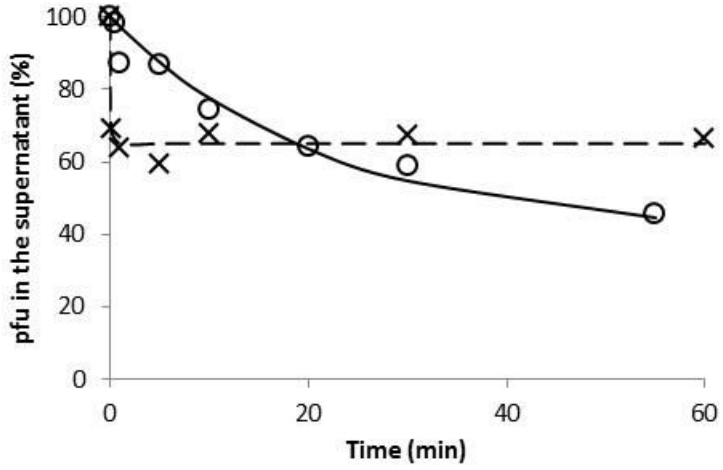


Figure 6-19: Adsorption of phages B1 (O) and B2 (X) to *L. plantarum* in MRS medium at pH6. The full line represents the model for phage B1 and the dashed line represents the model for phage B2.

6.5 Combined mathematical model

When developing a mathematical model it is important to use experiments to reinforce or discard the concepts and hypotheses established. The combinations of all parameters determined with the model constructed for the multiple organisms in the system were tested on top of empirical data.

6.5.1 Infection of non-susceptible/susceptible bacterial population

In order to better understand the behaviour of the host culture when infected by phages, a population of *L. plantarum* was infected with both phages B1 and B2. A high inoculation load (10%) was applied to allow a more accurate evaluation of bacterial response to phage infection. The model was then fit to the experimental data. The results can be found in **Figure 6-20**. As can be seen in **Figure 6-20**, the host population infected with phages grew for the first 3 hours before declining. This decrease was limited and was followed by a *plateau*. The non-susceptible/susceptible host model was used to describe this phenomenon. From an analysis of least squares and residuals, the equilibrium constant K_{eq} and the initial ratio of susceptible to non-susceptible cells were found to be of value 0.2.

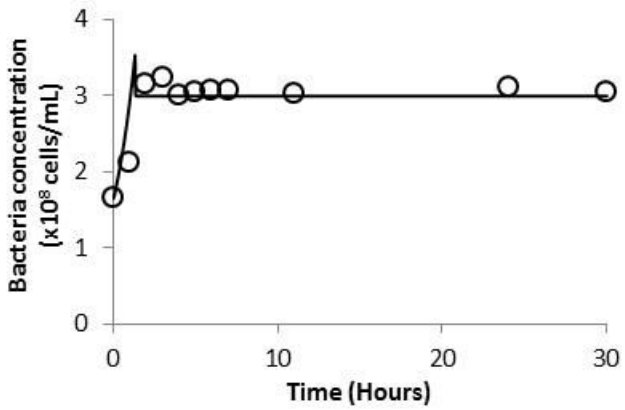


Figure 6-20: Infection of a *L. plantarum* population (O) by phages B1 and B2 at higher inoculation load. The full line represents the non-susceptible/susceptible bacterial populations model.

6.5.2 Phage amplification

Phage amplification was also assessed for the development of the model. Bacterial growth and phage production – measured through optical density and phage titer respectively – were monitored throughout the infection process. The experiment involved only one phage, B1, because it would not be possible to differentiate between phage strains during infection by both phages. The results of the amplification of phage B1 at two different initial values of MOI can be seen in **Figure 6-21.** In both cases, a significant increase in bacterial population can be seen after 20h. This increase was likely due to bacteria resistant to phage B1. The remaining bacterial population was further infected with phage B2 on agar plates and plaques resulting from host lysis were formed (data not shown). This reinforces the resistant population hypothesis. The equilibrium constant K_{eq} and the initial ratio of susceptible to non-susceptible cells concentrations were found to be 10^5. Again, this parameter was determined using least squares analysis.

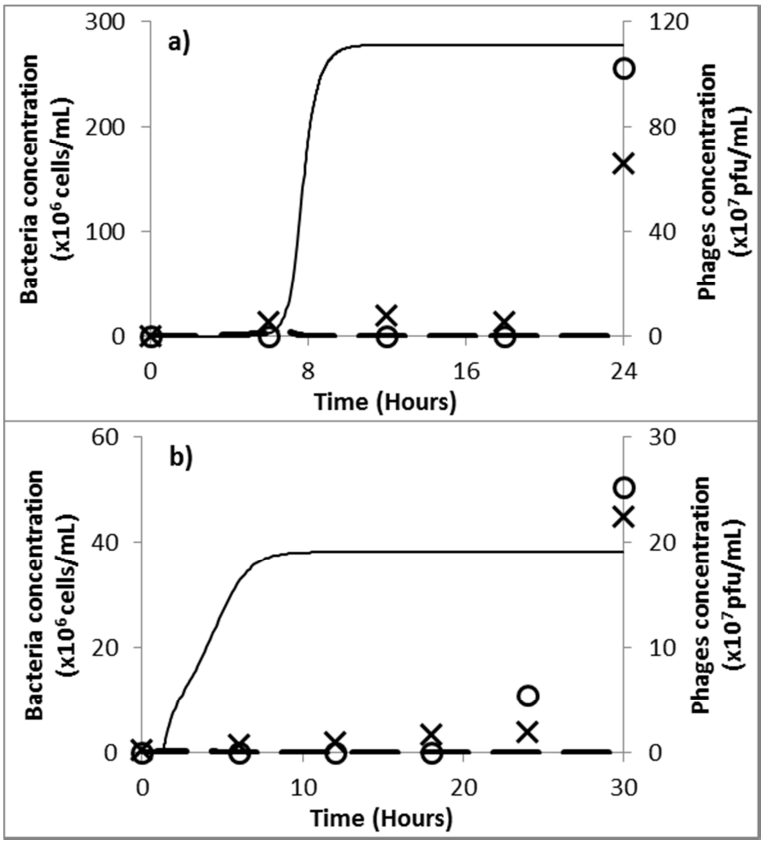


Figure 6-21: Amplification of phage B1 (X) in its host *L. plantarum* (O). Full line represents the model for phage B1 concentration and dashed line represents the model for *L. plantarum* cells concentration. Data is reported for amplifications at initial MOI of 0.1 (a) and 100 (b).

6.5.3 Complete model

Using all the parameters determined above (see **Table 6-1**) and the model described in **Section 5**, the developed model was compared to the experimental results presented in **Figure 6-12**, system containing *S. cerevisiae*, *L. plantarum*, phage B1 and phage B2 in MRS medium at pH 6. This comparison is shown in **Figure 6-22**. The model provides an acceptable description of the population dynamics. This is true for all cases investigated, when only yeasts were present, when yeasts and bacteria composed the

system and when phages were added. Again yeasts concentration – **Figure 6-22 a)** – was composed by the sum of yeasts from buds. The equilibrium constant K_{eq} and initial concentration ratio between susceptible and non-susceptible bacterial hosts applied for this model was 0.2.

Parameter	Value	Unit	Source
$\mu_{max - SC}$	0.0108	min^{-1}	Literature
$K_{Monod - SC}$	0.000108	g/mL	Literature
$Yi_{SC/glucose}$	$5.9x10^{9}$	cells/g of glucose	Experiment
$\mu_{max - LP}$	0.0137	min^{-1}	Literature
$K_{Monod - LP}$	0.00733	g/mL	Literature
$Yi_{LP/glucose}$	$4.8x10^{11}$	cells/g of glucose	Best-fit
$k_{ads - B1}$	$2.76x10^{-10}$	mL/min.cell	Best-fit
ε_{B1}	0.57	-	Best-fit
$k_{Lt - B1}$	0.029	min^{-1}	Experiment
b_{B1}	154	pfu/cell	Experiment
U_{B1}	80	min	Experiment
$k_{ads - B2}$	$9.99x10^{-8}$	mL/min.cell	Best-fit
ε_{B2}	0.35	-	Best-fit
$k_{Lt - B2}$	0.115	min^{-1}	Experiment
b_{B2}	21	pfu/cell	Experiment
U_{B2}	80	min	Experiment
K_{eq}	0.2	-	Best-fit

Table 6-1: Parameters applied in the complete model.

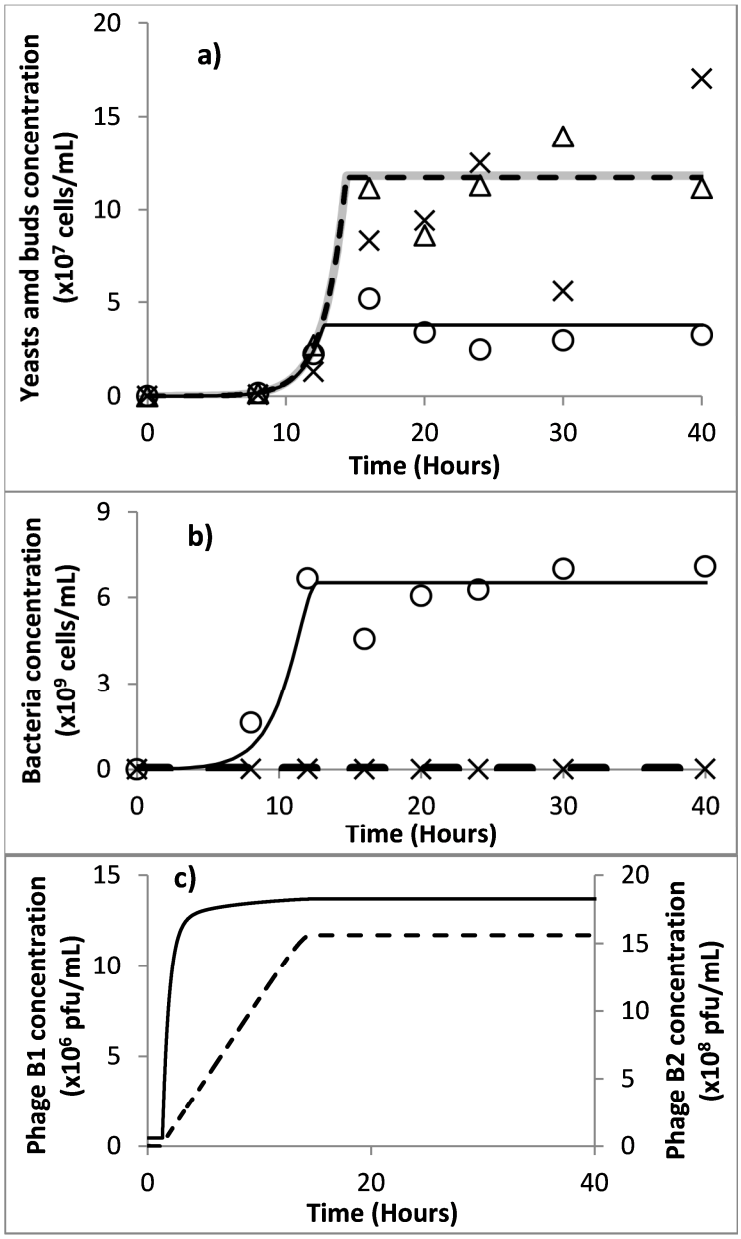


Figure 6-22: Simulation of the model with the population dynamics experiment. a) concentration of yeasts and buds, b) concentration of bacterial cells, and c) modeled concentration of phage B1 (full line) and phage B2 (dashed line). Data for a) and b) is reported for cases where yeasts were grown alone (Δ, model represented by grey line); in the presence of bacteria (O, model represented by full line); and in the presence of bacteria and phages B1 and B2 (×, model represented by dashed line).

7. DISCUSSION

Beyond demonstrating the efficiency of using bacteriophages as alternative antibacterial agents, the results obtained in this study help elucidate some of the conditions that impact the success of yeast fermentation processes. Furthermore, the study showed that the response of each biological entity involved affects the system as a whole.

7.1 Impact of pH on aerobic cultures

pH is an important parameter in the production of biofuels by yeast. It is used, among other purposes, to help control the level of bacterial contaminants in fermentation media. However, besides abating the growth of undesirable bacteria, pH also affects other biological entities present in the system. At lower pH, yeast often displays lower biofuel yields, and pH has been shown to affect phage infections.

The fact that the growth of *S. cerevisiae* in M9 minimal medium was not significantly affected by pH over the range tested (**Figure 6-1**) is an indication of its robustness. This was expected for this strain as it was developed specifically for commercial applications in biofuel production. Moreover, as stated by Russell (2003), although the ideal pH for yeast growth is 5.0 to 5.2, brewing and distilling strains are capable of good growth in the pH range of 3.5 to 6. It must be noted, however, that in the present study the experiment was carried out aerobically. Under aerobic conditions yeasts are able to perform the complete TCA cycle and oxidative phosphorylation that produce 36 molecules of ATP per molecule of glucose, whereas only 2 ATP molecules are released under anaerobic fermentation (Russell, 2003). According to Ingledew (2009b), acidic environment causes stress to yeast cells,

requiring it to waste ATP molecules to pump dissociated hydrogen molecules out of the cell. Hence, it can be assumed that aerobic conditions allowed the yeast to produce enough energy to exclude the H^+ ions from the cell and to grow normally.

Interestingly, for all pH values tested, a decrease in optical density was observed near the 24h mark. Because OD_{600} measures the scattering of light caused by cells in the medium, cell number, size and shape all affect this measurement. Hence, the reasons for this decrease in optical density could be either a difference in cells size and shape or a decrease in the number of cells. Change in morphology and difference in cell size during growth is not an uncommon phenomenon in microorganisms (Clark and Ruehl, 1919), however, in all experiments conducted for this research where pictures of yeasts were taken, no significant changes in cell size or morphology were observed. Therefore, a reduction in cells number is the most probable cause of this decrease. One possible explanation for the rapid reduction in amount of cells at that time is that yeasts could have switched the metabolic pattern from fermentation to respiration. Even though energy yield is lower, fermentation is yeasts' preferred metabolic state when substrate is readily available, since kinetics are generally faster in comparison to respiration (Mohammed Al-mhanna, 2010; van Dijken *et al*., 1993; van Urk *et al*., 1989). When substrate levels are reduced, the organism switches pathways to respiration in order to increase energy yield. This, however, leads to slower metabolic kinetics. This phenomenon is exclusive to yeasts and is called *Crabtree effect* (Russell, 2003). It can then be inferred that after approximately 24h of fermentation, nutrient levels reached a point that triggered the respiration pathway, which, consequently, reduced the observed rate of growth. In fact, it was confirmed by industrial contacts in the bioethanol field (Grochowalski, personal communication, May

29, 2013) that fluctuating growth rates are often encountered in industrial fermentations.

On the other hand, the yield of the lactic acid bacterium *L. plantarum* clearly decreased at lower pH (**Figure 6-2**). From the pattern observed, it can be inferred that this strain has an optimum pH value and a range of effective growth closer to pH6. Commonly, the range covers 2-3 pH units (Madigan *et al*., 2011a). Bacteria can use different mechanisms to survive in a medium at non-optimal pH; these vary from exchanging ions for protons with antiport transport system to synthesizing an array of new proteins (Willey *et al*., 2008). Such mechanisms require energy and, consequently, affect yield.

The infection of *L. plantarum* by either or both phages tested, B1 and B2, was also susceptible to pH, as can be seen in **Figure 6-3** to **Figure 6-6**. Several factors, alone or in conjunction, can explain the decrease in phage infectivity in acidic environments. As explained previously, in general, phage lytic infection cycle involves the adsorption of phage organelles to receptors on the bacterial wall, followed by injection of the genetic material. Phage's nucleic acid then takes over the bacterial machinery to produce progeny. It is followed by bacterium lysis and release of new phages. This mechanism of infection applies for the replication of the lytic phages B1 and B2.

The adsorption process has been shown to be considerably inhibited in acidic environments (phages T1 and T2 - Puck and Tolmach, 1954), however this pattern is not observed in all cases (lactic streptococcal phages - Thompson, 1959). Acidic conditions were also seen to affect the assembly of phage's genetic material (phage T7 - Richter and Loewen, 1982) and even phage's burst size (phage PBS2 - Price and Fogt,

1973). Therefore, further investigation is needed to identify the actual mechanisms affected by low pH in this system.

In a study on the influence of environmental factors on phage development, Briggiler Marcó *et al.* (2010) observed that the adsorption of both phages B1 and B2 to their host is not affected at pH values ranging from 5 to 7 in MRS medium at a temperature of 37^{o}C. The study didn't evaluate pH values lower than pH5. The researchers also investigated phage inactivation under acidic conditions. For both phages, inactivation was observed at pH4 and lower and no viable phages remained at pH2. While the experiments performed by Briggiler Marcó *et al.* (2010) were conducted in a different medium and at a different temperature than the present study, the results can still provide clues to the phenomena observed in this system.

In M9 medium, phage B1 infectivity was highest at pH 6 (**Figures 6-3** and **6-6a)**). This phage was, to a lesser extent, also effective at pH 7 (**Figure 6-6a)**) and not effective at pH below 6. The results of Briggiler Marcó *et al.* (2010) showed a major reduction in phage infectivity only for pH less than 4. The difference in experimental conditions could have changed the pH values causative of the drastic reduction in infectivity. Explanations for the reduction observed can be changes in the behaviour of the host at pH lower than 6 or changes in adsorption characteristics. The latter is more predictable as ion contents and phage stability differ for each medium. However, as demonstrated by Briggiler Marcó *et al.* (2010), the virtually inexistent phage infectivity at values below pH4 was likely caused by inactivation.

The differences in infectivity of phage B2 at different pH values were more attuned to the results of Briggiler Marcó *et al.* (2010). **Figure 6-4** shows a clear decrease, due to the presence of phages, in host yield at all pH tested, with the

exception of pH 7. Additionally, **Figure 6-6b)** illustrates that the action of the phage was most effective, and almost constant, at pH ranging from 4 to 5, with significant infectivity also seen at pH6 and pH3.5. The reason for the lower infectivity at pH7 is likely to be related to the bacterial physiological state change - no reduction in adsorption efficiency nor phage inactivation were observed at these pH values in MRS medium (Briggiler Marcó *et al.* (2010)).

When introducing both phages together, their inhibition pattern was compounded. The results show excellent reduction in bacterial cell numbers for pH ranging from 4 to 6 (**Figure 6-5**). This was further confirmed by the infectivity of the combined phages remaining elevated and fairly constant over this pH range (**Figure 6-6c)**).

7.2 Populations dynamics of yeasts, bacteria and phages B1 and B2

The populations dynamics studies were conducted under several different sets of conditions: MRS or M9 medium; pH4 or pH6; bacterial inoculation levels of 10^5cells/mL or 10^7cells/mL; with or without agitation; and aerobic or anaerobic conditions. These different parameters were used to investigate populations dynamics and establish effective conditions for the model design.

7.2.1 Aerobic conditions

No significant differences were observed for aerobic growth of *S. cerevisae* in M9 minimal medium at pH4 (**Figure 6-7**) or pH6 (**Figure 6-8**). The concentration of yeasts and buds did not clearly change in the absence or presence of bacteria or phages. Comparing growth at both pH tested, slightly higher bud concentration was

seen at pH4 and bacteria reached higher yields at pH6. The addition of phages caused a reduction in bacterial concentration of approximately 1 order of magnitude at both pH values tested. The consistent yeast growth regardless of the presence of contaminating bacteria was likely due to the fact that M9 minimal medium, while advantageous for its known composition, was not favourable to the cultures, especially *L. plantarum*.

In fact, when similar experiments were performed in MRS, a medium which is often used in research on lactobacilli, results were more dramatic. MRS has the advantage of being a complex medium and thus more closely resembles industrial mashes used in yeast fermentations (de Man *et al*., 1960; Monceaux, 2009).

First of all, it is important to note that all bacterial and yeast concentrations were greater in MRS in comparison to M9. Secondly, **Figure 6-11a** clearly shows a reduction in yeasts concentration linked to greater bacterial concentration levels (**Figure 6-11b)**). This is consistent with the fact that yeasts and bacteria compete for the same resources, thus favourable conditions for the latter impede on the growth of the yeasts. The addition of phages to the system virtually wiped out bacteria, increasing yeasts yield (**Figures 6-11a)** and **b)**). Statistical analysis of variance and t-tests confirmed significant differences in concentrations when yeasts were grown alone (I - control) and when yeasts were grown in the presence of bacteria at high inoculation level (III). Bacterial elimination by phages was similar at higher and lower inoculation levels and reached more than 99.8%. The ratio of buds per yeast cell in this experiment remained practically constant for all cases (data not shown).

The impact of bacteria and yeasts competing for resources was further investigated through a dynamic study (**Figure 6-12**). The presence of bacteria lowered the yield of yeasts from $9x10^7$cells/mL to $2x10^7$cells/mL, a reduction of approximately

77%, and of buds from $7x10^7$buds/mL to 10^7buds/mL. The addition of phages caused full recovery of yeasts and buds yield. The whole trend of bacterial concentration in the presence of phages remained extremely low. In fact, phages were capable of reducing bacterial levels by 3 orders of magnitude after the first 30 hours and 2 orders of magnitude for the last 10 hours of experiment.

7.2.2 Anaerobic conditions

For anaerobic growth of *S. cerevisae* in M9 medium, the outcome was different from the one seen in the same medium under aerobic conditions. The presence of bacteria clearly reduced the final yeast concentration, and this reduction was greater with higher bacterial inoculation levels (**Figure 6-9** and **Figure 6-10**). This was observed at both values of pH tested, although more clearly at pH6. More importantly, the addition of phages reduced bacterial levels and increased final yeast concentrations, almost reaching the same concentrations as pure yeast cultures for the lowest bacterial load. This outcome was expected since bacteria affected by phages were not competing with the yeast for the resources present in the medium, most notably the substrate. Also of note, the effectiveness of the phages was greater when the lower bacterial inoculation load was used. It is important to observe that the initial contamination level of 10^5cells/mL is within the range usually encountered in industries (Leja and Broda, 2009; Skinner and Leathers, 2004; Sossa Urrego *et al*., 2009), and hence is more representative of a real case scenario. Higher initial bacterial concentrations were used as a worst-case scenario to mimic a more acute contamination problem in industry. It should also be noted that the ratio of bud to yeast remained constant in all cases tested (data not shown).

In MRS medium under anaerobic conditions, the impact of the presence of bacteria and phages on the final concentration of yeast (**Figure 6-14**) was similar to the effects observed under aerobic conditions (**Figure 6-11**). In fact, a 95% reduction in final yeast concentration was seen when bacteria were added to the system, while this reduction was only 37% with the addition of phages. Contamination (concentration of bacteria) was reduced by approximately 70% when phages were present. Statistical analysis of variance and t-tests confirmed significant differences between all concentrations obtained. This means that yeast concentration was significantly reduced in the presence of bacteria and that phages attacking *L. plantarum* enabled a significant recovery of yeasts, but not as high as the control (axenic yeast culture). The decrease in yeast concentration, even when phages were present, was probably due to anaerobic conditions or lack of agitation. Reduction in phage infection due to changes in aeration has been observed previously (Howes, 1965; Tanji *et al*., 2005). This is most often related to the correlation between growth rate of the host and burst size. Since the bacteria grow slower under anaerobic conditions, the phage burst size will be reduced and so is the observed infectivity. As well, agitation has been shown to affect phages adsorption (Koch, 1960). Hence, more investigation is needed to determine the actual impact of these factors on the reduction in infectivity in this system.

The results of these experiments prove that, under many different sets of conditions, bacteria compete with yeasts for resources and affect their growth and development, and that phages are capable of reducing bacterial levels to an extent that doesn't affect yeast growth. A better visualization of this conclusion and of the effective impact of the presence of phages can be seen in the sample pictures in **Figure 6-13**.

7.3 Ethanol production

To assess the feasibility of using phages to reduce bacterial contamination in yeast fermentation, especially in the context of biofuel production, it was important to investigate their impact on ethanol production. While the population dynamics studies showed that adding phages led to final yeast concentrations equivalent to those observed when yeast was grown alone, similar trends were observed in ethanol production.

Under aerobic conditions, major reduction in ethanol production (67%) was observed when bacteria were competing with yeasts for nutrients (**Figure 6-15a)**). When phages were added to the system, the ethanol production reached the same level as the control system (yeast alone). Statistical analysis of variance and t-tests confirmed these results. Measurements were taken after 24h of fermentation as this corresponded to the maximum in ethanol concentration. After this time, glucose was depleted and the organisms started to consume ethanol for their growth and metabolism (data not shown and Ramon-Portugal *et al.*, 2004).

Similar results were obtained under anaerobic conditions (**Figure 6-15b)**). Virtually no ethanol was produced by the yeast when competing with bacteria for nutrients. However, the addition of phages allowed significant ethanol production (60% of the production in the control experiment). Statistical analysis (variance analysis and t-tests) showed significant difference in all values. It means that, just like the results obtained for yeast concentrations (**Figure 6-14a)**), ethanol synthesizing by yeasts was significantly reduced by bacteria and that phages attacking *L. plantarum* enabled a significant improvement in ethanol production. However, ethanol yield in

the presence of phages was not as high as the control – again, probably the lack of agitation or aeration in the system reduced the efficiency of the phage infectivity.

The averages of ethanol production in these experiments didn't surpass 1.2% (v/v). While lower than industrial production levels, the quantities fall within expectation because of the comparatively low amount of glucose present in the medium (around 20g/L). As the glucose molar mass is 180g/mol, molar concentration of glucose in the medium was 0.11mol/L. Stoichiometry of the reaction of glucose into ethanol dictates that one molecule of glucose is converted into two molecules of ethanol, so 0.22mol/L of ethanol was expected upon full conversion. Ingledew (2009a) states that approximately 10% of the substrate is used by the yeast for its metabolism and multiplication. Hence, 90% conversion can be expected, which would correspond to a production of 0.2mol/L. Converting ethanol molar concentration into mass (molar mass of 46g/mol) and further mass into volume (density of 0.789g/cm^3), the expected yield of ethanol was 1.17% (v/v), in accordance with the results obtained. One should however consider that MRS medium has other carbon sources that can be utilized by yeasts for metabolism and even ethanol production. But, as glucose is the main substrate of the medium, the calculation on its basis is sufficient to show that ethanol yield was fairly high. Also of note, the ethanol produced was not observed to be inhibitory towards the organisms.

7.4 Mathematical model

The results for the individual growth of *S. cerevisiae* and *L. plantarum* (**Figures 6-16** and **6-17,** respectively) illustrated that Monod kinetics was an appropriate model for these situations.

The model developed to describe the one step growth experiments of the phages was also in accordance with empirical results (**Figure 6-18**). Unlike models developed specifically for one step growth curves (Rabinovitch *et al*., 1999), the parameters (latent period, lysis time and burst size) were obtained from the experiments. The model was then applied to verify the hypotheses that: 1) the lytic process follows first-order kinetics with a time delay and 2) the ratio of final infected cells to initial infected cells is 1/100 (or that lysis occur in 99% of the infected cells). From the good fit of the model, it can be concluded that these hypotheses are reasonable.

The parameters determined experimentally from the one step growth curves presented some divergences in relation to previous reports. The parameters determined by Nes *et al*. (1988) for phage B2 in MRS at 30^{o}C (latent period of 75min, lysis time of 90min and burst size of 12-14 phages per infective centre) were somewhat different from those reported here, with the exception for latent period. The differences can be due to the pH of the medium used in their experiment (not pH6). In addition, their burst size was reported as a function of infective centres, which tend to underestimate the burst size in comparison to more accurate measures reported per infected cell, as in the present study. The experiments performed by Briggiler Marcó *et al.* (2010) also indicated different results (latent period of 30min and lysis time of 75min for both phages, and burst size of 83pfu per infective centre for phage B2 and 60pfu per infective centre for phage B1). While these results somewhat differ from those reported here, so were the experimental conditions (pH different than 6, 37^{o}C, burst size reported per infective centre). In fact, it has been shown that environmental

factors, such as pH and temperature, can greatly influence phages infection parameters (Kutter *et al*., 1994).

The adsorption behaviours observed for both phages (**Figure 6-19**) are also significantly different from those previously reported (Briggiler Marcó *et al.*, 2010; 2012). In this study, adsorption of phage B1 was relatively slow and reached an efficiency of approximately 60%. Phage B2 adsorbed to the host at a much faster rate, but had an adsorption efficiency of only 35%. In the reported studies, phage B1 had a faster adsorption rate and a higher efficiency. In the case of phage B2, adsorption rate was apparently slower in comparison to this study but the efficiency was higher. As explained previously, these divergences can be due to the different study conditions (medium at a different pH and temperature of 37^{o}C).

The first-order adsorption efficiency model (Storms *et al*., 2010, 2012) adopted to simulate the adsorption behaviour of both phages proved to be accurate and robust for this system. The simple first-order reaction mechanism model (Hyman and Abedon, 2009) was also tested but did not fit the data accurately (data not shown).

It is important to note that the conditions in which the model was developed (aerated MRS medium in shake flasks, 30^{o}C and agitation of 150rpm) can be considered not to have diffusion limitation.

7.5 Combined mathematical model

To assess the validity of the complete model for yeast, bacteria and phages present in the system, all parameters and equations were combined and compared to experimental data (**Figure 6-22**)**.** The results obtained reinforced the hypotheses and assumptions established for this model.

7.5.1 Susceptible and non-susceptible bacterial cells

The model developed in this study defines the concept of bacterial cells that are susceptible and non-susceptible to phage infection depending on their physiological state. It predicts that both states are transient and that the transformation is reversible. The expected consequence of this concept is that, in a batch system with phage infecting a host, after a certain period of time, all inoculated susceptible bacteria will be killed and the non-susceptible group will compose the only bacteria in the system. However, the non-susceptible bacteria will constantly become susceptible and be infected by phages. Therefore, phages will keep being formed. This pattern of phages being amplified while bacterial population doesn't increase can be observed in **Figure 6-21**.

Figure 6-20 demonstrated that the bacterial population not only didn't increase after 4h of phage attack, but actually remained constant. The observed *plateau* is certainly not a regular stationary phase caused by lack of nutrients, as glucose was still present in the system. A plausible explanation for that phenomenon is that the pool of susceptible bacteria was depleted and the rates of non-susceptible bacterial growth (cell division) and transformation into susceptible bacteria were equal. For this to happen the reaction rate constants for bacterial transformation (k_1 and k_2) have to be factors of the bacterial growth rate – determined in the model to be $\mu_{bacteria}$. This observation was tested using different relationships for k_1 and k_2 in several simulations (*e.g.* $k_1=k_2\neq\mu$, $k_1=fk_2\neq\mu$, $k_1=k_2=\mu$, $k_1=fk_2=\mu$, $f_1k_1=f_2k_2\neq\mu$, etc. – where f is the factor of bacterial growth rate) (data not shown). According to the experimental results and the simulations tested, the hypothesis that k_1 and k_2 were related to $\mu_{bacteria}$ was confirmed. Actually, they were found to be proportional to $\mu_{bacteria}$. Hence the model

$f_1k_1=f_2k_2=\mu_{bacteria}$ was found to be the most adequate. As explained in **Section 5.3**, the relation between k_1 and k_2 can be expressed through K_{eq} (**Equation 5-14**). Therefore, K_{eq} is precisely the factor of $\mu_{bacteria}$ that differentiates k_1 from k_2.

It is hypothesized that K_{eq}, which correlates both the factor that differentiates k_1 from k_2 and the ratio between susceptible and non-susceptible bacterial concentrations at equilibrium, is specific for each bacteria-host system. K_{eq} of 0.2 was determined from the experiment of *L. plantarum* infected by both phages (**Figure 6-20**). This indicates that the bacterial population inoculated has five times more non-susceptible cells than susceptible cells at the onset of infection and that the rate constant for the transformation of susceptible bacteria to non-susceptible (k_2) is five times greater than k_1. This value of K_{eq} was applied in the complete population dynamics system and showed to fit satisfactorily (**Figure 6-22**), reinforcing the hypothesis. In the experiment involving only one phage (**Figure 6-21**) the best K_{eq}, according to least squares evaluation, was found to be 10^5. For that experiment, this means that all bacteria inoculated were susceptible and that (k_1) is 10^5 times higher than k_2. When comparing K_{eq} determined for the experiment involving both phages (**Figure 6-20**) to the one for only phage B1 (**Figure 6-21**), a major difference can be seen. This observation stresses two points: 1) how combining phages (cocktails) can greatly increase the efficiency of infections, and 2) how K_{eq} is specific for each system evaluated.

7.5.2 Limitations of the model

After 40h of experiment, the yeast populations, in the cases where yeasts were grown alone and in the presence of bacteria and phages B1 and B2, increased in

numbers. This phenomenon can be visualized in **Figure 6-12**, but is not demonstrated in **Figure 6-22**. The increase was likely caused by yeasts consuming ethanol as carbon source, growing in a diauxic fashion, once all glucose was consumed (Mohammed Al-mhanna, 2010). The model didn't follow this trend because it did not include ethanol consumption. Likewise, increases in bacterial concentrations were observed after 40h of fermentation in cases where yeasts were grown in the presence of bacteria and in the presence of bacteria and phages B1 and B2. The cause of this increase is not clear at the moment. A possible explanation is that bacteria were able to consume substances released by the yeasts as they consumed ethanol. In fact, HPLC measurements demonstrated an increase in the concentration of an unknown substance during ethanol decrease (data not shown).

7.5.3 Population dynamics

Despite these limitations, it can be seen that the combined model displayed good fit to the population dynamics data (**Figure 6-22**). The model was thus able to predict the populations of yeast and bacteria in all cases tested. This is important as it could become the base for important tools in the determination of conditions to control contamination levels in yeast fermentations. Moreover, this model could be translatable to other fields of phage application, such as phage production, phage therapy, recombinant phage systems or host-phage interaction studies.

Another important aspect of the model is its capacity to describe multiple phage populations in a system (**Figure 6-22c)**). Considering the experimental difficulties in the differentiation and quantification of different phages in systems where multiple phage species attack a single host, the model provides clues to the dynamics of the

different populations. In the present case, the model showed that phage B2 is expected to amplify to a much higher extent than phage B1 (around 2 orders of magnitude). Even though phage B2 has a lower burst size in comparison to B1, its faster rate of adsorption and shorter lysis time were responsible for its population taking over. The final titer calculated for both phages together was 1.58×10^{9}pfu/mL.

8. CONCLUSION

The present work demonstrated that phages are suitable antimicrobial candidates to be applied in industrial yeast fermentation processes and that the mathematical model developed simulates well the dynamics of the organisms involved in the system. More specifically, the following conclusions can be drawn from this work:

The inhibition of a robust commercial yeast by *Lactobacillus plantarum* was significant with regards to yeasts growth and ethanol production. This inhibition was greater in a medium suitable for lactobacilli (MRS) and under anaerobic conditions. Under those conditions, ethanol production was practically non-existent in the presence of high contamination level.

The presence of phages allowed the levels of yeasts growth and ethanol production to be similar to those of pure yeast cultures under aerobic conditions and extensive recovery under anaerobic conditions. It was also observed that, expectedly, a cocktail of virulent phages was more effective at killing bacteria than a single phage. Moreover, bacterial resistance was facilitated when only one phage was present.

The concept of bacterial populations that are transitorily non-susceptible to phage infections was reinforced with the results observed. It could be also seen that susceptibility varied for each system studied (*e.g.* phage B1 alone or phages B1 and B2 together). Overall, the model suited the population dynamics studied adequately.

9. FUTURE WORK AND RECOMMENDATIONS

Although this work covered several aspects of the application of phages in yeast fermentation processes, many avenues could be taken to provide further insight on the topic.

First, the phages cocktail can be improved with more viruses that are potentially able to reduce bacterial contamination levels even further. **Section 2.2.6** presented several *L. plantarum* phages that were recently isolated and that could be members of the cocktail composition. In addition, a similar study as the one presented here could be conducted with other lactic acid bacteria that are common contaminants in bioethanol processes, such as other lactobacilli species, lactococci and pediococci. Several phages that could compose a cocktail against these bacteria have been already isolated.

Another study on phages isolation suitable to be performed is the induction of temperate phages from bacterial contaminants isolated from industrial processes. It is well known that an enormous amount of bacteria carry prophages integrated in their genome. Some chemical substances, such as mitomycin C, are able to induce the lytic cycle. Although these temperate phages wouldn't be highly virulent against the hosts that carried the prophage, it is possible that they would be strictly lytic against other related strains.

Furthermore, although MRS was shown to be a suitable medium to simulate the mash commonly utilized in industrial fermentation, it would be pertinent to validate these studies in industrial media. It is indeed expected that phages are effective in industrial mashes as well, but the parameters of infection are likely to change in relation to those determined in this study. In addition, because the

characteristics of the media applied in industries change considerably for each feedstock used (Monceaux, 2009), population dynamics is expected to be different for each case. Hence, it is important to study the efficiency of phages infection in industrial media, at least in the mashes obtained from corn and sugar-cane.

The application of other predators of bacteria, such as bacteriovorus, could also be investigated. *Bdellovibrio*, an example of bacteriovorus, is a genus of bacteria that prey on other bacteria. This small bacterium with very thick flagellum penetrates the cell wall of the host, nourishes from its cytoplasmic constituents, replicates in its periplasm and kills it (Madigan *et al*., 2011b). Preliminary advantages of the application of *Bdellovibrio* in relation to phages are: they have high motility and are less specific towards their host. However, they are obligate aerobes, attack only Gram-negative bacteria and are not obligatory predators. Nevertheless, research on this topic would be relevant.

Finally, regarding the model development, the concept of the transitory non-susceptibility state of a host can be further investigated in other phage-host systems. This concept may be present in several other bacterial infection systems.

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