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Optimization of biomass production of an OTA-degrading Pediococcus parvulus

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Dissertação de Mestrado
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Professor **Doutor Luís João Abrunhosa Pereira**
Doutora **Isabel Maria Pires Belo**

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Abstract

Lactic acid bacteria (LAB) are considered beneficial to health due to their use in the production of fermented foods and because they have several probiotic properties. Also, certain LAB strains have the ability to detoxify mycotoxins which make them a promising solution to reduce the levels of mycotoxins in food and feed products. *Pediococcus parvulus* is a LAB with the ability to detoxify ochratoxin A (OTA), which is one of the most important mycotoxins found in agricultural commodities. The present work aimed to optimize the biomass production of *P. parvulus* using batch and fed-batch fermentation processes. Initially, the composition of culture medium and operational conditions were optimized in flaks. The biomass production was evaluated by testing three culture media (MRS, TGE and GYP), wherein with MRS was obtained the maximal biomass concentration of 0.81 $g{\cdot}L^{-1}$. Lactose was also tested as an alternative carbon source but *P. parvulus* did not metabolized this monosaccharide. Temperature, pH control and L-(+) cysteine were the factors that had the most relevant effect on bacteria growth. At this level, the highest concentration of biomass achieved (1.14 $g{\cdot}L^{-1}$) was obtained in batch culture in bioreactor using MRS broth supplemented with 1.0 $g{\cdot}L^{-1}$ of L-(+) cysteine wherein pH and temperature were maintained at 5.2 and 30 °C, respectively. After selecting the best conditions for *P. parvulus* cultivation, different batch and fed-batch fermentation process were studied. In those experiments, no significant difference in biomass production were observed between batch and fed-batch fermentation, since they achieved biomass concentrations of 1.14 $g{\cdot}L^{-1}$ and 1.19 $g{\cdot}L^{-1}$, respectively. Additionally, it was observed that *P. parvulus* was inhibited by lactic acid making it difficult to obtain high biomass yields with a unique and simple process. To overcome this problem, a fed-batch with cells-recycling through centrifugation was finally studied. Using this process, the biomass production was further enhanced being achieved a final biomass concentration of 2.24 $g{\cdot}L^{-1}$ and 3.19 $g{\cdot}L^{-1}$, respectively, with one and two cells-recycling steps. This maximum concentration was approximately 3-fold better than the one obtained in batch cultures. Besides, the OTA-degrading capacity of *P. parvulus* was not affect by the different operational conditions and modes of operations. Based on this results it will be interesting, in future studies, to evaluate the performance of a cell-recycling fed-batch culture system that uses a microfiltration membrane unit to continuously recycle the cells.

Resumo

As bactérias láticas são consideradas benéficas para a saúde devido à sua aplicação em produtos alimentares fermentados e às suas propriedades probióticas. Outra possível vantagem destes microrganismos prende-se ao facto de certas bactérias láticas apresentarem a capacidade de degradar micotoxinas, sendo deste modo, uma solução promissora para reduzir os níveis de micotoxinas em produtos alimentares e rações animais. *Pediococcus parvulus* é uma bactéria lática com a capacidade de degradar ocratoxina A (OTA), que é uma das mais importantes micotoxinas encontradas nos produtos agrícolas. O estudo realizado teve com principal objetivo a otimização da produção de biomassa de *P. parvulus* recorrendo a processos de cultura descontínuo e semi-contínuo. Inicialmente, a composição do meio de cultura e as condições de operação foram otimizadas em matraz. A produção de biomassa foi avaliada por crescimento da bactéria em três meios de cultura distintos (MRS, TGE e GYP), obtendo-se uma maior concentração de biomassa (0,81 $g{\cdot}L^{-1}$) com o meio MRS. A lactose foi também testada como fonte alternativa de carbono, verificando-se, contudo que *P. parvulus* não metaboliza este monossacarídeo. A temperatura, o pH e L- (+) cisteína foram fatores que tiveram um efeito relevante no crescimento da bactéria. A maior concentração de biomassa (1,14 $g{\cdot}L^{-1}$) foi obtida em bioreator usando o meio MRS suplementado com 1,0 $g{\cdot}L^{-1}$ de L-(+) cisteína onde o pH e a temperatura foram mantidos a 5,2 e 30 °C, respetivamente. Depois de selecionadas as melhores condições para o crescimento de *P. parvulus*, diferentes processos de fermentação descontínuo e semi-contínuo foram estudados. Nestes ensaios iniciais não foram observadas diferenças significativas na produção de biomassa entre os modos descontínuo e semi-contínuo, tendo-se obtido concentrações de biomassa de 1,14 $g{\cdot}L^{-1}$ e 1,19 $g{\cdot}L^{-1}$, respetivamente. Adicionalmente, foi observado que o crescimento de *P. parvulus* era inibido pelo ácido lático formado, tornando-se difícil obter concentrações elevadas com um único e simples processo. Para superar este problema, foi testada uma fermentação semi-contínua com reciclagem das células por centrifugação. Usando este processo, a produção de biomassa foi melhorada tendo-se obtido uma concentração final de biomassa de 2,24 $g{\cdot}L^{-1}$ e 3,19 $g{\cdot}L^{-1}$, respetivamente, com uma e duas etapas de reciclagem das células. Esta concentração máxima foi 3 vezes superior à obtida em cultura descontínua. Além disto, a capacidade de degradação da OTA pela *P. parvulus* não foi afetada pelas diferentes condições e modos de operação. Tendo em consideração os resultados obtidos, a avaliação do desempenho de um sistema de cultura semi-contínua com uma membrana de microfiltração para reciclagem contínua das células seria uma hipótese a ser futuramente testada.

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List of Abbreviations

ATP	Adenosine triphosphate
CAS	Chemical abstract specification
D	Dilution rate (h^{-1})
DCW	Dry Cell Weight
dX/dt	Biomass production rate
dS/dt	Substrate consumption rate
EMM	Edinburgh Minimal Medium
ESP	Exopolysaccharides
F	Flow rate ($mL \cdot h^{-1}$)
FDA	Food and Drug Administration
GHP	Hydrophilic polypropylene
GRAS	Generally recognized as safe
GYP	Glucose Yeast Peptone
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IUPAC	International Union of Pure and Applied Chemistry
LAB	Lactic acid bacteria
MRS	Man Rogosa Sharpe
OD	Optical density
OTA	Ochratoxin A
PP	Polypropylene
q_s	Specific substrate uptake rate ($g \cdot g^{-1} \cdot h^{-1}$)
rpm	Revolutions per minute
S_o	Substrate concentration in feed solution ($g \cdot L^{-1}$)
t	Time
TGE	Tryptone Glucose Extract
V	Volume
USA	United States of America
X	Biomass concentration ($g \cdot L^{-1}$)

$Y_{X/S}$	Biomass yield ($g \cdot g^{-1}$)
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})

CHAPTER 1

INTRODUCTION

Mycotoxins are toxic secondary metabolites produce by filamentous fungi that occur in many agriculture products and are frequently detected in processed food. The occurrence of mycotoxins in food is potentially dangerous for public health because of the diversity of their toxic effects. In addition to the human's risks, mycotoxins cause significant economic losses in livestock production. In crops, contaminated commodities are often submitted to treatments that reduce their nutritive quality. In animals, contaminated feed may cause feed rejection and cause many livestock losses due to its toxicity (Dalié *et al.*, 2010; Zain, 2011).

OTA is one of the most important mycotoxins that can be found in food and feed. It is produced by several species of *Aspergillus* and *Penicillum* and it is mainly found in cereals, coffee, spices, red wine and meats. OTA is considered dangerous for health of humans and animals, because, besides of being carcinogenic it is nephrotoxic and has other relevant toxicological properties. So, it is recommended to reduce as much as possible its presence on food and feed, in order to minimize exposure to this mycotoxin. Several measures have been implemented with the aim of preventing their formation or reducing their presence in agricultural products through destruction or inactivation. The application of good agriculture practices and storage are some of the preventive measures most recommended. However, when the food products are contaminated, the decontamination of mycotoxins is possible by physical, chemical or biological methods (Abrunhosa *et al.*, 2010). Presently, biological methods of detoxification have been sought to control OTA. The ability of microorganisms to degrade mycotoxins have been studied. Lactic acid bacteria is one of the biological agents that are able to detoxify mycotoxins (Bianchini and Bullerman, 2009).

LAB are generally considered beneficial microorganisms due to their health and nutritional benefits, having probiotics properties and a potential to improve food nutritional characteristics. LAB produce a variety of antimicrobial compounds responsible by their antifungal activity such as bacteriocins and organics acid. Also LAB are traditionally used in the production of fermented food products and used in animal feed as silage inoculum to improve forages preservation (Bernardeau *et al.*, 2006; Naidu *et al.*, 2010; Weinberg *et al.*, 2004). A less known property of LAB is the ability of some strains to detoxify mycotoxins.

Pediococcus parvulus is a LAB which is able to detoxify OTA (Abrunhosa *et al.*, 2014). Although there has not much information, it is known that some *Pediococcus* strains have antifungal and probiotic properties (de Palencia *et al.*, 2009; Rouse *et al.*, 2008). Since *P. parvulus* is able to biodegrade OTA under anaerobic conditions, they may be susceptible to use as silage inoculants or feed additives and, therefore, to bring some additional advantages to animal's health. The use of the

OTA-degrading LAB in animal nutrition can become a valuable practice, because OTA levels that are detected in feed are of most concern for livestock production.

Due to the potential biotechnological application of *P. parvulus* in reducing the risks associated with OTA, it is of interest the production of large amounts of bacteria that could be used as commercial starter cultures.

The main objective of this work is to optimize the biomass production of *P. parvulus*. In an initial phase, the composition of the culture medium and the fermentations conditions were optimized in order to achieved high biomass concentration without the loss of OTA-degrading capacity. The optimization of cell growth conditions was done in batch cultures performed in Erlenmeyer flasks. In a second phase, several strategies like batch and fed-batch cultures performed in bioreactor were implemented, in order to optimize biomass productivity without losing the OTA-degrading capacity.

CHAPTER 2

LITERATURE REVIEW

2.1. Mycotoxins

The term mycotoxin emerged in the 1960 in the wake of an unusual veterinary crisis in England where approximately 100,000 turkeys died. The death of turkeys was associated with their feed because it contained peanut meal contaminated with toxic secondary metabolites produced by *Aspergillus flavus*, which were named aflatoxins. This event led scientists to consider the discovery of new fungal secondary metabolites that could be dangerous to human health (Bennett and Klich, 2003; Zain, 2011).

Mycotoxins are one of the important classes of naturally occurring toxicants in human food and animal feed. Mycotoxins are low molecular weight compounds produced by the secondary metabolism of filamentous fungi, such as *Aspergillus*, *Fusarium* and *Penicillium* spp. Mycotoxins are present in several food products such as cereals, fruits, oil seeds, spices, beverages (wine and beer), meat, dairy products and other products (Bhat *et al.*, 2010; Zain, 2011).

2.1.1. Production

Mycotoxins occur more frequently in hot and humid climates, favorable to fungal growth, but they can also be found in zones of temperate climate (Zain, 2011).

The mycotoxin contamination in food products can occur at different stages of the food chain (Bennett and Klich, 2003). Several factors contribute to the presence of mycotoxins in food and feed, which are often out of the control of human. The factors can be extrinsic, such as environmental conditions related to storage, or intrinsic, such as fungal strain specificity and interaction of the mycotoxigenic fungi with substrate (Zain, 2011). Mycotoxins are produced when fungal contamination of crops occurs before harvest in the field, during harvest, during storage and sometimes during food processing, particularly in some of fermentation processes (Hamed and Shier, 2009; Zain, 2011). Others factors influencing mycotoxin contamination of crops are the stress factors during plant growth, late harvesting of crops, high humidity and poor storage practices (Zain, 2011).

2.1.2. Risks and economic impact

Mycotoxins constitute a risk for health because they are present in diverse food products. Human health risks are usually associated with the direct consumption of food products, but can also occur by the dermal and inhalation routes. Human are exposed to mycotoxins by two different

routes: directly, via foods of plant origin; or indirectly, through contaminated food of animal origin (Juodeikiene *et al.*, 2012; Zain, 2011). Mycotoxins have carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic and neurotoxic properties (Dalié *et al.*, 2010; Milićević *et al.*, 2010). The severity of these effects depends, among other factors, on the toxicity of the compound, the concentration and duration of exposure, age, and physiologic state of the individual and the presence of other mycotoxins (synergistic effects) (Milićević *et al.*, 2010).

In addition, feed contaminated with mycotoxins pose a health risk to animals and as a consequence may cause significant economic losses due to increased veterinary care costs, reduced livestock production and disposal of contaminated feed.

Currently, more than 400 mycotoxins are known, but the most important in terms of impact and risk to human and animal health are aflatoxins, fumonisins, ochratoxins, patulin, zearalenone and trichothecenes (Dalié *et al.*, 2010; Milićević *et al.*, 2010; Zain, 2011). In Table 2.1, it is represented the most important mycotoxins found in foods from the point of view of health, as the main producers, the most contaminated commodities and their effects.

Table 2.1 - The most important mycotoxins found in food, producing fungal species, the commodities most frequently contaminated, as well as their pathological effects (Bhat *et al.*, 2010; Zain, 2011).

Mycotoxin	Fungal species	Food commodity	Pathological effects
Aflatoxins	*Aspergillus flavus, Aspergillus parasiticus*	Maize, wheat, rice, sorghum, tree nuts, figs	Hepatocellular cancer, kwashiorkor, Reye's syndrome, liver lesions
Fumonisins	*Furasium verticillioides*, *Furasium proliferatum*	Maize, cornflour, dried figs, herbal tea	esophageal carcinoma
Ochratoxin A	*Aspergillus ochraceus*, *Penicillium verrucosum*, *Aspergillus carbonarius*	Cereals, coffee, cocoa, dried fruit, spices, wine	Endemic nephropathy, urothelial tumors
Deoxynivalenol	*Furasium graminearum*, *Furasium culmorum*	Cereals, cereal products	Nausea, vomiting, diarrhea
Patulin	*Penicillium expansum*	Apples, pears	Damage of gastrointestinal, respiratory systems, DNA
Zearalenone	*Furasium graminearum*, *Furasium culmorum*	Cereals	Premature puberty, cervical cancer

2.1.3. Control strategies

Since mycotoxins have been recognized as a potential threat to human and animal health, many countries have established limits in food and feed to safeguard the health of consumers. The implementation of legislation is one of the most important measures used to protect consumers from the harmful effects of mycotoxins since it imposes limits to the presence of some mycotoxins in diverse food products and avoids its commercialization (Zain, 2011). Nonetheless, there are also other possibilities to avoid the harmful effect of contaminated food and feed. For example, measures can be taken to prevent fungal and mycotoxin contamination, different decontaminations methods can be applied to food and feed containing mycotoxins and absorption of mycotoxins into the digestive tract may be inhibited by using specifics adsorbents (Halász *et al.*, 2009).

Many strategies have been developed to prevent mycotoxin contamination in the field as well as during storage. The pre-harvest measures can avoid the fungal growth and formation of mycotoxins through, for example, the implementation of good agriculture practices and utilization of resistant varieties. On the other hand, the improvement of drying and storage conditions of food products are important post-harvest strategies to prevent mycotoxins formation (Milićević *et al.*, 2010; Zain, 2011).

When it is not possible to avoid mycotoxin contamination, decontamination and detoxification can be used to remove or reduce the content of mycotoxins before the use of commodities for food and feed purposes. Decontamination of mycotoxins is possible by physical, chemical or biological methods. However, any decontamination strategy used, should follow some requisites, such as: it must destroy or remove mycotoxins, it must not produce toxic residues, it should not adversely affect the desirable physical and sensory properties of the product, it must be capable of destroying fungal spores and mycelium and it has to be technically and economically feasible (Halász *et al.*, 2009; Kabak *et al.*, 2006).

2.1.3.1 Physical methods

The physical methods used for the decontamination can include the cleaning, mechanical sorting and separation of contaminated feed, washing steps, density segregation, thermal inactivation and adsorption. The utilization of adsorbents is the most applied method for protecting animals against the action of mycotoxins, in which the absorbents mixed with the feed are supposed to bind the mycotoxins efficiently in the gastro-intestinal tract. These processes aim the reduction of

mycotoxins levels in contaminated food. However, the efficacy of physical treatments is very expensive and limited and depends on the level of contamination (Huwig *et al.*, 2001; Kabak *et al.*, 2006).

2.1.3.2. Chemical methods

Various chemicals (hydrochloric acid, ammonia, hydrogen peroxide, ozone) have been tested for detoxification of mycotoxins but only a limited number of methods are effective against mycotoxins without reducing nutritive value of food or producing toxic derivatives with undesirable sensory properties. Furthermore, chemical methods need additional cleaning treatments and are therefore very expensive and time consuming (Kabak *et al.*, 2006).

2.1.3.3. Biological methods

Although the different methods on use have been successful, most of them have important disadvantages, such as extensive implications in the loss of important nutrients and high costs. Therefore, the biological decontamination is the better strategy for the removal of mycotoxin under mild conditions, without significant losses in nutritive value and sensory properties of decontaminated food and feed (Halász *et al.*, 2009; Kabak *et al.*, 2006). Biological detoxification of mycotoxins by enzymes and/or microorganisms (bacteria, fungi and yeast) offers a very specific, irreversible and environmentally friendly way of detoxification (Karlovsky, 1999). However, the toxicity of products of enzymatic degradation and undesired effects of fermentation with non-native microorganisms for the quality of food are key points that need to be keep in mind during the biological control (Shetty and Jespersen, 2006).

One of the most frequently used strategies for biodegration of mycotoxins includes isolation of microorganisms able to degrade mycotoxins and treatment of food or feed with appropriate fermentation process. Other strategy is the knowledge of enzymes that take part in degradation of mycotoxins, which opens new approaches to fight the problem, such as producing genetically modified microorganisms commonly used in food production and their use for production of enzymes capable of degrading the mycotoxins (Juodeikiene *et al.*, 2012).

2.2. Ochratoxin A

Ochratoxin A (OTA) is one of the most important mycotoxins and it is found in diverse food and feed products. OTA occurs naturally in many plant products such as wheat, barley, coffee, beans, cocoa and dried fruits and it is also detected in products based on cereals, spices, wine, beer, grape juice and animal products (meat, eggs and milk) (Abrunhosa *et al.*, 2010; Coronel *et al.*, 2011)

OTA was discovered in 1965 as secondary metabolite produced by *Aspergillus ochraceus*, later it was discovered that other species of genus *Aspergillus* and *Penicillium* had the capacity to produce OTA (Abrunhosa *et al.*, 2010).

OTA (Figure 2.1) is composed by a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-*R*-methylisocoumarin molecule, ochratoxin α (OTα), and by a of L-*β*-phenylalanine molecule, which are linked by an amine bond. The empirical formula is $C_{20}H_{18}O_6NCl$ and the molecular weight is 403.82 Da. The IUPAC formula of OTA is L-phenylalanine-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl]-(*R*)-isocoumarin and its chemical abstract specification (CAS) is 303-47-9 (Abrunhosa *et al.*, 2010; Ringot *et al.*, 2006).

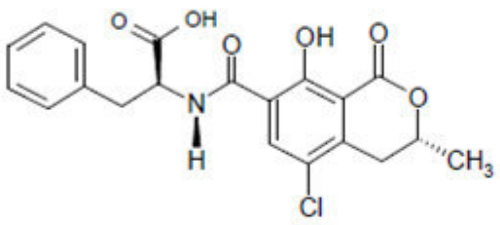

Figure 2.1 - Chemical structure of OTA (Adapted of Abrunhosa *et al*, 2010).

2.2.1. Biosynthetic pathway

The OTA biosynthesis pathway is not yet completely established. However, experimental studies with radioactive labeled precursors showed that L-*β*-phenylalanine derives from the shikimate pathway and the isocoumarin derives from the pentaketide pathway. The synthesis of the isocoumarin polyketide results in the condensation of one acetyl-CoA unit to four malonate units, probably by the action of a polyketide synthase (O'Callaghan *et al.*, 2003). The polyketide chain is then modified forming the ochratoxin α, which is coupled to L-*β*-phenylalanine by the action of an ochratoxin A synthetase. Although not knowing at what exact point of the biosynthesis, the chlorine

atom is incorporated through the action of cloroperoxidases, forming OTA (Harris and Mantle, 2001; Ringot *et al.*, 2006). OTA production depends on different factors such as temperature, water activity (aw) and nutrients (Ringot *et al.*, 2006).

2.2.2. Toxicity

OTA is considered one of the mycotoxins more dangerous for heath of humans and animals. OTA was classified by IARC as possibly carcinogenic to humans (group 2B), but it has also other toxicological properties such as nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic effects (Coronel *et al.*, 2011; El Khoury and Atoui, 2010). OTA disturbs cellular physiology in multiple ways, but the primary effects are associated with enzymes that participate in phenylalanine metabolism, especially by inhibiting the enzymes responsible for the synthesis of the phenylalanine tRNA complex. Furthermore, it inhibits mitochondrial ATP production and stimulates lipid peroxidation (Bennett and Klich, 2003).

In addition, OTA is a cumulative toxic compound, because it is easily absorb through the stomach and the small intestine and it is hardly eliminated through the biliary and urinary routes. Accumulation occurs in blood, liver and kidney. OTA bounds strongly to serum proteins, mainly albumin, limiting its transfer from the blood to the hepatic and renal cells. This characteristic explains its long half-life observed in some species, which is of 35.5 days in the case of humans (Abrunhosa *et al.*, 2010; Ringot *et al.*, 2006).

The toxicity of OTA depends on its concentration changes over time in the organism, on the dynamic interactions that is establishes with biological targets and on their downstream biological effects (Ringot *et al.*, 2006).

2.2.3. Elimination strategies

Due to OTA toxicity, the presence of OTA in food and feed products should be reduced as much as possible to minimize human and animal exposure to this mycotoxin.

The use of good agricultural practices, the correct application of fungicides and the proper storage of commodities are preventive methods which are fundamental to avoid the contamination of commodities (Amézqueta *et al.*, 2009). Despite

the application of these measures, when environmental conditions are favorable, commodities can be contaminated by OTA requiring decontamination or detoxification measures to eliminate or reduce the levels of the mycotoxin.

The physical and chemical methods are generally effective in the reduction or elimination of OTA, but the toxicological safety in the final product is not always guaranteed (Abrunhosa *et al.*, 2010). Biological methods use microorganisms, which can decompose, transform and adsorb OTA to detoxify contaminated food and also use enzymes capable to hydrolyze OTA. The main pathway to detoxify OTA involves the hydrolysis of the amine bond that links the L-*β*-phenylalanine molecule to the ochratoxin α (OTα) moiety (Figure 2.2), whose products are non-toxic (Abrunhosa *et al.*, 2010; Karlovsky, 1999).

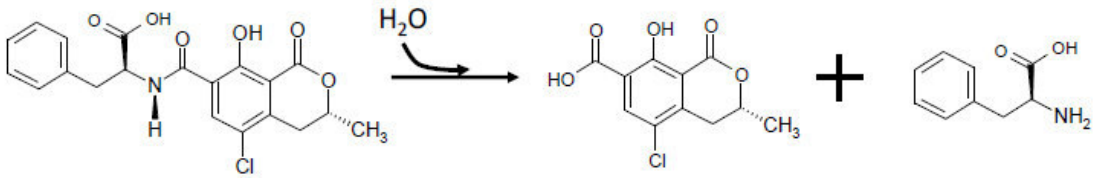

Figure 2.2 - Hydrolysis of OTA in OTα and L-*β*-phenylalanine (Adapted of Abrunhosa *et al*, 2010).

Several enzymes have been described as capable of degrading OTA. Carboxypeptidase A was the first protease reported with capacity to hydrolyze OTA (Pitout, 1969). Other enzymes that can efficiently degrade OTA are lipases obtained from *Aspergillus niger* (Stander *et al.*, 2000) and some commercial proteases (Abrunhosa *et al.*, 2006).

Several microorganisms are able to degrade OTA, such as bacteria, yeast, protozoa and filamentous fungi. The ability to eliminate OTA has been observed for bacteria: *Phenylobacterium immobile* and *Acinetobacter coloaceticus*, which respectively degraded OTA present in medium containing 0.1 and 10 mg·L^{-1} OTA, after incubation at 25 °C. In both cases, OTα is one of the final products of reaction. Some lactic acid bacteria, such as certain *Pediococcus parvulus* strains, have the ability to biodegrade OTA into OTα, when cultivated in MRS medium supplemented with OTA (Abrunhosa *et al.*, 2014). Certain fungi belonging to *Aspergillus*, *Botrytis* and *Rhizopus* genera are also able to degrade OTA up to more than 95% (Abrunhosa *et al.*, 2010; Piotrowska and Zakowska, 2005).

2.3. Lactic acid bacteria

Lactic acid bacteria can be defined as a group of gram-positive, non-sporulating bacteria with nonaerobic habit but aerotolerant, which produce lactic acid as the major end-product during fermentation. LAB are a group of bacteria very demanding in terms of nutritional requisites and support very low pH values, with acidity tolerance variable between strains. LAB are present in many diverse environments (fermented food and beverages, plants, fruits, soil, wastewater) and make also part of intestinal microflora (Patrick, 2012; Rattanachaikunsopon and Phumkhachorn, 2010).

The LAB group is composed of 13 genera: *Carnobacterium*, *Enterococcus*, *Lactoccoccus*, *Lactobacillus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Paralactobacillus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Patrick, 2012). The classification of LAB into different genera is based on morphology, mode of glucose fermentation, growth at different temperatures, acid or alkaline tolerance, ability to grow at high salt concentrations and configuration of lactic acid produced. LAB can be divided into two groups based on the end-products formed during the fermentation of glucose: homofermentative or heterofermentative. Homofermentative bacteria convert sugars almost only to lactic acid and heterofermentative bacteria produce not only lactic acid but also ethanol and carbon dioxide (Rattanachaikunsopon and Phumkhachorn, 2010). LAB have diverse potential applications that can go from the production of fermented foods to its use in animal feed as silage inoculums to improve forages preservation (Bernardeau *et al.*, 2006; Naidu *et al.*, 2010; Weinberg *et al.*, 2004). LAB are also considered important microorganisms because of their health and nutritional benefits, having attractive probiotic properties and a great potential to improve food nutritional characteristics (Naidu *et al.*, 2010; Patrick, 2012). LAB are also capable to prevent microbial growth producing some substances that are able to control pathogenic bacteria and undesirable microflora (Dalié *et al.*, 2010).

LAB are named according to their ability to produce lactic acid as the major product of sugar fermentation. Lactic acid has been used for fermentation and preservation of human food, but it is also widely used in the cosmetic, pharmaceutical and chemical industries (Castillo Martinez *et al.*, 2013). Lactic acid is recognized a chemical GRAS (Generally Recognized as Safe) by FDA (United Sates Food and Drug Administration) and it is widely used in food industry for flavouring, pH regulation, improve microbial quality, and mineral fortification. Furthermore, lactic acid is used in food industry to provide products with an increased shelf-life (Wee *et al.*, 2006).

Lactic acid can be produced by microbial fermentation or chemical synthesis and can be either of L(+) and D(-) form (Dalié *et al.*, 2010). In microbial fermentation, the pure L(+)-lactic acid and D(-)-lactic acid can be obtained when microorganism capable to produce only one of the isomers are used (Castillo Martinez *et al.*, 2013; Wee *et al.*, 2006).

2.3.1. Antifungal activities of LAB

The use of LAB to increase food safety and the quality of a large range of fermented foods is due to the ability of those bacteria to inhibit the growth of other microorganisms (Jeevaratnam *et al.*, 2005). Their antimicrobial activity comes from their capacity to produce organic acids and, therefore, to lower the intracellular pH, and to produce antimicrobial agents such as ethanol, carbon dioxide and bacteriocins (Rattanachaikunsopon and Phumkhachorn, 2010).

LAB produce a great variety of antimicrobial compounds which suggests further potential applications for food and feeds preservation. The preservative effect exerted by LAB is mainly due to production of organic acid (lactic acid) which result in lower pH. Several compounds have been isolated with the capacity to eliminate fungal growth, including organic acids, reuterin, fatty acids and cyclic dipeptides (Schnürer and Magnusson, 2005).

According to Cabo *et al.* (2002), certain LAB have antifungal activity against to *Penicillium discolor* due to the acetic acid. However, reuterin presents a broad antimicrobial spectrum. It was originally isolated from *Lactobacillus reuteri* and it is one of the most intensively studied low-molecular-mass inhibitory compounds of LAB (Axelsson *et al.*, 1989; Talarico *et al.*, 1988). Hidroxylated fatty acids have also antifungal activity against a broad spectrum of yeast and moulds (Sjögren *et al.*, 2003).

2.3.2. Bacteriocins

Bacteriocins are antimicrobial peptides which inhibit the growth of some bacteria. These substances, synthesized in ribosomes, are cationic peptides which exhibit hydrophobic or amphiphilic properties and whose target for their activity is in most cases the bacterial membrane (Cleveland *et al.*, 2001; Jeevaratnam *et al.*, 2005). Bacteriocins have been isolated from a diversity of LAB and they are commonly divided into four main groups. They can be classified on the basis of their molecular mass, thermo and enzymatic stability, sensitivity, presence of posttranslational modified amino acids and mode of action (Juodeikiene *et al.*, 2012; Klaenhammer, 1993).

Bacteriocins of Class I or lantibiotics consist of small (<5 kDa) post-translationally modified peptides which contain modified thioester amino acids such as lanthionine or methyllanthionine. They are divided into two types based on structural similarities. Class Ia bacteriocins consist in elongated, flexible and positively charged peptides that form pores in target membrane. The most extensively characterized of this group is nisin, which is produced by *Lactococcus lactis* subsp *lactis*. Class Ib bacteriocins are globular peptides, more rigid, which have negative charge or no net change. Bacteriocins of class II are also small (<10 kDa), heat stable and they do not contain lanthionine peptides. These substances can be classified into two subclasses. Subclass IIa, a pediocin-like or Listeria-active bacteriocin, which have an N-terminal consensus sequence Tyr–Gly–Asn–Gly–Val and two cysteines. Subclass IIb refers to two-component bacteriocins that requires two peptides to work synergistically in order to have an antimicrobial activity, as for example, lactacin F and lactococcin (Cleveland *et al.*, 2001; Deegan *et al.*, 2006). Class III bacteriocins include heat labile proteins which are large molecular mass (>30 kDa) (Rattanachaikunsopon and Phumkhachorn, 2010). Enterolysin produced by *Enterococcus faecalis* is one bacteriocins of this group (Nilsen *et al.*, 2003). Class IV bacteriocins consist in complex bacteriocins with other macromolecules (Klaenhammer, 1993). However, this two end classes are not well characterized (Cleveland *et al.*, 2001; Rattanachaikunsopon and Phumkhachorn, 2010).

More than 100 peptide bacteriocins produced by LAB have been described (Hammami *et al.*, 2010). In general, the bacteriocins produced by LAB associated with food belong to class I and class II (Jeevaratnam *et al.*, 2005). Nisin is the most characterized bacteriocin, is the only that has been approved for commercial use in many countries and has an inhibitory spectrum against Gram-positive bacteria, including food pathogens such as *Listeria monocytogenes* and spoilage bacteria such as *Clostridium* species. Nisin is approved for use as a component of the preservation procedure for processed or fresh cheeses and canned foods (Delves-Broughton, 2005). Other commercially produced bacteriocins is pediocin PA-1 produced by *Pediococcus acidilactici* (Cleveland *et al.*, 2001). This bacteriocin belong the Class IIa that have attracted particular attention due to their activities and potential applications. Pediocins are produced by *Pediococcus* spp. and have anti-listerial activity. They are not very effective to spores but can inhibit *L. monocytogenese* as effectively as nisin and they are more effective in some food such as meat (Jeevaratnam *et al.*, 2005; Papagianni and Anastasiadou, 2009).

Thus, the LAB bacteriocins have many attractive characteristics that make them susceptible candidates for use as food preservative. However, bacteriocin activity can be affected by several

factors including interaction with other bacteriocins, constituents from the cells and from the growth medium, and concentration of exogenous enzymes (Campos *et al.*, 2006). Whereby, it is important of testing the effectiveness of bacteriocins in food for which they are intended to be applied against the target and nontarget bacteria (Hartmann *et al.*, 2011).

2.4. Lactic acid bacteria and mycotoxins

One of properties of LAB is the ability of some strains to detoxify mycotoxins. Although it is a less well known property, several studies show the ability of LAB to remove mycotoxins. El-Nezami *et al.* (2002b) showed that some *Lactobacillus* strains can remove deoxynivalenol from liquid medium. Other articles showed that *Lactobacillus* strains were capable to detoxify mycotoxins such as OTA and patulin (Fuchs *et al.*, 2008). Some LAB have also showed the ability to remove zearalenone, fumonisins and aflatoxins (El-Nezami *et al.*, 1998; El-Nezami *et al.*, 2002a; Niderkorn *et al.*, 2006).

The mechanisms of action of LAB on mycotoxins are not yet fully understood. However, the main mechanism described involves the adsorption of mycotoxins by cell walls (Abrunhosa *et al.*, 2010; Shetty and Jespersen, 2006). Others mechanisms may involve the inhibition of mycotoxin biosynthesis by LAB and their biodegradation (Abrunhosa *et al.*, 2014; Dalié *et al.*, 2010).

2.4.1. Ochratoxin A

Several studies reported the ability of some LAB to detoxify OTA. For example, some *Lactobacillus rhamnosus* strains were able to eliminate OTA by 36% to 76% depending on conditions (Turbic *et al.*, 2002). Fuchs *et al.* (2008) tested several *Lactobacillus* strains, with particular attention for *L. acidophilus* that caused decreases of OTA superiors to 95% in buffer solutions (pH 5.0) containing 0.5 and 1 mg·L^{-1} OTA when incubated at 37 °C for 4h. Also, Piotrowska and Zakowska (2005) demonstrated that *L. acidophilus* and *L. rhamnosus* caused OTA reductions of 70% and 87% on 1 mg·L^{-1} OTA culture medium after 5 days at 37 °C. Mateo *et al.* (2010) reported the capacity to eliminate OTA of *Oenococcus oeni*, having found reductions higher than 60% in culture medium containing 2 µg·L^{-1} OTA when incubated at 28 °C for 14 days. Also, Abrunhosa *et al.* (2014) demonstrated the ability of *Pediococcus parvulus* to eliminate between 72 to 100% of OTA present in MRS media supplement with 1 µg·L^{-1} through its biotransformation into OTα after an incubation period of 7 days at 30 °C.

Currently, the mainly mechanism involved in OTA detoxification by LAB is OTA adsorption to the cells walls. The involvement of cell-binding mechanisms was confirmed because OTA adsorbed by the cells was recovered from the bacteria pellets through extraction, crude cell-free extracts were not able to degrade OTA and degradation products were not detected. Studies evidencing adsorption effects, such as Piotrowska and Zakowska (2005) verified that significant levels of the OTA were present in the centrifuged bacteria cells. In addition, it was verified that heat and acid treated cells from LAB were more effective in removing OTA than viable cells (Mateo *et al.*, 2010; Turbic *et al.*, 2002). The chemistry and the molecular basis of mycotoxin binding is not yet fully understood. Limited literature suggests that the peptidoglycan part of the cell wall is involved in the surface binding of mycotoxin. The fact of the bacteria with heat and acid treatments being more effective in removing OTA is due to protein denaturation since it leads to the exposure of more hydrophobic surfaces (Dalié *et al.*, 2010; Shetty and Jespersen, 2006). However, some authors consider that metabolism may also be involved, because Fuchs *et al.* (2008) indicate that viable cells of *L. acidophilus* removed OTA more efficiently than unviable.

The binding between mycotoxins and LAB is of a reversible nature and the stability of the complexes formed depends on the bacterial strain, bacterial treatment and environmental conditions (Dalié *et al.*, 2010).

2.5. *Pediococcus parvulus*

Pediococcus parvulus is a gram-positive and catalase negative bacteria that forms pares or tetrads. *P. parvulus* is a facultative anaerobe cocci and homofermentative bacteria that produce lactic acid as the major end product. The glucose is transported into the pediococcal cell via a permease and undergoes glycolysis using the Embden-Meyerhorf pathway yielding pyruvate. The pyruvate is reduced to lactic acid with the complete oxidation of NADH to NAD. Since lactic acid is the only end product of glucose metabolism, two molecules of lactic acid are produced from one molecule of glucose (Fugelsang and Edwards, 2006; Gunther and White, 1961; Raccach, 1999). In addition, other hexoses such as fructose and maltose are also fermented by *P. parvulus*. These sugars enter the Embden-Meyerhorf pathway after isomeration or phosphorylation (Velasco *et al.*, 2007). *P. parvulus* have probiotic properties, which include cholesterol-lowering and immunomodulatory properties as a result of produced exopolysaccharides (EPS) (de Palencia *et al.*, 2009; Lindström *et al.*, 2013; Mårtensson *et al.*, 2005). The ESP, β-glucan, produced by *P. parvulus* play an important

role in the rheology, texture and consistency of fermented milks and other fermented products, being therefore of interest to the food industry (Velasco *et al.*, 2009; Vuyst, 2000). Like many other LAB, *P. parvulus* also produce bacteriocins, designated pediocins, which are responsible for inhibitory effects on microorganisms (Schneider *et al.*, 2006). In addition to the characteristics mentioned, the species has some antifungal effects and is a potential candidate in production of functional foods (Garai-Ibabe *et al.*, 2010; Magnusson *et al.*, 2003). Some strains are also able to biodegrade OTA (Abrunhosa *et al.*, 2014; Rodrigues, 2011).

2.6. Lactic acid bacteria growth

Batch and fed-batch fermentation strategies can be used to achieve high cell density and so to improve productivity of biomass, as well as of metabolites.

In batch operation, all nutrients and the inoculum are placed in a closed system, and no supply of substrate or removal of samples is made during the course of process. Basics controls for temperature, dissolved oxygen and pH are applied during the course of batch operation and are normally held constant. The batch operation does not require much supporting equipment compared to a continuous operation and is therefore used for small-scale operations, including experimental studies of reaction kinetics (Lim and Shin, 2013). The batch operation offers advantages as a low risk of contamination compared with fed-batch and continuous operations. However, the initial substrate concentration can have an effect of inhibition resulting on low biomass productivity (Ratledge and Hristiansen, 2006).

The fed-batch operation is the most common industrial process that achieves a high cell density, as well as metabolic products. Initially, a batch mode of operation is used and the fed-batch mode of operation starts when the culture are fed either intermittently or continuously via one or more feed streams, without removal of broth from reactor during the fermentation period. Fed-batch mode of operation enables control of substrate concentration in optimal range without inhibition effect of high initial substrate concentration, so it can be a promising strategy for intensification of biomass production (Lim and Shin, 2013). As mentioned above, a disadvantage of fed-batch process is that it is more susceptible to contamination and is relatively more labor intensive.

Studies evidence that biomass of LAB produced by fed-batch fermentation is higher than that achieved by batch fermentation. For example, Hwang *et al.* (2011) showed that biomass of *Lactobacillus plantarum* LP02 was significantly improved at 28h of fed-batch fermentation, 9.45 g

dry cell weight (DCW)·L^{-1}, over a constant feeding rate of 20 mL·L^{-1} of feeding solution. In batch fermentation, only 2.2 g DCW·L^{-1} was reached in a 5 L fermentor after the completely consumption of glucose. Guerra *et al.* (2005) demonstrate also that fed-batch culture is characterized by production of higher biomass yields of *Pediococcus acidilactici* (6.57 g·L^{-1}) compared with the batch process (<1.76 g·L^{-1}). Thus, fed-batch fermentation mode might be a promising strategy to increase process productivity.

In LAB production, the choice of medium is important, because LAB are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acids and vitamins (Zannini *et al.*, 2005). Especially, nitrogen sources play an important role on growth of LAB (Altaf *et al.*, 2007). Other factors can affect growth rates and biomass yields such as the temperature, pH and oxygen concentration (Zannini *et al.*, 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals and media

De Man-Rogosa-Sharpe (MRS) broth, MRS agar, tryptone and ´Lab-Lemco´ Powder (beef extract) were obtained from Oxoid (England). Tomato juice, D-(+)-glucose anhydrous, peptone, Edinburgh Minimal Medium (EMM) growth medium without dextrose were obtained from Himedia (India). Lactose and yeast extract used were obtained from Difco (USA) and Tween 80 and L-(+) cysteine hydrochloride monohydrate were purchased to from Fisher Chemical (USA). Sodium acetate tryhydrate ($CH_3COONa.3H_2O$), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) and sodium hydroxide were obtained from Merk (Germany). Manganese (II) sulphate monohydrate ($MnSO_4.H_2O$) and MES hydrate were purchased from Sigma. Aldrich (USA) and Sodium chloride (NaCl) came BHD Prolabo (France).

3.2. Microorganism

Pediococcus parvulus UTAD 473 is the microorganism used in these experiments. It was obtained from the UTAD-LAB collection. This LAB was isolated from red wines of the Douro region. Stock cultures of LAB were stored at -20 °C in 1 mL Eppendorf containing MRS medium and 20% (v/v) of glycerol.

3.3. Media composition and Batch cultures in flasks

3.3.1 Growth conditions

All experiments were carried out in 500 mL Erlenmeyer flasks containing 300 mL of medium on an orbital shaker (150 rpm) at 30 °C, until reaching the stationary phase. All medium cultures were sterilized at 121 °C for 20 minutes on autoclave.

Samples of 7 mL were taken aseptically every 3 hours (except in period of night, approximately during 12 hours) to determine cell growth, pH and the consumption of glucose and respective production of lactic acid. For chemical analysis, 1.5 mL of each sample was collected and centrifuged. The supernatant from centrifuged samples were filtered into a clean 2 mL vial using syringe filters with pore size 0.22 µm. Then the samples were preserved at -20 °C until HPLC

analysis. Additionally, at the end of fermentations it was collected aseptically a sample of 10 mL to test the ability of *P. parvulus* to degraded OTA and to determine the viability of bacteria.

3.3.2. Culture medium

The MRS, tryptone glucose extract (TGE) and glucose yeast peptone (GYP) sodium acetate minerals salts broth, which are universally used to cultivate different LAB strains (Table 3.1), were tested to evaluate the growth of *P. parvulus*. MRS broth was supplemented with 20% (w/v) Tomato juice, which improved the performance of MRS. To compare the effect of three culture medium, batch cultures were inoculated to reach an initial concentration of $1x10^7$ CFU·mL^{-1}.

Table 3.1 - Composition of MRS, TGE and GYP medium for cultivation of *P. parvulus*.

MRS		TGE		GYP	
Glucose	20.0 g·L^{-1}	Glucose	20.0 g·L^{-1}	Glucose	20.0 g·L^{-1}
`Lab-lemco` powder	8.0 g·L^{-1}	`Lab-lemco` powder	3.0 g·L^{-1}	Peptone	10.0 g·L^{-1}
Peptone	10.0 g·L^{-1}	Tryptone	5.0 g·L^{-1}	Yeast Extract	10.0 g·L^{-1}
Yeast Extract	4.0 g·L^{-1}			$CH_3COONa._3H_2O$	10.0 g·L^{-1}
$CH_3COONa._3H_2O$	5.0 g·L^{-1}			$FeSO_4.7H_2O$	10.0 mg·L^{-1}
$MgSO_4.7H_2O$	0.2 g·L^{-1}			$MgSO_4.7H_2O$	0.2 g·L^{-1}
$MnSO_4.H_2O$	0.05 g·L^{-1}			$MnSO_4.H_2O$	10.0 mg·L^{-1}
$C_6H_{17}N_3O_7$	2.0 g·L^{-1}			NaCl	10.0 mg·L^{-1}
K_2HPO_4	2.0 g·L^{-1}				
Tween 80	1.0 mL·L^{-1}				
Tomato juice	20% (w/v)				

3.3.3. Effect of carbon source

In order to verify the influence of carbon source on *P. parvulus* growth, two monosaccharides were tested, lactose and glucose. To compare the effect of glucose and lactose, two MRS cultures were prepared containing 20 g·L^{-1} of respective carbon source. The cultures were inoculated to reach an initial concentration of $1x10^7$ CFU·mL^{-1}.

3.3.4. Effect of temperature, glucose, tomato juice and beef extract

In order to characterize how some factors affect *P. parvulus* growth, the composition of the basal medium culture (MRS) was optimized by comparing different levels of factors. The factors tested were glucose, tomato juice, beef extract and temperature. The levels of factors used in the experimental design are listed in Table 3.2. Nine experiments were performed according Table 3.3, which also shows the levels of the factors for each experiment. All culture media were inoculated to reach an initial concentration of $1x10^7$ CFU·mL^{-1}. And the temperature of each experiment was different as indicated in Table 3.3. The experimental design were performed using a Taguchi L9 orthogonal array with Qualitek-4 software (Nutek, Bloomfield Hills, USA).

Table 3.2 - Levels of temperature, glucose, tomato juice and beef extract used in the experimental design.

	Factor	Level 1	Level 2	Level 3
1	Temperature (°C)	30	35	37
2	Glucose (g·L^{-1})	10	20	30
3	Tomato juice (%w/v)	10	20	30
4	Beef extract (g·L^{-1})	5	10	20

Table 3.3 - Experimental design.

Experiment	Temperature (°C)	Glucose (g·L^{-1})	Tomato juice (w/v%)	Beef extract (g·L^{-1})
I	30	10	10	5
II	30	20	20	10
III	30	30	30	20
IV	35	10	20	20
V	35	20	30	5
VI	35	30	10	10
VII	37	10	30	10
VIII	37	20	10	20
IX	37	30	20	5

3.3.5. Effect of different factors

In addition to the factors already tested on *P. parvulus* growth, the individual influence of nutrients peptone, Tween 80, $FeSO_4.7H_2O$, cysteine, yeast extract and minerals salts (EMM) supplementation in MRS broth was studied. The influence of these factors was tested by preparing batch cultures on flasks with MRS broth supplemented independently with the following: 10.0 $g{\cdot}L^{-1}$ peptone; 2.0 $g{\cdot}L^{-1}$ Tween 80; 0.01 $g{\cdot}L^{-1}$ $FeSO_4.7H_2O$; 1.0 $g{\cdot}L^{-1}$ L cysteine; 6.0 $g{\cdot}L^{-1}$ yeast extract and 12.35 $g{\cdot}L^{-1}$ EMM, respectively. Furthermore, initial pH of MRS broth and the buffering effect were evaluated. For that, batch cultures with MRS broth with a pH adjusted to 4.2 and 5.2 by adding HCl 37% were prepared. The buffer effect was studied by preparing MRS in MES-NaOH 0.1 M buffer (pH6.4). MES-NaOH 0.1 M buffer was prepared by mixing 150 mL of MES 0.1 M and 77.4 mL of NaOH 0.1 M, which were diluted to a total of 300 mL and by adjusting pH to 6.4 with HCl (6N). All flasks batch cultures were inoculated to reach an initial concentration of $1x10^6$ $CFU{\cdot}mL^{-1}$.

3.4. Batch and Fed-batch cultures in bioreactor

3.4.1. Biolab bioreactor

To conduct batch and fed-batch fermentations a bioreactor (Biolab, B. Braun, Germany) was used. The equipment comprised a glass vase with a maximum capacity of 2 L, an agitator with two turbine of six blades, a disperser to promote aeration, a motor with controller stirring speed and a unit of measurement and temperature control (FerMarc 240, Electrolab, United Kindgom), whose control is done with a heating mantle.

For measurement of pH we used a pH electrode (Mettler Toledo, Switzerland) connected to a pH controller (FerMac 260 Electrolab, United Kingdom). For measurement oxygen concentration an oxygen probe (Mettler Toledo, Switzerland) connected to an external meter (Mettler Toledo, Switzerland), whose signals were acquired by a LABtech Notebook software (Datalab Solution, USA) was used.

The bioreactor with culture medium was sterilized on autoclave at 110 °C during 30 minutes. The temperature meter, oxygen probe and pH electrode were disinfected separately with a 70% (v/v) ethanol solution. The calibration of oxygen probe was performed before being disinfected.

Figure 3. 1 - Photography of Biolab bioreactor.

3.4.2. Growth conditions

. Inocula for these experiments were prepared by transferring 1 mL stock culture to 70 mL of medium in a 250 mL Erlenmeyer flasks. Inocula were incubated at 30 °C for 24 hours on an orbital shaker before bioreactor inoculation at 10% of total volume, with a final concentration approximately of $1x10^{10}$ CFU·mL^{-1}. The medium for inoculum preparation or cell growth was MRS broth (glucose concentration at 20 g·L^{-1}) supplemented with 20% (w/v) tomato juice and 1.0 g·L^{-1} L-(+) cysteine.

All experiments were carried out in a 2 L bioreactor Biolab at 30 °C with a stirring speed of 150 rpm. The culture pH was maintained at 5.2 by addition of NaOH 5 M. Samples of 4 mL were collected every 3 hours (except during the night period, approximately during 12 hours) and were subsequently analysed to determine cell growth, consumption of glucose and production of lactic acid. At the end of fermentations, an additional sample of 10 mL was taken to analyse the degradation of OTA and to determine bacteria viability.

3.4.3. Batch cultures

Two batch cultures of 49 hours (Batch-I and Batch-II) were performed in the bioreactor using an initial culture media volume of 700 mL. In Batch-I the pH was not controlled, being possible to follow the change of pH with the *P. parvulus* growth. A third batch culture (Batch-III) was carried out but with MRS broth with a glucose concentration of 60 g·L^{-1}.

Additionally, two successive batch cultures (Batch-IV and Batch-V) were also performed in the bioreactor. For each of them, the process was initiated with a batch culture with an initial culture media volume of 600 mL. After 24 hours, the entire medium was collected from the bioreactor to

an Erlenmeyer flask of 1 L using a peristaltic pump. The medium was divided into 250 mL centrifuge tubes and centrifuged (4053 xg, 15 minutes at 20 °C). Supernatants were discarded and the pellet was resuspended in 600 mL of MRS broth with a glucose concentration of 60 $g \cdot L^{-1}$. This medium was added to the bioreactor and a second batch culture (Batch-V) was carried for 48 hours. The bacteria recycling process was done in aseptic conditions.

3.4.4. Fed-Batch cultures

All fed-batch cultures were preceded by a batch culture using MRS with an initial glucose concentration of 20 $g \cdot L^{-1}$.

In the first fed-batch experiment (Fed-I), feeding was started after 30 hours of batch culture. The initial volume of batch was 600 mL. The feeding medium, composed of 600 mL MRS broth with a glucose concentration of 100 $g \cdot L^{-1}$ and supplemented with 20% tomato juice and 1.0 $g \cdot L^{-1}$ cysteine, was pumped to the bioreactor at a constant feeding flow rate (F) of 18 $mL \cdot h^{-1}$ using a peristaltic pump (Reglo Analog, Switzerland). In a constant feeding flow rate, the dilution rate (D) varies according to the equation 3.1:

$$D = \frac{F}{V} \qquad \text{(Equation 3. 1)}$$

where D is the dilution rate (h^{-1}), F is the flow rate ($mL \cdot h^{-1}$) and V is the volume of medium in bioreactor (mL).

Glucose was added to the bioreactor with a substrate feeding rate of 2.6 $g \cdot L^{-1} \cdot h^{-1}$, which was calculated according to the equation 3.2:

$$D.S_o = qs.X \qquad \text{(equation 3. 2)}$$

where, D is the dilution rate (h^{-1}), S_o is the substrate concentration in feed solution ($g \cdot L^{-1}$), q_s is the specific substrate consumption rate ($g \cdot g^{-1} \cdot h^{-1}$) and X is the biomass concentration ($g \cdot L^{-1}$).

In the second fed-batch experiment (Fed-II), the initial broth volume of batch culture was at 600 mL. After 24 hours, the medium was collected from bioreactor, centrifuged as mentioned above for Batch IV and resuspended in 600 mL MRS broth without glucose and supplemented with 20% tomato juice and 1.0 $g \cdot L^{-1}$ cysteine. Then this medium was added to the bioreactor and a constant feeding flow rate of 18 $mL \cdot h^{-1}$ was set. The feeding solution consisted in 600 mL of MRS broth, tomato juice and cysteine concentrated five times. The fed-batch culture ended after 30 hours of feed.

The third fed-batch experiment (Fed-III) was identical to second fed-batch, but two successive fed-batch cultures were performed. The initial batch culture was performed as in Fed-II. Two successive fed-batch cultures were carried out after the batch culture as done for Fed-II experiment. Each fed-batch culture takes 26 hours to complete.

3.5. Analytical methods

3.5.1. Cell dry weight

To determine cell concentration the optical density (OD) was measured using a microplate ELISA reader (Synergy HT, Biotech, USA) at a wavelength of 600 nm and converted to cell dry weight ($g \cdot L^{-1}$) with a calibration curve.

The biomass calibration curve was prepared from a cell suspension with an OD of 2. Using this cell suspension, several successive dilutions were prepared (1:2 to 1:128) and their OD read. Then, 10 mL of each dilution were vacuum filtered using an across membrane (RC, 0.2 µm), washed with 5 mL of distilled water, dried at 105 °C for 24 hours and weighed. The same membranes were previously weighed after being dried as described. The dry weight was calculated as the difference between the initial and final weight of the membrane. The calibration curve was thus obtained by graphical representation of OD in terms of dry weight (Annexe A.1).

3.5.2 pH

The samples pH of flasks batch cultures was read using a digital bench top pH meter (Sentek Model 922).

3.5.3. Glucose and lactic acid concentration

Glucose and lactic acid concentration were quantified using a High-Performance Liquid Chromatography (HPLC) with Refractive Index (RI) detection. HPLC system was comprised of a Jasco 880-PU pump, a Jasco AS-2057 Plus autosampler, a K-2300 Knauer RI detector and an Eldex CH-150 column heater. The instrument and the chromatographic data were managed by a Varian Star 800 data system interface and a Star Workstation chromatography data system, respectively. The chromatographic separation was performed on a MetaCarb 67H column (300 mm x 6.5 mm) for a 20 min isocratic run. The mobile phase was 5 mM H_2SO_4 that was previously filtered (GHP, 0.2 µm)

and degassed. The flow rate was 0.7 mL·min^{-1} and the column temperature was maintained at 60 °C. The injection volume was 20 µL.

Glucose and lactic acid quantification was carried out by comparing area of peaks with respective calibration curves prepared with concentrations of 0.5 g·L^{-1} to 40.0 g·L^{-1} (Annexe A.2).

3.5.4. Cell viability

Cell viability was determined by plating serially dilutions of samples in MRS agar plates. Number of viable *P. parvulus* (CFU·mL^{-1}) was estimated by counting the number of colony forming unit (CFU) formed after an incubation period of 96 hours at 30 °C.

3.5.5. Biodegradation of OTA

To test the ability of *P. parvulus* to degrade OTA into OTα, 5 mL MRS broth supplemented with 1 µg·mL^{-1} of OTA (MRS-OTA) was prepared. The 10 mL sample taken at the end of each experiment was centrifuged and the pellet was resuspended with 5 mL MRS-OTA. Tubes were incubated at 30 °C for 5 days with periodic agitation (once a day). After the incubation period, 5 mL of acetonitrile/methanol/acetic acid (78:20:2, v.v.v) was directly added and vortexed for 1 minute. A 2 mL sample was collected and filtered into a clean 2 mL vial using a syringe filter (PP, 0.45 µm). Samples were preserved at -20 °C until HPLC analysis.

OTA was analysed by HPLC with fluorescence detection. HPLC system was comprised of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler and a Jasco FP-920 fluorescence detector (λ_{exc}=333 nm and λ_{em}=460 nm; gain=100). The instrument and the chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively. The chromatographic separation was performed on a C_{18} reversed phase YMC-Pack ODS-AQ analytical column (250 mm x 4.6 mm, I.D. 5 µm) that was fitted with a pre-column with the same stationary phase. The compounds were eluted using acetonitrile/water/acetic acid (99:99:2) at a flow rate of 0.8 mL·min^{-1} for a 21 min isocratic run. The injection volume was 50 µL and the column temperature was maintained at 35 °C. The mobile phase was previously filtered (GHP, 0.2 µm) and degassed.

OTA standards with 0.5 - 7.5 µg·mL^{-1} were prepared by serially diluting a primary OTA stock solution (25 µg·mL^{-1}) and used to elaborate the calibration curve. OTA quantification was performed

by measuring the peak area and by comparing it to the respective OTA calibration curve. OTα was quantified in equivalents of OTA.

3.6. Kinetic parameters calculations

The kinetics parameters were estimated after treatment of experimental data. For batch and fed-batch cultures, the specific growth rate (μ), the specific substrate uptake rate (q_s) and the biomass yield ($Y_{x/s}$) were calculated according the equations shown in Table 3.4.

In batch culture the maximum specific growth rate (μ_{max}) was determined from the slope of the line ln (x) vs time, whose line equation results of integration of equation of μ.

Table 3.4 – Equations used in determination of the specific growth rate (μ), specific substrate uptake rate (q_s) and biomass yield ($Y_{x/s}$) in batch and fed-batch culture. D – dilution rate (h^{-1}); dS/dt – Substrate consumption rate ($g \cdot L^{-1} \cdot h^{-1}$); dX/dt – Biomass production rate ($g \cdot L^{-1} \cdot h^{-1}$); F – flow rate ($L \cdot h^{-1}$); S_o – substrate concentration in feed solution ($g \cdot L^{-1}$); t – time (h); Vi – volume of medium at initial of fed-batch culture (L); Vf – volume of medium at the end of fed-batch culture (L); X – Biomass concentration ($g \cdot L^{-1}$); ΔX – Difference between final biomass concentration (Xf) and initial biomass concentration (Xi); ΔS – Difference between initial concentration of glucose (Si) and final glucose concentration (Sf).

	Batch culture	Fed-batch culture
μ (h^{-1})	$1/X\ (dX/dt)$	$1/X\ (dX/dt) + D$
q_S ($g \cdot g^{-1} \cdot h^{-1}$)	$\mu / Y_{x/s}$	$D(S_o-S)/X - 1/X\ (dS/dt)$
$Y_{x/s}$ ($g \cdot g^{-1}$)	$\Delta X /(-\Delta S) = (Xf - Xi)/(Si - Sf)$	$(Xf \cdot Vf - Xi \cdot Vi) /(F \cdot S_o \cdot t + Si \cdot Vi - Sf \cdot Vf)$

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Batch culture in flasks

4.1.1. Culture medium

Culture media MRS, TGE and GYP are all used for cultivation of LAB. The MRS medium is used for the general cultivation of LAB (Atlas, 1996). TGE medium is recommended for the general cultivation and enumeration of bacteria (Atlas, 1996), while GYP is used for cultivation of *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Pediococcus acidilatici* and *Pediococcus pentosaceus* (Atlas, 1996; Tanasupawat and Daengsubha, 1983; Tanasupawat *et al.*, 1992).

Cultivation of *P. parvulus* was initially carried out using MRS, TGE and GYP to verify which of these media had more effect on cell growth. The cellular growth was monitored in the three media for 97 hours and the results are presented in Table 4.1.

Table 4.1 - Values of final biomass, maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), specific substrate uptake rate (q_s) and final lactic acid concentration for MRS, TGE and GYP batch cultures in flasks.

	Biomass ($g \cdot L^{-1}$)	μ_{max} (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)	q_s ($g \cdot g^{-1} \cdot h^{-1}$)	Lactic acid ($g \cdot L^{-1}$)
MRS	0.78	0.13	0.07	1.70	21.59
TGE	0.03	0.01	0.04	0.14	1.57
GYP	0.06	0.01	0.03	0.41	5.13

As shown in Table 4.1, it was verified that MRS is the best medium for *P. parvulus* growth, since with this medium the cells grew and consumed the substrate faster, leading to highest cellular and lactic acid final concentrations. Moreover, the conversion yield of substrate to biomass was higher in MRS than in other medium tested. Therefore, MRS was the medium chosen for the cultivation of *P. parvulus* in subsequent studies.

The cell growth, substrate and product kinetics and the changes of pH during the cultivation in MRS are shown in Figure 4.1. The cell grew with a maximum specific growth rate of 0.13 h^{-1} reaching a maximum biomass concentration of 0.81 $g \cdot L^{-1}$ after 69 hours. After this point, cells entered in the stationary phase and cell growth was practically inexistent until the end of fermentation, which finished with a biomass concentration of 0.78 $g \cdot L^{-1}$. The exponential phase was preceded of a lag phase of 21 hours, which can be explained by the low size of the inoculum used. The glucose concentration decreased gradually with a specific uptake rate of 1.70 $g \cdot g^{-1} \cdot h^{-1}$.

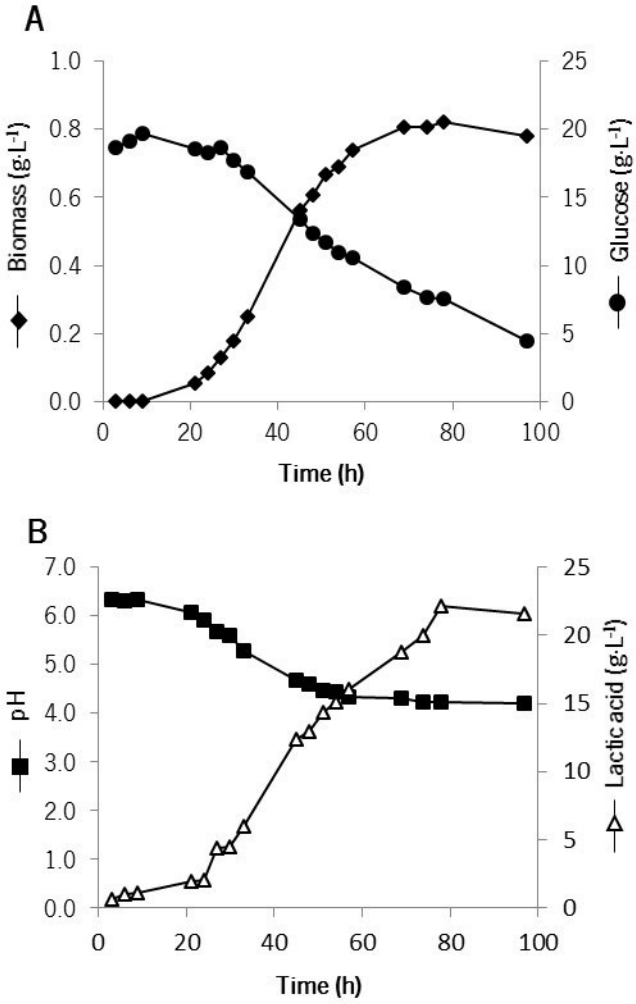

Figure 4.1 - (A) *P. parvulus* growth and glucose consumption, (B) lactic acid production and pH change during cultivation on MRS batch culture in flasks.

Furthermore, during cells growth lactic acid was produced until it reached a final concentration of 21.59 $g \cdot L^{-1}$. With the production of lactic acid, a decrease in culture pH was observed. As shows in Figure 4.1.B, the pH of the culture, which initially was 6.3, decreased gradually until it reaches 4.2. The decreased of culture pH was more significant during the exponential growth phase, between 21 and 57 hours.

For this experiment, the number of viable cells obtained was 2.6×10^9 $CFU \cdot mL^{-1}$ and the percentage of OTA eliminated by *P.* parvulus after a 5 days cultivation period in MRS-OTA was of 82%.

P. parvulus grows better in MRS medium since it is a complex nutritional medium with all the nutritional requirements need for LAB normal growth and metabolic activity. The MRS medium contain glucose that is the main source of carbon and energy. From this carbohydrate, LAB can

obtained their energy by substrate phosphorylation and can produced lactic acid by homofermentative pathway that is based on glycolysis (Wright and Axelsson, 2012). In addition to glucose, MRS medium contains different nitrogen sources as beef extract (´Lab-Lemco´ Powder), peptone and yeast extract. These nitrogen sources are known to contain a wide range of amino acids and peptides that can satisfy requirements of most LAB strains. They can also be sources of carbon, minerals and vitamins (van Niel and Hahn-Hägerdal, 1999). The magnesium and manganese sulphates provide metals ions, Mg^{2+} and Mn^{2+}, which play an important role in the growth and metabolic activity of LAB (Fitzpatrick *et al.*, 2001; Hébert *et al.*, 2004). Tween 80 provides fatty acids need to LAB growth (Corcoran *et al.*, 2007). Sodium acetate, triammonium citrate and dipotassium phosphate are commonly used in LAB media as buffering agents. These are included in the MRS because LAB produced lactic acid during growth, which decrease pH and consequently leads to a slower growth. Finally, the MRS was supplemented with tomato juice to improve LAB growth. The tomato juice acts as a carbon source, minerals and vitamins of the B complex of which stimulate the growth of LAB (Fugelsang and Edwards, 2006). Yang *et al.* (2007) showed that LAB grow better in MRS contained tomato juice compared with the other broths, suggesting that tomato juice favours the reproduction of LAB. For example, in work performed by Saguir *et al.* (2009) LAB strains were grown in MRS with tomato juice 15%.

The simple broth TGE had not the nutritional requirements needed for *P. parvulus* growth, since only a biomass of 0.03 $g \cdot L^{-1}$ was achieved. According to Altuntas *et al.* (2010), *Pediococcus acidilactic* is able to growth in TGE medium, however they supplement TGE with other micronutrients and Tween 80.

In GYP medium, *P. parvulus* growth reached a maximum biomass of 0.06 $g \cdot L^{-1}$. Although the composition of GYP medium is more complete at the level of nutritional requirements, this medium does not appear to promote *P. parvulus* growth sufficiently to obtain higher amounts of biomass.

4.1.2. Carbon source

In order to evaluate the effect of carbon source on *P. parvulus* growth, cultivation of bacteria was carried out using MRS broth in which glucose was replaced by lactose (MRS-lactose). Figure 4.2 shows the cell growth, substrate and product kinetics for this experiment.

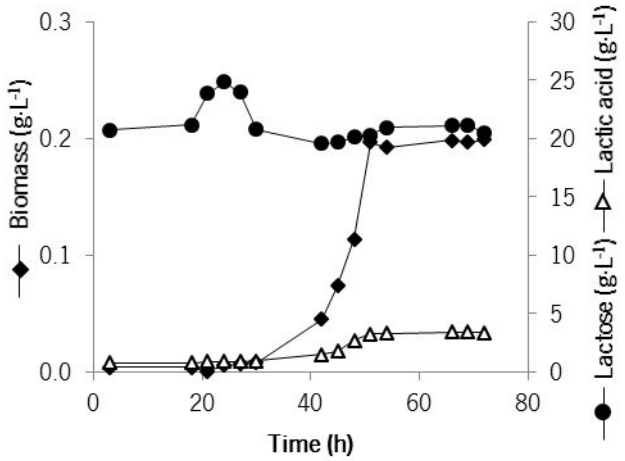

Figure 4.2 – *P. parvulus* growth, lactose and lactic acid kinetics during cultivation on MRS contained 20 g·L^{-1} lactose.

In this experiment it was verified a long lag phase of approximately 40 hours. This may have been due to the necessary adaptation of cells to medium or to unavailability of metabolize sugars. The cells grew with maximum specific growth rate of 0.14 h^{-1} and reached its maximum value of 0.20 g·L^{-1}.

Comparing with the cell growth on MRS containing glucose (MRS-glucose), it can be verified that the maximum cell concentration was greater than biomass obtained in MRS-lactose. This slight growth may have been due to the presence of some residual glucose in medium components like tomato juice and beef extract. The fact that lactose concentration and pH remains constant throughout the cultivation, reinforces that *P. parvulus* do not metabolize lactose. Indeed *P. parvulus* cannot use lactose as carbon source and the acid lactic is produced from glucose, carbon source commonly preferred by a larger number of LAB strain (Fugelsang and Edwards, 2006; Sheeladevi and Ramanathan, 2011; Velasco *et al.*, 2007; Walling *et al.*, 2005).

The number of viable cells obtained was of 9.9x10^{7} CFU·mL^{-1} and *P.* parvulus only eliminated 42% of OTA from MRS-OTA after 5 days of cultivation. According Abrunhosa *et al.* (2014), the OTA biodegradation rate by *P. parvulus* is dependent of the concentration of inoculum. So the inoculum size and the loss of viability during the cultivation period may have affected the ability to biodegrade OTA and explain the percentage of OTA eliminate in this experiment

4.1.3. Temperature, glucose, tomato juice and beef extract effects

The temperature, glucose, tomato juice and beef extract were chosen as factors for further optimization studies and were assigned for each one of three levels, according to Table 3. 2. In Table

4.2, the final biomass obtained in the experiments designed with Taguchi L-9 orthogonal array is show.

Table 4.2 – Final biomass concentration obtained in the experiments designed using Taguchi L-9 orthogonal array.

Experiment	Biomass ($g \cdot L^{-1}$)
I	0.84
II	0.87
III	0.62
IV	0.62
V	0.69
VI	0.57
VII	0.13
VIII	0.11
IX	0.03

The maximum cell mass concentration was obtained in experiment II with 20 $g \cdot L^{-1}$ glucose, 20% tomato juice, 10 $g \cdot L^{-1}$ beef extract and 30 °C of temperature. The biomass obtained in each experiment ranged according to the effect of factors combination but also according to the individual influence of each factor under study.

The obtained experimental data was processed in the Qualitek-4 software with the bigger is better quality characteristics to identify the individual influence of each factor on the biomass production (Figure 4.3).

The difference between average value of each factor at higher and lower level indicated the relative influence of factor. The temperature was the factor with more influence on the biomass production. Its highest effect was observed at level 1 (30 °C) with the maximum biomass of 0.78 $g \cdot L^{-1}$. The other factors showed to be less influent in the biomass production.

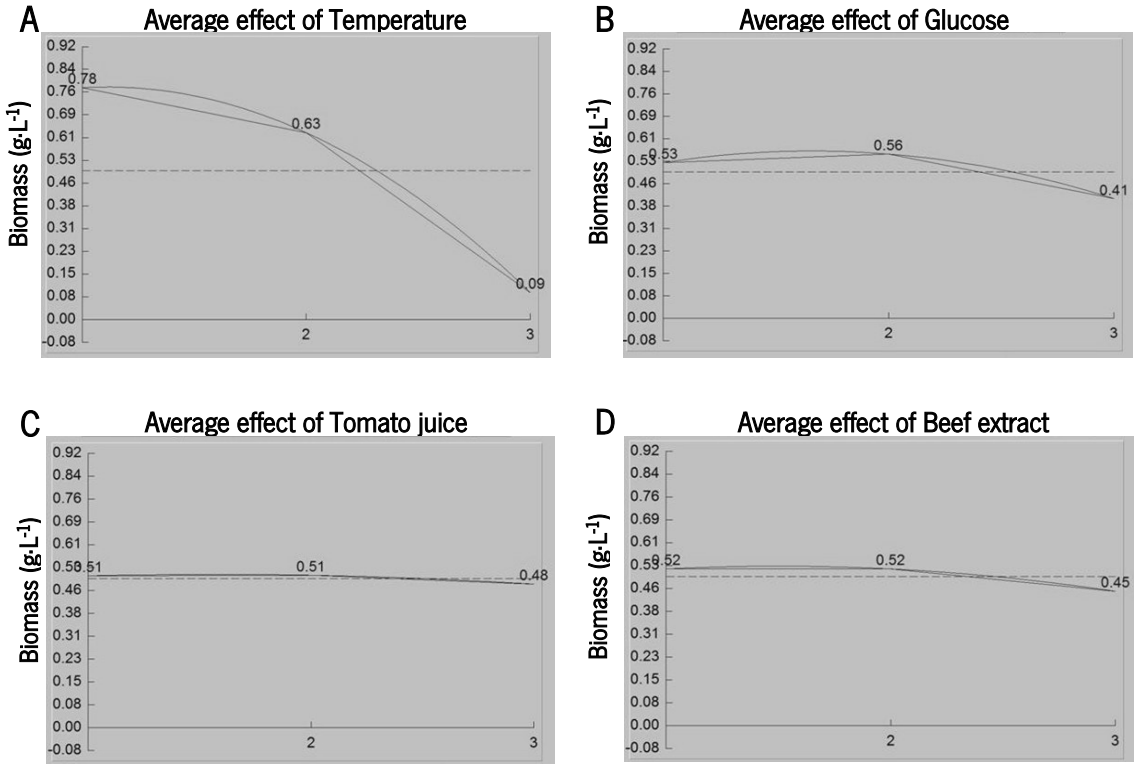

Figure 4.3 – Effect of of (A) temperature, (B) glucose, (C) tomato juice and (D) beef extract at selected levels on biomass production. Assigned levels 1, 2 and 3 are described in Table 3.2.

Environmental factors can influence the normal growth and metabolic activity of LAB, as temperature and pH (Velasco *et al.*, 2006; Zhang *et al.*, 2012). However, there is an optimum growth temperature for each LAB strain and temperature of 30 °C corresponds to optimum growth temperature of *P. parvulus* (Gunther and White, 1961).

The medium constituents, as glucose, tomato juice and beef extract can also influence the bacteria growth. According Walling *et al.* (2005), higher initial glucose concentrations stimulate exopolysaccharide production by improving *P. damnosus* IOEB8801 growth. However, for the glucose concentrations tested no significant influence on *P. parvulus* growth was observed in this experiment. Similarly, it was not found a significant impact of tomato juice and beef extract on growth at concentration used. Nevertheless, both are nutrients sources, containing peptides and free amino acids in addition to essential growth factors such as mineral and vitamins which may satisfy requirements of LAB and can stimulate the growth (Babu *et al.*, 1992; Fugelsang and Edwards, 2006; Seesuriyachan *et al.*, 2011). According to Seesuriyachan *et al.* (2011), biomass production by *Lactobacillus confusus* is not affected by the presence of beef extract. In turn, Walling *et al.* (2005)

and Dueñas *et al.* (2003) describe that the concentrations of nitrogen sources have a significant effect on biomass levels.

In all experiments, it was observed the ability to biodegrade OTA by *P. parvulus*. The bacteria was able to eliminate between 60 and 74% of OTA from MRS-OTA after a 5 day cultivation period.

4.1.4. Other factors

The individual influence of some nutrients in MRS was also tested. Batch cultures cultivation of *P. parvulus* were carried out using MRS supplemented independently with each factor mentioned in a two steps experiment. In the first step, the nutrients peptone, Tween 80, MES-NaOH, L-(+) cysteine and yeast extract were tested. In the second step, $FeSO_4.7H_2O$, minerals salts (EMM) and the initial pH were the factors studied. In Table 4.3 and 4.4, main results obtained for each experiment are presented. The results show maximum biomass produced, maximum specific growth rate, biomass yield, specific substrate uptake rate, final lactic acid concentration, number of viable bacteria and the percentage of OTA eliminate by *P. parvulus*.

Table 4.3 - Values of final biomass, maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), specific substrate uptake rate (q_s), final lactic acid concentration, cell viability and percentage of OTA eliminated for MRS medium supplemented with: 10.0 g·L^{-1} peptone; 2.0 g·L^{-1} Tween 80; MRS diluted in MES-NaOH; 1.0 g·L^{-1} L-(+) cysteine and 6.0 g·L^{-1} yeast extract, respectively. MRS medium without supplement is used as control.

	Biomass (g·L^{-1})	μ_{max} (h^{-1})	$Y_{x/s}$ (g·g^{-1})	q_s (g·g^{-1}·h^{-1})	Lactic acid (g·L^{-1})	CFU·mL^{-1}	OTA (%)
MRS	0.39	0.08	0.06	1.29	14.60	3.6x10^8	58
Peptone	0.58	0.09	0.05	1.64	20.23	8.1x10^8	64
Tween 80	0.39	0.08	0.06	1.37	14.62	3.5x10^8	56
MES-NaOH	0.38	0.06	0.05	1.33	16.26	2.6x10^8	50
L-(+) cysteine	1.02	0.12	0.08	1.47	23.90	4.1x10^8	70
Yeast extract	0.43	0.09	0.07	1.15	16.12	5.0x10^8	60

Table 4.4 - Values of final biomass, maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), specific substrate uptake rate (q_s), final lactic acid concentration, cell viability and percentage of OTA eliminated for MRS medium supplemented with: 0.01 g·L^{-1} $FeSO_4.7H_2O$; 12.35 g·L^{-1} EMM; initial pH 5.2 and pH 4.2, respectively. MRS medium without supplement is used as control.

	Biomass (g·L^{-1})	μ_{max} (h^{-1})	$Y_{x/s}$ (g·g^{-1})	q_s (g·g^{-1}·h^{-1})	Lactic acid (g·L^{-1})	CFU·mL^{-1}	OTA (%)
MRS	0.31	0.06	0.08	0.81	9.82	1.8x10^8	60
$FeSO_4.7H_2O$	0.30	0.06	0.07	0.79	10.14	1.2x10^8	60
EMM	0.22	0.06	0.14	0.46	8.18	6.4x10^8	70
pH 5.2	0.35	0.07	0.05	1.27	10.08	3.5x10^8	66
pH 4.2	0.08	0.03	0.00	0.00	3.43	7.0x10^7	78

Analysing the results of Table 4.3, it is possible to conclude that L-(+) cysteine has a significant impact on *P. parvulus* growth, since the highest biomass concentration and specific growth rate were obtained when MRS medium was supplemented with this amino acid. In this case, the lag phase was shorter than in the control experiment, supporting further the benefits of using L-(+) cysteine. This result was in accordance with several reports, where the growth of *Leuconostoc*, *Pediococcus* and *Lactobacillus* strains was improved with L-(+) cysteine (Dicks and Endo, 2009; Garai-Ibabe *et al.*, 2010; Hwang *et al.*, 2011). L-(+) cysteine favoured the growth of some LAB strains, because it is an amino acid containing sulphur which can acts as a source of amino nitrogen for fastidious microorganisms as LAB (Dave and Shah, 1997).

The peptone and yeast extract also had a positive effect on *P. parvulus* growth, since higher cells concentration and specific maximum growth rate were also obtained relatively to control. Several reports describe the effect of these nitrogen sources on the LAB growth. Seesuriyachan *et al.* (2011) showed that *Lactobacillus confusus* growth does not suffer a significant impact when peptone was supplied from 0 to 10 g·L^{-1} into to medium. However, they also observed that cell growth was enhanced by adding yeast extract. Additionally, with *Pediococcus damnosus* a higher growth rate was observed in MRS supplemented with 1.7% bacteriological peptone than with MRS with 2.5% yeast extract, but in both, similar final OD was obtained (Nel *et al.*, 2001).

Addition of 2 g·L^{-1} of Tween 80 in MRS had no impact on *P. parvulus* growth. This observation is consistent with Nel *et al.* (2001), which reported that more than 1% (v/v) of Tween 80 result in a slight decrease in *P. damnosus* growth. The buffer effect intended with the addition of MES-NaOH was not achieved, since the lactic acid had during the fermentation resulted in a pH decrease.

In Table 4.4, it can be verified that EMM and the initial pH of 4.2 had a negative effect in bacteria growth. According to some authors, the pH of culture media may have an important effect on the metabolic activity of LAB and pH 4.0 can significantly reduce their growth rate (Velasco *et al.*, 2006). The addition of 12.35 $g \cdot L^{-1}$ EMM to MRS medium was studied because this supplement provides minerals and trace elements that could stimulate the bacteria growth, but this effect was not observed.

In all experiments, *P. parvulus* showed the ability to biodegrade OTA with a percentage of OTA elimination ranging from 58 to 78%.

4.2. Batch and Fed-batch cultures in 2 L bioreactor

According to the results obtained in flasks, the culture medium selected for the cultivation of *P. parvulus* was MRS medium supplemented with 1.0 $g \cdot L^{-1}$ of L-(+) cysteine.

In bioreactor, it was possible to evaluate the effect of pH control on the kinetics of cell growth and cellular metabolism in regard to glucose consumption and lactic acid production. Cultivations were carried out using MRS medium with L-(+) cysteine under uncontrolled (Batch-I) and controlled pH (Batch-II) conditions. Figure 4.4 represents the kinetic of *P. parvulus* growth, the glucose concentration, lactic acid production and the changes of pH in cultivations under uncontrolled and controlled pH conditions, respectively.

The bacteria grew exponentially in both cultures with different rates without any significant lag phase. Cells grew with maximum specific growth rate of 0.09 h^{-1} and 0.12 h^{-1} for uncontrolled and controlled pH cultures, respectively. In controlled pH culture, the maximum biomass obtained was 1.14 $g \cdot L^{-1}$ and the biomass yield was 0.05 $g \cdot g^{-1}$, while in uncontrolled pH culture the maximum biomass was only 0.78 $g \cdot L^{-1}$ and the biomass yield obtained was 0.06 $g \cdot L^{-1}$. In uncontrolled pH culture, the glucose concentration decreased gradually with specific substrate uptake rate of 1.60 $g \cdot g^{-1} \cdot h^{-1}$, the decreased of pH was observed as a result of lactic acid production, which reached a final concentration of 17.27 $g \cdot L^{-1}$ of lactic acid. However, in controlled pH culture the glucose was completely consumed after 28 hours, with a specific substrate uptake rate of 2.65 $g \cdot g^{-1} \cdot h^{-1}$ and more lactic acid was produced (23.57 $g \cdot L^{-1}$) comparing to uncontrolled pH culture.

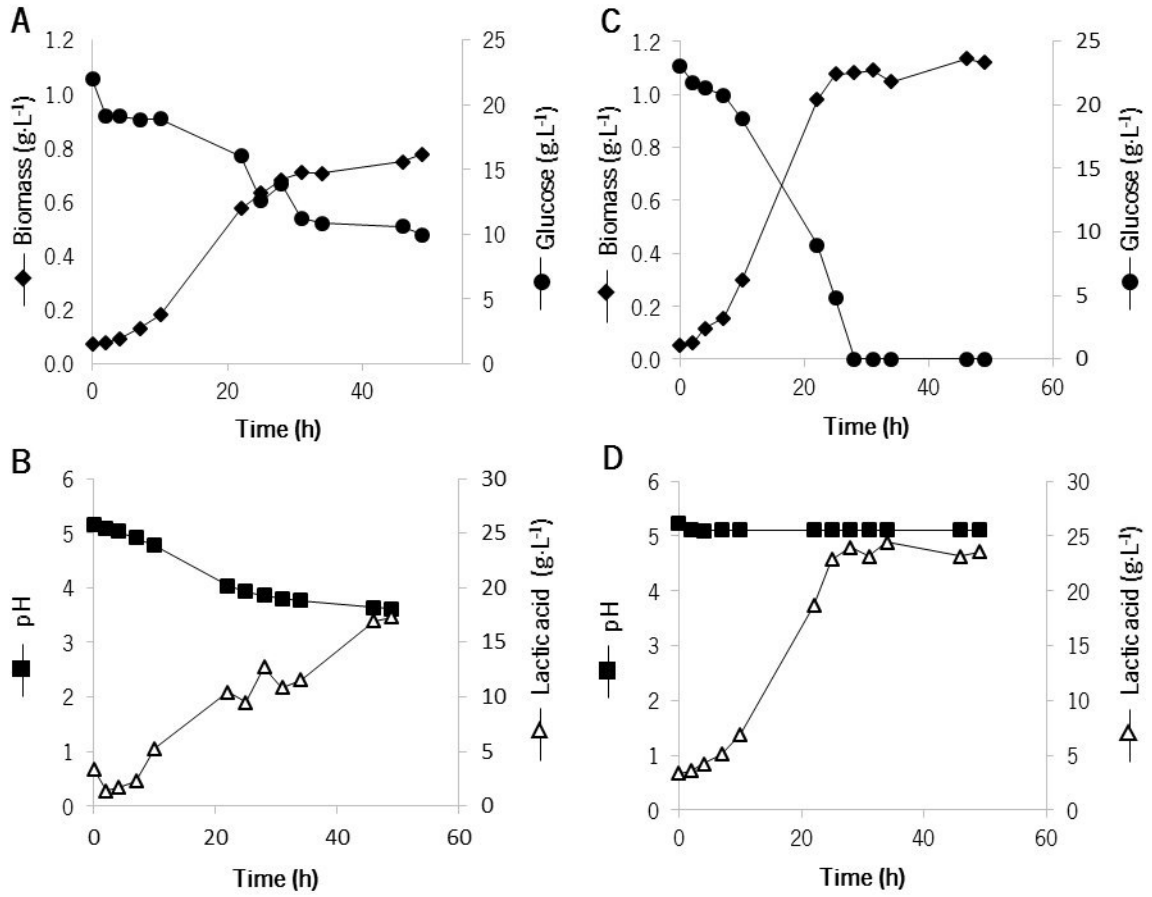

Figure 4.4 - *P. parvulus* growth, glucose consumption, lactic acid production and change in pH during cultivation in Biolab bioreactor at uncontrolled (A and B) and controlled (C and D) pH.

From these results, it is possible to conclude that controlled pH condition was more favourable for biomass production. According Velasco *et al.* (2006), the higher biomass yields of *P. parvulus* is obtained when the cultures were pH controlled at 5.2. This was verified in this experiment and it was also observed a positive effect in cellular metabolism. As results of controlled pH, better metabolic activity of glucose consumption and lactic acid production was observed, because the glucose was completely consumed and was produced more lactic acid compared to uncontrolled pH culture.

In uncontrolled and controlled pH conditions, the number of viable cells obtained was of 4.70×10^8 CFU·mL^{-1} and 3.80×10^8 CFU·mL^{-1}, respectively. Although in control pH culture a higher cell mass concentration has been obtained, the final number of viable cells was not much different to the uncontrolled pH culture, since NaOH used in this study may had some effect on cells viability. The use of ammonium hydroxide instead of the sodium hydroxide resulted in higher cell yields in other studies (Zannini *et al.*, 2005).

On the other hand, the percentage of OTA eliminated by bacteria after cultivation period in MRS-OTA was of 99% and 100% for uncontrolled and controlled pH culture, respectively. The ability of *P. parvulus* UTAD 473 to eliminated completely OTA from the culture medium was previously described by Abrunhosa *et al.* (2014).

When compared to the batch cultures in flasks, batch cultures in bioreactor did not showed significant differences in growth kinetics, however it is easier to control growth conditions. For example, the dissolved oxygen measurement was possible in order to understand the behaviour of bacteria with and without aeration. Figure 4.5 shows the evolution of dissolved oxygen through the time with the uncontrolled pH culture (Batch-I). During the fermentation, the dissolved oxygen concentration in the culture medium did not dropped below 80%, indicating that the bacteria is not using oxygen in its metabolism of sugars. Although, *Pediococcus* do not require high oxygen availability, this is an aerotolerant species.

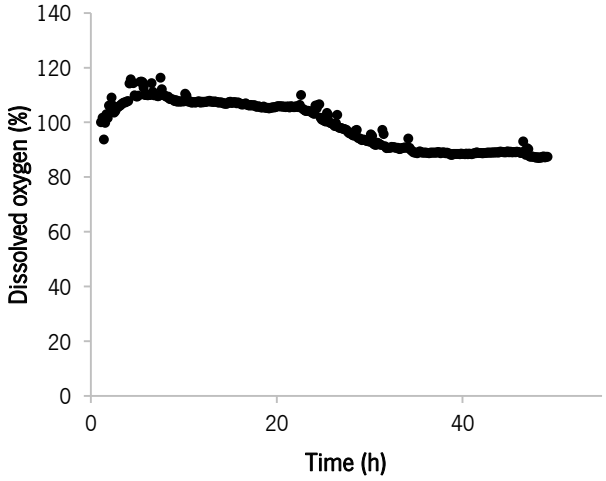

Figure 4.5 - Time course of dissolved oxygen concentration during *P. parvulus* cultivation in Biolab bioreactor at uncontrolled pH conditions (Batch-I).

In order to increase biomass productivity, other strategies for *P. parvulus* cultivation were performed such as the fed-batch culture.

Based on data obtained from the previous experiments, fed-batch cultures were conducted with controlled pH of culture. Fed-batch experiments were designed to improve cell mass during the cultivation by different strategies. In the first strategy (Fed-I), feeding was carried out after 30 hours using MRS medium with 100 $g \cdot L^{-1}$ of glucose and added to the bioreactor using a peristaltic pump at constant flow-rate, with initial dilution rate and specific feeding rate of 0.03 h^{-1} and 2.6 $g \cdot g^{-1} \cdot h^{-1}$,

respectively. This feeding rate was calculated based on the data of glucose consumption rate which was obtained in the Batch-II (controlled pH condition). Figure 4.6 shows the cell growth and the glucose and lactic acid profiles during Fed-I culture. Table 4.5 shows the kinetic parameters obtained in Fed-I culture.

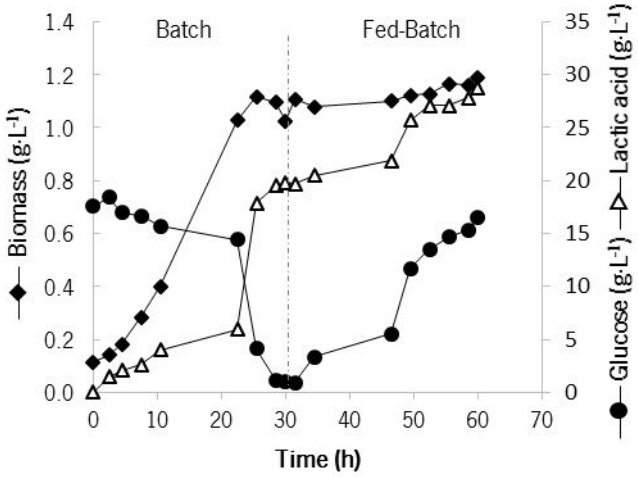

Figure 4.6 – *P. parvulus* growth, glucose and lactic acid kinetics during cultivation in fed-batch culture (Fed-I).

Table 4. 5 - Values of final biomass, maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), specific substrate uptake rate (q_s) and final lactic acid concentration during cultivation in fed-batch culture (Fed-I).

	Biomass ($g \cdot L^{-1}$)	μ_{max} (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)	q_s ($g \cdot g^{-1} \cdot h^{-1}$)	Lactic acid ($g \cdot L^{-1}$)
Batch	1.10	0.13	0.06	2.36	19.80
Fed-batch	1.19	0.03	0.02	2.21-0.62	29.08

The fed-batch was initiated with a batch culture, whose kinetic parameters were similar to Batch-II culture. After 30 hours the glucose limitation was observed and the feeding was initiated. At the end of fed-batch, it was obtained a maximum biomass of 1.19 $g \cdot L^{-1}$ that is not very different from the one obtained in batch culture. However, comparing the total quantity of biomass produced in each operating modes an increase of approximately 2-fold was obtained (0.63 g of cells under batch culture and 1.25 g of cells under fed-batch culture). Additionally, it was observed an accumulation of glucose suggesting that it was not entirely metabolized by the bacteria. The other nutrients from feeding solution may be not sufficient to stimulate the growth of bacteria. However, it was observed

a slight increase of the production of lactic acid as a result of normal metabolic activity of cells. The accumulation of lactic acid may have inhibited the cells growth, since its increase was proportional to the increase of glucose.

Regarding the fed-batch culture, it is possible to observe a decrease in dilution rate (D) over time, since it dropped from 0.028 h^{-1} until 0.015 h^{-1}. According to equation 3.1 (section Materials and Methods), this decrease is expected because the flow rate remained constant and the volume of the reactor increased over time.

In Fed-I culture, *P. parvulus* showed the ability to biodegrade 99% of OTA present in MRS-OTA and it was obtained a number of cells viable of $6.0x10^8$ CFU·mL^{-1}.

A third batch culture (Batch-III) was performed, with an initial glucose concentration of 60 g·L^{-1} to evaluate, in one hand, if lactic acid also inhibit the growth of *P. parvulus* under this operation mode batch culture, and in another hand to justify or not the use of fed-batch culture. Figure 4.7 shows *P. parvulus* growth and the glucose and lactic acid kinetics during the cultivation described for Batch-III culture.

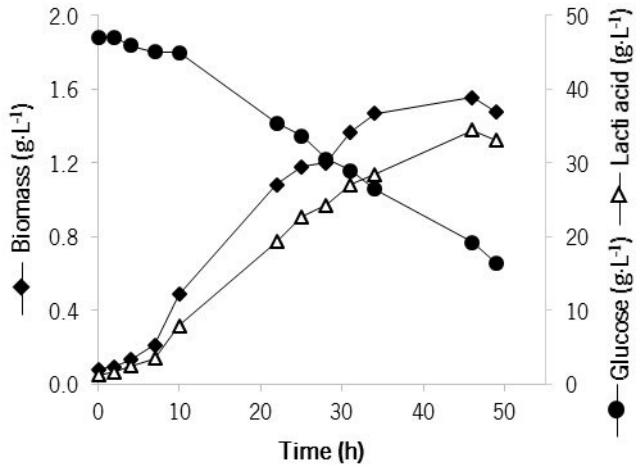

Figure 4.7 - *P. parvulus* growth, glucose and lactic acid kinetics during cultivation in Batch culture with an initial glucose concentration of 60 g·L^{-1} (Batch-III).

The cells grew with specific growth rate of 0.12 h^{-1}, reaching the maximum cell concentration of 1.47 g·L^{-1}. The glucose decreased gradually with a specific substrate uptake rate of 2.65 g·g^{-1}·h^{-1} and, consequently, the lactic acid concentration increased and reached a concentration of 33.07 g·L^{-1}. The biomass yield obtained was of 0.05 g·L^{-1}. For this culture, the number viable cells was $6.6x10^8$ CFU·mL^{-1} and bacteria was able to biodegrade 99% of OTA present in MRS-OTA.

Comparing the results of Batch-III culture with the results obtained in Batch-II culture, no differences in kinetics parameters were observed. Nonetheless, in Batch-III culture, more biomass and lactic acid was obtained. Due to the higher initial concentration of glucose in Batch-III culture it would be expected a higher quantity of biomass produced, which was observed despite not very significant (0.74 g of cells under Batch-II culture and 0.96 g of cells under Batch-III culture). Comparing Batch-III with Fed-I culture, more biomass and lactic acid concentration was also obtained. Although in Fed-I culture less biomass was produced, the total quantity of biomass produced is higher than that produced in Batch-III (1.25 g of cells under Fed-I culture and 0.96 g under Batch-III culture), because there is an increased volume of medium during feeding. These results suggest that cells may have been inhibited by lactic acid present in the medium.

In order to increase the biomass production, another fed-batch culture was performed. The Fed-II culture involved a batch culture of 24 hours that was followed by a fed-batch culture of 30 hours. In this case, the operation mode was changed in order to use only the cells to start the fed-batch, thus removing the fermentation broth that contained lactic acid. The feeding medium consisted of MRS medium concentrated five times. Figure 4.8 shows *P. parvulus* growth and the glucose and lactic acid kinetics during the cultivation described for Fed-II culture. Table 4.6. shows the kinetics obtained during the batch and fed-batch mode in Fed-II culture.

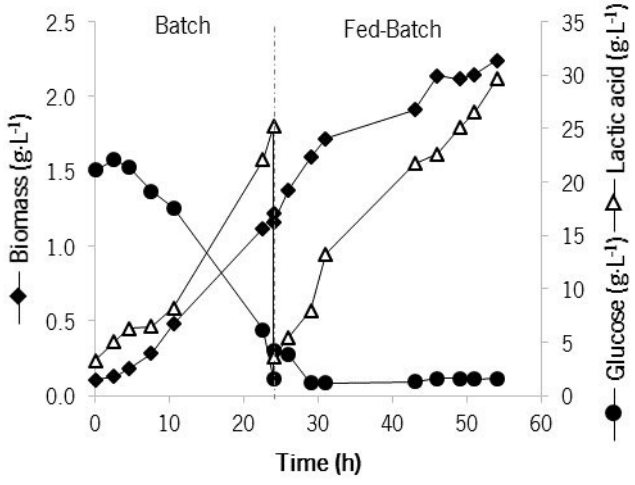

Figure 4.8 - *P. parvulus* growth, glucose and lactic acid kinetics during cultivation in fed-batch culture (Fed-II).

Table 4.6 - Values of final biomass, maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), specific substrate uptake rate (q_s) and final lactic acid concentration during cultivation in fed-batch culture (Fed-II).

	Biomass ($g \cdot L^{-1}$)	μ_{max} (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)	q_s ($g \cdot g^{-1} \cdot h^{-1}$)	Lactic acid ($g \cdot L^{-1}$)
Batch	1.22	0.15	0.06	2.55	25.18
Fed-batch	2.24	0.09	0.04	1.60-0.54	29.65

The batch culture mode of Fed-II presented similar kinetics parameters to previous batch cultures. After 24 hours, when there was only a residual concentration of glucose, the fed-batch was initiated and the cells grew with a maximum specific growth rate of 0.09 h^{-1}. At the end of culture, the biomass reached a maximum of 2.24 $g \cdot L^{-1}$, corresponding the total quantity of biomass produced to 2.07 g of cells (an increase of approximately 3-fold was obtained from batch culture). The fact of feed being composed of concentrated medium ensured that cells received sufficient nutrients to stimulate *P. parvulus* growth. Additionally, it was observed during the fed-batch phase the permanence of a residual concentration of glucose and the production of lactic acid. The removal of lactic acid from medium at the end of the batch phase allowed that cells were not inhibited by produced lactic acid and that they could continue to grow exponentially during the fed-batch phase. For Fed-II culture, the number of viable cells obtained was of 9.5×10^9 $CFU \cdot mL^{-1}$ and bacteria degraded 74% of OTA presbent in MRS-OTA after the incubation period. As observed in Fed-I culture, it was also possible to observe a decrease in D over time, which ranged from 0.02 h^{-1} to 0.01 h^{-1}.

Based on the results obtained in the previous experiments, a new strategy was performed in order to increase further the production of biomass. So, two successive batch with cells recycling were performed. Figure 4.9 shows the kinetic of cells growth and the changes of substrate and product in the successive batch culture (Batch-IV and Batch-V). The kinetic parameters obtained on the two successive batch cultures are shown in Table 4.7.

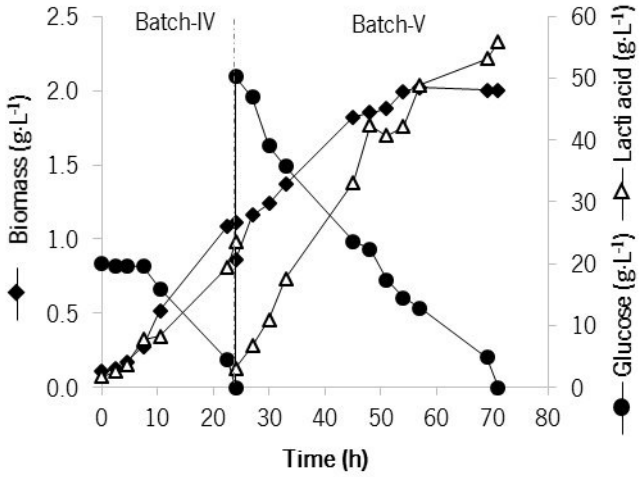

Figure 4.9 - *P. parvulus* growth, glucose and lactic acid kinetics during cultivation in two successive batch cultures (Batch-IV and Batch-V).

Table 4.7 - Values of final biomass, maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), specific substrate uptake rate (q_s) and final lactic acid concentration during cultivation in two successive batch cultures (Batch-IV and Batch-V).

	Biomass ($g \cdot L^{-1}$)	μ_{max} (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)	q_s ($g \cdot g^{-1} \cdot h^{-1}$)	Lactic acid ($g \cdot L^{-1}$)
Batch-IV	1.12	0.17	0.05	3.40	23.58
Batch-V	2.02	0.02	0.02	1.02	55.86

With these successive batch cultures a maximum biomass of 2.02 $g \cdot L^{-1}$ was reached, which is similar to the biomass obtained in previous Fed-II culture (2.24 $g \cdot L^{-1}$). However, the total quantity of biomass produced was 2-fold lower than the one obtain in Fed-Batch-II culture (1.12 g of cells under two successive batch culture and 2.07 g of cells under Fed-II culture). The first batch culture (Batch-IV) with an initial glucose concentration of 20 $g \cdot L^{-1}$, was carried out under the same conditions of batch mode in Fed-II culture. After 24 hours, cells were removed from medium through centrifugation. Then, the second batch was performed with an initial glucose concentration of 60 $g \cdot L^{-1}$. In both batch cultures, the glucose was completely consumed and consequently there was lactic acid production. In Batch-V culture, the lactic acid concentration achieved was more pronounced probably because the initial glucose concentration was also higher. However, comparing with Batch-III culture, the values of the maximum specific growth rate, the biomass yield and the specific substrate uptake rate were lower and the lactic acid production was higher, suggesting that the bacteria metabolism was more directed to the production of lactic acid or other compounds like ESP

than for the production of biomass. According to Walling *et al.* (2005), higher initial glucose concentrations can stimulated ESP production by improving *P. damnosus* growth as well as keeping more glucose available for ESP synthesis. The number of viable cells got at the end of the two cultures was $4.4x10^9$ CFU·mL^{-1} and bacteria was able to eliminated completely OTA present in MRS-OTA after the cultivation period.

Another strategy implemented was successive fed-batchs, which consisted initially of a batch culture and subsequently of two fed-batch (Fed-III). Between each one, cells were harvested by centrifugation, to remove fermentation medium and lactic acid, and resuspended in a fresh medium. Figure 4.10 shows the kinetic of cells growth and the glucose and lactic acid profiles during *P. parvulus* cultivation in Fed-III culture. In Table 4.8 the kinetics parameters obtained for *P. parvulus* growth in different culture mode in Feed-III culture are shown.

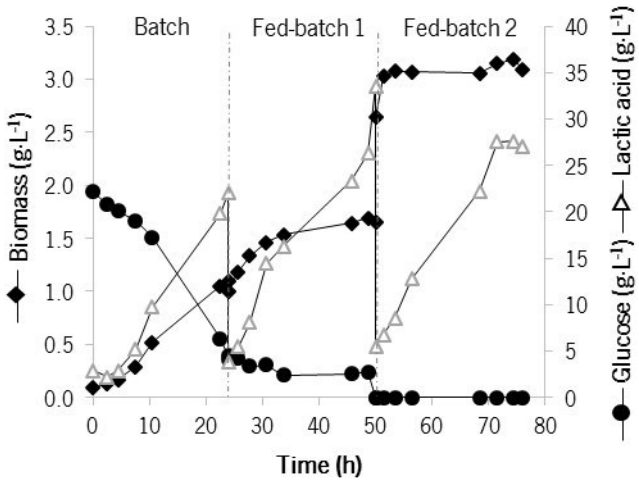

Figure 4.10 - *P. parvulus* growth, glucose and lactic acid kinetics during cultivation in fed-batch culture (Fed-III).

Table 4.8 - Values of final biomass, maximum specific growth rate (μ_{max}), biomass yield ($Y_{x/s}$), specific substrate uptake rate (q_s) and final lactic acid concentration during cultivation in fed-batch culture (Fed-III).

	Biomass (g·L^{-1})	μ_{max} (h^{-1})	$Y_{x/s}$ (g·g^{-1})	q_s (g·g^{-1}·h^{-1})	Lactic acid (g·L^{-1})
Batch	1.10	0.16	0.06	2.74	22.00
Fed-batch 1	1.69	0.09	0.03	1.94-0.70	33.57
Fed-batch 2	3.19	0.03	0.03	0.64-0.39	27.01

For the batch culture mode of this experiment, the kinetics obtained were very similar to the one obtained in previous experiments since the same conditions were used. During the first 24 hours, the cells grew until they reached a maximum biomass of 1.10 $g \cdot L^{-1}$, corresponding the total quantity of biomass produced of 0.60 g of cells. After removing medium containing lactic acid, as well as other compounds produced during the batch fermentation, it was initiated the first fed-batch (Fed-Batch 1). In the Fed-Batch 1, the cells grew with the maximum specific growth rate of 0.09 h^{-1}, reaching a maximum biomass of 1.69 $g \cdot L^{-1}$, corresponding the total quantity of biomass produced of 1.52 g of cells (an increase of approximately 2.5-fold was obtained from batch culture). After 26 h of feeding a new fed-batch was initiated, after removing again medium containing lactic acid. At this moment an increase in the biomass concentration was observed, because the Fed-Batch 2 was initiated with a lower volume than the final volume of Fed-Batch 1. During Fed-batch 2, the growth rate was not significant and the biomass concentration remained almost the same. It would be expected that the biomass concentration increased more vigorously during this second fed-batch period, since the initially number of cells was higher. However, this was not observed. Nonetheless, in both fed-batch cultures the lactic acid production was observed and the glucose concentration remained low. In Fed-Batch 2 the low values of kinetic parameters suggest that the bacteria metabolism was directed preferentially to the lactic acid production than to the production of biomass. However, at the end of the successive fed-batch it was achieved a maximum biomass concentration of 3.19 $g \cdot L^{-1}$, corresponding the total quantity of biomass produced to 2.69 g (an increase of approximately 1.7-fold was obtained from Fed-Batch 1).

For both fed-batch cultures, it was also possible to observe a decrease in dilution rate (D) over time, which ranged from 0.02 h^{-1} to 0.01 h^{-1}. The number of viable cells obtained was of 2.0×10^{10} $CFU \cdot mL^{-1}$ and bacteria degraded 90% of OTA present in MRS-OTA after the incubation period.

For all fed-batch cultures performed it was found a decrease of specific growth rate and glucose consumption rate during *P. parvulus* cultivation. These may be due to production and accumulation of metabolites, as lactic acid, which inhibit cells growth and, thus, the consumption of glucose. This observation confirms what was reported by Velasco *et al.* (2006), who affirmed that lactic acid is a severe growth inhibitor in *P. parvulus* cultures. They tested *P. parvulus* 2.6 growth in the presence of various initial lactic acid concentrations (between 7 and 37 $g \cdot L^{-1}$) and they verified that lactic acid affected the growth rate since it increased the length of exponential growth phase.

Lactic acid inhibition mechanism may involve the solubility of the non-dissociated form within the cytoplasm membrane and the insolubility of the ionised form. This cause the acidification of the cytoplasm and causes changes in the transmembrane pH gradient, resulting in inhibition of nutrient transport (Gonçalves *et al.*, 1997; Wee *et al.*, 2006). So to alleviate the inhibitory effect of lactic acid during the fermentation it is imperative that it be removed. In this study, we used centrifugation in fed-batch culture (Fed-II and Fed-III) and two successive batch.

CHAPTER 5

CONCLUSION

The principal objective of this work was to optimize the biomass production of *P. parvulus* without changes of OTA-degrading capacity. For this, the culture medium, different concentrations of nutrients and the effect of factors were tested in order to achieve higher biomass concentration.

MRS broth supplemented with 20% (w/v) tomato juice was initially found to be the best media for *P. parvulus* growth. This result confirmed that the genus *Pediococcus* has rather complex nutritional requirements and MRS is a complex medium recommended for the cultivation of most LAB.

P. parvulus did not metabolized lactose, thus glucose was the monosaccharide used as carbon and energy source. The temperature was the factor with more influence on bacteria growth, being the optimum growth temperature achieved at 30 °C. Furthermore, glucose, tomato juice and beef extract concentrations on medium did not significantly affect the impact in biomass production. However, L-(+) cysteine at a concentration of 1.0 $g \cdot L^{-1}$ stimulated strongly the growth of *P. parvulus*. Other nutrients such as peptone and yeast extract also had a positive effect on *P. parvulus* growth, although their effect was not so significant. Thus, it was concluded that MRS supplemented with 20% of tomato juice and 1.0 $g \cdot L^{-1}$ of L-(+) cysteine was the most appropriate media for the cultivation of *P.parvulus* in order to obtain more biomass. After this optimization, experiments at bioreactor scale were performed.

Initially, the maximum biomass concentration achieved in batch culture in bioreactor was of 1.14 $g \cdot L^{-1}$, being necessary to control pH of culture medium. After the implementation of several strategies to increase the biomass production, a final maximum concentration of 3.19 $g \cdot L^{-1}$ was obtained using a two steps fed-batch culture process with cell-recycling.

According to the results obtained from the different cultures conducted in the bioreactor it was also possible to conclude that the growth of *P. parvulus* in nutrient medium was very poor, when compared with other lactic acid bacteria, and that the lactic acid is a severe growth inhibitor of this microorganism. This inhibitor effect was only avoided in experiments where cells were separated from lactic acid through centrifugation and recycled into a fresh medium as done in the two fed-batch culture with cell-recycling. However, from the strategies of culture used it was observed a low biomass production, due to the fermentative metabolism of microorganism.

Furthermore, it can be concluded that OTA-degrading capacity of *P. parvulus* was not affected by the composition of media and fermentations conditions studied.

According to the results obtained in this work, it would be interesting, in future works, to study the influence of some additional factors such as:

- The use of calcium carbonate (Ca_2CO_3) in order to determinate if it is better than NaOH;
- The development of new feeding strategies in order to increase the biomass productivity;
- The performance of cell-recycling fed-batch culture with more cycles in order the increase the biomass concentration;
- The use of a microfiltration membrane instead of centrifugation in order to recycle cells continuously;
- The development of a method of separation and purification of lactic acid from fermentation broths, since the lactic acid is one the most important organic acid used in a range of industrial and biotechnological applications;
- The implementation of a continuous fermentation in terms of avoiding the lactic acid inhibition that occurs in batch and fed-batch fermentations by diluting the product, thus both products, biomass and lactic acid, would be continuously produced and separated.

CHAPTER 6

REFERENCES

Abrunhosa, L., Inês, A., Rodrigues, A.I., Guimarães, A., Pereira, V.L., Parpot, P., Mendes-Faia, A., Venâncio, A., (2014) Biodegradation of ochratoxin A by *Pediococcus parvulus* isolated from Douro wines. International Journal of Food Microbiology 188, 45-52.

Abrunhosa, L., Paterson, R., Venâncio, A., (2010) Biodegradation of Ochratoxin A for Food and Feed Decontamination. Toxins 2, 1078-1099.

Abrunhosa, L., Santos, L., Venâncio, A., (2006) Degradation of Ochratoxin A by Proteases and by a Crude Enzyme of *Aspergillus niger*. Food Biotechnology 20, 231-242.

Altaf, M., Naveena, B.J., Reddy, G., (2007) Use of inexpensive nitrogen sources and starch for L(+) lactic acid production in anaerobic submerged fermentation. Bioresource Technology 98, 498-503.

Altuntas, E.G., Cosansu, S., Ayhan, K., (2010) Some growth parameters and antimicrobial activity of a bacteriocin-producing strain *Pediococcus acidilactici 13*. International Journal of Food Microbiology 141, 28-31.

Amézqueta, S., González-Peñas, E., Murillo-Arbizu, M., López de Cerain, A., (2009) Ochratoxin A decontamination: A review. Food Control 20, 326-333.

Atlas, R.M., (1996) Handbook of Microbiological Media, Second Edition ed. CRC Press

Axelsson, L.T., Chung, T.C., Dobrogosz, W.J., Lindgren, S.E., (1989) Production of a Broad Spectrum Antimicrobial Substance by *Lactobacillus reuteri*. Microbial Ecology in Health and Disease 2, 131-136.

Babu, V., Mital, B.K., Garg, S.K., (1992) Effect of tomato juice addition on the growth and activity of *Lactobacillus acidophilus*. International Journal of Food Microbiology 17, 67-70.

Bennett, J.W., Klich, M., (2003) Mycotoxins. Clinical Microbiology Reviews 16, 497-516.

Bernardeau, M., Guguen, M., Vernoux, J.P., (2006) Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiology Reviews 30, 487-513.

Bhat, R., Rai, R.V., Karim, A.A., (2010) Mycotoxins in Food and Feed: Present Status and Future Concerns. Comprehensive Reviews in Food Science and Food Safety 9, 57-81.

Bianchini, A., Bullerman, L.B., (2009) Biological Control of Molds and Mycotoxins in Foods. Mycotoxin Prevention and Control in Agriculture. American Chemical Society, pp. 1-16.

Cabo, M.L., Braber, A.F., Koenraad, P.M.F.J., (2002) Apparent Antifungal Activity of Several Lactic Acid Bacteria against *Penicillium discolor* Is Due to Acetic Acid in the Medium. Journal of Food Protection 65, 1309-1316.

Campos, C.a., Rodríguez, Ó., Calo-Mata, P., Prado, M., Barros-Velázquez, J., (2006) Preliminary characterization of bacteriocins from *Lactococcus lactis*, *Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (*Psetta maxima*). Food Research International 39, 356-364.

Castillo Martinez, F.A., Balciunas, E.M., Salgado, J.M., Domínguez González, J.M., Converti, A., Oliveira, R.P.D.S., (2013) Lactic acid properties, applications and production: A review. Trends in Food Science & Technology 30, 70-83.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., (2001) Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology 71, 1-20.

Corcoran, B.M., Stanton, C., Fitzgerald, G.F., Ross, R.P., (2007) Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. Microbiology 153, 291-299.

Coronel, M.B., Marin, S., Tarragó, M., Cano-Sancho, G., Ramos, A.J., Sanchis, V., (2011) Ochratoxin A and its metabolite ochratoxin alpha in urine and assessment of the exposure of inhabitants of Lleida, Spain. Food and Chemical Toxicology 49, 1436-1442.

Dalié, D.K.D., Deschamps, A.M., Richard-Forget, F., (2010) Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. Food Control 21, 370-380.

Dave, R.I., Shah, N.P., (1997) Effect of cysteine on the viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures. International Dairy Journal 7, 537-545.

de Palencia, P.F., Werning, M.L., Sierra-Filardi, E., Duenas, M.T., Irastorza, A., Corbi, A.L., Lopez, P., (2009) Probiotic properties of the 2-substituted (1,3)-beta-D-glucan-producing bacterium *Pediococcus parvulus* 2.6. Applied And Environmental Microbiology 75, 4887-4891.

Deegan, L.H., Cotter, P.D., Hill, C., Ross, P., (2006) Bacteriocins: Biological tools for bio-preservation and shelf-life extension. International Dairy Journal 16, 1058-1071.

Delves-Broughton, J., (2005) Nisin as a food preservative. Food Australia 57, 525-527.

Dicks, L.M.T., Endo, A., (2009) Taxonomic Status of Lactic Acid Bacteria in Wine and Key Characteristics to Differentiate Species. South African Journal of Enology and Viticulture 30

Dueñas, M., Munduate, A., Perea, A., Irastorza, A., (2003) Exopolysaccharide production by *Pediococcus damnosus* 2.6 in a semidefined medium under different growth conditions. International Journal of Food Microbiology 87, 113-120.

El-Nezami, H., Kankaanpaa, P., Salminen, S., Ahokas, J., (1998) Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, Aflatoxin B1. Food and Chemical Toxicology 36, 321-326.

El-Nezami, H., Polychronaki, N., Salminen, S., Mykkänen, H., (2002a) Binding Rather Than Metabolism May Explain the Interaction of Two Food-Grade *Lactobacillus* Strains with Zearalenone and Its Derivative ά-Zearalenol. Applied And Environmental Microbiology 68, 3545-3549.

El-Nezami, H.S., Chrevatidis, A., Auriola, S., Salminen, S., Mykkänen, H., (2002b) Removal of common *Fusarium* toxins in vitro by strains of *Lactobacillus* and *Propionibacterium*. Food Additives and Contaminants 19, 680-686.

El Khoury, A., Atoui, A., (2010) Ochratoxin A: General Overview and Actual Molecular Status. Toxins 2, 461-493.

Fitzpatrick, J.J., Ahrens, M., Smith, S., (2001) Effect of manganese on *Lactobacillus casei* fermentation to produce lactic acid from whey permeate. Process Biochemistry 36, 671-675.

Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M., Knasmüller, S., (2008) Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. Food and Chemical Toxicology 46, 1398-1407.

Fugelsang, K.C., Edwards, C.G., (2006) Wine Microbiology: Practical Applications and Procedures. Springer

Garai-Ibabe, G., Dueñas, M.T., Irastorza, A., Sierra-Filardi, E., Werning, M.L., López, P., Corbí, A.L., Fernández de Palencia, P., (2010) Naturally occurring 2-substituted (1,3)-β-d-glucan producing *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the production of functional foods. Bioresource Technology 101, 9254-9263.

Gonçalves, L.M.D., Ramos, A., Almeida, J.S., Xavier, A.M.R.B., Carrondo, M.J.T., (1997) Elucidation of the mechanism of lactic acid growth inhibition and production in batch cultures of *Lactobacillus rhamnosus*. Appl Microbiol Biotechnol 48, 346-350.

Guerra, N.P., Bernárdez, Paula F., Agrasar, Ana T., Macías, C.L., Castro, Lorenzo P., (2005) Fed-batch pediocin production by *Pediococcus acidilactici* NRRL B-5627 on whey. Biotechnology and Applied Biochemistry 42, 17-23.

Gunther, I.L., White, H.R., (1961) The Cultural and Physiological Characters of the Pediococci. Journal of General Microbiology 26, 185-197.

Halász, A., Lásztity, R., Abonyi, T., Bata, Á., (2009) Decontamination of Mycotoxin-Containing Food and Feed by Biodegradation. Food Reviews International 25, 284-298.

Hamed, K.A., Shier, W.T., (2009) Mycotoxin Contamination of Agricultural Products in the Southern United States and Approaches to Reducing it from Pre-harvest to Final Food Products. Mycotoxin Prevention and Control in Agriculture. American Chemical Society, pp. 37-58.

Hammami, R., Zouhir, A., Le Lay, C., Ben Hamida, J., Fliss, I., (2010) BACTIBASE second release: a database and tool platform for bacteriocin characterization. BMC Microbiology 10, 1-5.

Harris, J.P., Mantle, P.G., (2001) Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry 58, 709-716.

Hartmann, H.A., Wilke, T., Erdmann, R., (2011) Efficacy of bacteriocin-containing cell-free culture supernatants from lactic acid bacteria to control *Listeria monocytogenes* in food. International Journal of Food Microbiology 146, 192-199.

Hébert, E.M., Raya, R.R., Giori, G.S.d., (2004) Nutritional Requirements of *Lactobacillus delbrueckii* subsp. *lactis* in a Chemically Defined Medium. Curr Microbiol 49, 341-345.

Huwig, A., Freimund, S., Käppeli, O., Dutler, H., (2001) Mycotoxin detoxication of animal feed by different adsorbents. Toxicology Letters 122, 179-188.

Hwang, C.-F., Chen, J.-N., Huang, Y.-T., Mao, Z.-Y., (2011) Biomass production of *Lactobacillus plantarum* LP02 isolated from infant feces with potential cholesterol lowering ability. African Journal of Biotechnology 10, 7010-7020.

Jeevaratnam, K., Jamuna, M., Bawa, A.S., (2005) Biological preservation of foods – Bacteriocins of lactic acid bacteria. India Journal of Biotechnology 4, 446-454.

Juodeikiene, G., Bartkiene, E., Viskelis, P., Urbonaviciene, D., Eidukonyte, D., Bobinas, C., (2012) Fermentation Processes Using Lactic Acid Bacteria Producing Bacteriocins for Preservation and Improving Functional Properties of Food Products. InTech

Kabak, B., Dobson, A.D.W., Var, I., (2006) Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed: A Review. Critical Reviews in Food Science and Nutrition 46, 593-619.

Karlovsky, P., (1999) Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. Natural Toxins 7, 1-23.

Klaenhammer, T.R., (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS microbiology reviews 12, 39-85.

Lim, H.C., Shin, H.S., (2013) Fed-Batch Cultures: Principles and Applications of Semi-Batch Bioreactors. Cambridge University Press

Lindström, C., Xu, J., Oste, R., Holst, O., Molin, G., (2013) Oral administration of live exopolysaccharide-producing *Pediococcus parvulus*, but not purified exopolysaccharide, suppressed *Enterobacteriaceae* without affecting bacterial diversity in ceca of mice. Applied And Environmental Microbiology 79, 5030-5037.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., Schnürer, J., (2003) Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology Letters 219, 129-135.

Mårtensson, O., Biörklund, M., Lambo, A.M., Dueñas-Chasco, M., Irastorza, A., Holst, O., Norin, E., Welling, G., Öste, R., Önning, G., (2005) Fermented, ropy, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. Nutrition Research 25, 429-442.

Mateo, E.M., Medina, Á., Mateo, F., Valle-Algarra, F.M., Pardo, I., Jiménez, M., (2010) Ochratoxin A removal in synthetic media by living and heat-inactivated cells of *Oenococcus oeni* isolated from wines. Food Control 21, 23-28.

Milićević, D.R., Škrinjar, M., Baltić, T., (2010) Real and Perceived Risks for Mycotoxin Contamination in Foods and Feeds: Challenges for Food Safety Control. Toxins 2, 572-592.

Naidu, A.S., Bidlack, W.R., Clemens, R.A., (2010) Spectra of Lactic Acid Bacteria (LAB) Probiotic Spectra of Lactic Acid Bacteria (LAB). Critical Reviews in Food Science and Nutrition Probiotic 39, 13-126.

Nel, H.A., Bauer, R., Vandamme, E.J., Dicks, L.M.T., (2001) Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1. Journal of Applied Microbiology 91, 1131-1138.

Niderkorn, V., Boudra, H., Morgavi, D.P., (2006) Binding of *Fusarium* mycotoxins by fermentative bacteria in vitro. Journal of Applied Microbiology 101, 849-856.

Nilsen, T., Nes, I.F., Holo, H., (2003) Enterolysin A , a Cell Wall-Degrading Bacteriocin from *Enterococcus faecalis* LMG 2333. Applied And Environmental Microbiology 69, 2975-2984.

O'Callaghan, J., Caddick, M.X., Dobson, A.D.W., (2003) A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiology 149, 3485-3491.

Papagianni, M., Anastasiadou, S., (2009) Pediocins: The bacteriocins of Pediococci. Sources, production, properties and applications. Microbial cell factories 8, 1-16.

Patrick, O.M., (2012) Lactic Acid Bacteria in Health and Disease. Rwanda Journal of Health Sciences 1, 39-50.

Piotrowska, M., Zakowska, Z., (2005) The elimination of ochratoxin A by lactic acid bacteria strains. Polish Journal of Microbiology 54, 279-286.

Raccach, M., (1999) *Pediococcus* In: Robinson, R.K. (Ed.), Encyclopedia of Food Microbiology. Elsevier, Oxford, pp. 1641-1647.

Ratledge, C., Hristiansen, B., (2006) Basic biotechnology, 3rd ed. Cambridge University Press

Rattanachaikunsopon, P., Phumkhachorn, P., (2010) Lactic acid bacteria : their antimicrobial compounds and their uses in food production. Annals of Biological Research 1, 218-228.

Ringot, D., Chango, A., Schneider, Y.-J., Larondelle, Y., (2006) Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chemico-Biological Interactions 159, 18-46.

Rodrigues, A., (2011) Selecção de Bactérias Lácticas para o controlo de micotoxinas e caracterização dos mecanismos envolvidos. School of Engenharia. University of Minho.

Rouse, S., Harnett, D., Vaughan, A., Sinderen, D.v., (2008) Lactic acid bacteria with potential to eliminate fungal spoilage in foods. Journal of Applied Microbiology 104, 915-923.

Saguir, F.M., Campos, I.E.L., Maturano, C., de, M.C.M., Nadra, (2009) Identification of dominant lactic acid bacteria isolated from grape juices. Assessment of its biochemical activities relevant to flavor development in wine. International Journal of Wine Research 1, 175-185.

Schneider, R., Fernández, F.J., Aguilar, M.B., Guerrero-Legarreta, I., Alpuche-Solís, A., Ponce-Alquicira, E., (2006) Partial characterization of a class IIa pediocin produced by *Pediococcus parvulus* 133 strain isolated from meat (Mexican “chorizo”). Food Control 17, 909-915.

Schnürer, J., Magnusson, J., (2005) Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science & Technology 16, 70-78.

Seesuriyachan, P., Kuntiya, A., Hanmoungjai, P., Techapun, C., (2011) Exopolysaccharide production by *Lactobacillus confusus* TISTR 1498 using coconut water as an alternative carbon source: the effect of peptone, yeast extract and beef extract. Songklanakarin Journal of Science and Technology 33 (4), 379-387.

Sheeladevi, A., Ramanathan, N., (2011) Lactic Acid Production Using Lactic Acid Bacteria under Optimized Conditions. International Journal of Pharmaceutical & Biological Archive 2 (6), 1686-1691.

Shetty, P.H., Jespersen, L., (2006) *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends in Food Science & Technology 17, 48-55.

Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J., Kenne, L., (2003) Antifungal 3-Hydroxy Fatty Acids from *Lactobacillus plantarum* MiLAB 14. Applied And Environmental Microbiology 69, 7554-7557.

Talarico, T.L., Casas, I.A., Chung, T.C., Dobrogosz, W.J., (1988) Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrobial Agents and Chemotherapy 32, 1854-1858.

Tanasupawat, S., Daengsubha, W., (1983) *Pediococcus* species and related bacteria found in fermented foods and related materials in Thailand. The Journal of General and Applied Microbiology 29, 487-506.

Tanasupawat, S., Ezaki, T., Suzuki, K.-I., Okada, S., Komagata, K., Kozaki, M., (1992) Characterization and Identification of *Labtobacillus pentosus* and *Lactobacillus plantarum* strains from fermentes foods in Thailand. The Journal of General and Applied Microbiology 38, 121-134

Turbic, A., Ahokas, J.T., Haskard, C.A., (2002) Selective in vitro binding of dietary mutagens, individually or in combination, by lactic acid bacteria. Food Additives and Contaminants 19, 144-152.

van Niel, E.W.J., Hahn-Hägerdal, B., (1999) Nutrient requirements of lactococci in defined growth media. Appl Microbiol Biotechnol 52, 617-627.

Velasco, S., Årsköld, E., Paese, M., Grage, H., Irastorza, A., Rådström, P., van Niel, E.W.J., (2006) Environmental factors influencing growth of and exopolysaccharide formation by *Pediococcus parvulus* 2.6. International Journal of Food Microbiology 111, 252-258.

Velasco, S.E., Areizaga, J., Irastorza, A., Duenas, M.T., Santamaria, A., Munoz, M.E., (2009) Chemical and rheological properties of the beta-glucan produced by *Pediococcus parvulus* 2.6. Journal of Agricultural and Food Chemistry 57, 1827-1834.

Velasco, S.E., Yebra, M.J., Monedero, V., Ibarburu, I., Dueñas, M.T., Irastorza, A., (2007) Influence of the carbohydrate source on β-glucan production and enzyme activities involved in sugar metabolism in *Pediococcus parvulus* 2.6. International Journal of Food Microbiology 115, 325-334.

Vuyst, L.D., (2000) Technology Aspects Related to the Application of Functional Starter Cultures. Food Technology and Biotechnology 38, 105-112.

Walling, E., Dols-Lafargue, M., Lonvaud-Funel, A., (2005) Glucose fermentation kinetics and exopolysaccharide production by ropy *Pediococcus damnosus* IOEB8801. Food Microbiology 22, 71-78.

Wee, Y.-j., Kim, J.-n., Ryu, H.-w., (2006) Biotechnological Production of Lactic Acid and Its Recent Applications. Food Technology and Biotechnology 44, 163-172.

Weinberg, Z.G., Muck, R.E., Weimer, P.J., Chen, Y., Gamburg, M., (2004) Lactic acid bacteria used in inoculants for silage as probiotics for ruminants. Applied Biochemistry and Biotechnology 118, 1-9.

Wright, A.V., Axelsson, L., (2012) Lactic acid bacteria: An Introduction. In: Lahtinen, S., Ouwehand, A.C., Salminen, S., Wright, A.V. (Eds.), Lactic acid bacteria: Microbiological and Functional Aspects, 4th ed. CRC Press, pp. 1-17.

Yang, H.Y., Gao, L.J., Wang, X.F., Wang, W.D., Cui, Z.J., (2007) Effects of cultivation conditions on the diversity of microbes involved in the conversion of rice straw to fodder. Journal of Environmental Sciences 19, 67-73.

Zain, M.E., (2011) Impact of mycotoxins on humans and animals. Journal of Saudi Chemical Society 15, 129-144.

Zannini, E., Santarelli, S., Osimani, A., Dell'aquila, L., Clementi, F., (2005) Effect of process parameters on the productiob of lactic acid bacteria in batch fermentation. Annals of Microbiology 33, 273-278.

Zhang, J., Zhang, Y., Liu, S.-n., Han, Y., Zhou, Z.-j., (2012) Modelling Growth and Bacteriocin Production by *Pediococcus acidilactici* PA003 as a Function of Temperature and pH Value. Applied Biochemistry and Biotechnology 166, 1388-1400.

CHAPTER 7

ANNEXES

Annexe A.1 – Calibration curve of biomass

As mentioned in section 3.5.1 (Materials and Methods), biomass quantification was estimated using the calibration curve built for *P. parvulus* UTAD 473 (Figure A.1). The calibration curve is represented by equation A.1:

$$y = 1.91x + 0.02,\ R^2 = 0.998 \qquad \text{(Equation A.1)}$$

where, y is the OD_{600nm} and x is the biomass concentration ($g \cdot L^{-1}$).

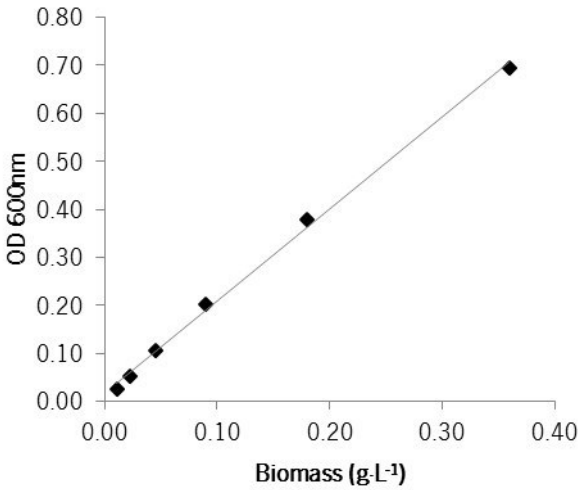

Figure A.1 – Calibration curve of biomass. Absorbance at 600 nm versus biomass concentration ($g \cdot L^{-1}$).

Annexe A.2 – Calibration curve of glucose and lactic acid

Glucose and lactic acid quantification was estimated using the calibration curve built using HPLC (Figure A.2 and A.3). The calibration curves of glucose and lactic acid are represented by equation A.2 and equation A.3, respectively:

$$y = 2.36e^5\,x,\ R^2 = 0.998 \qquad \text{(Equation A.2)}$$

$$y = 1.05e^5\,x,\ R^2 = 0.999 \qquad \text{(Equation A.3)}$$

where, y is the peaks area detected by HPLC and x is the concentration of compound ($g \cdot L^{-1}$).

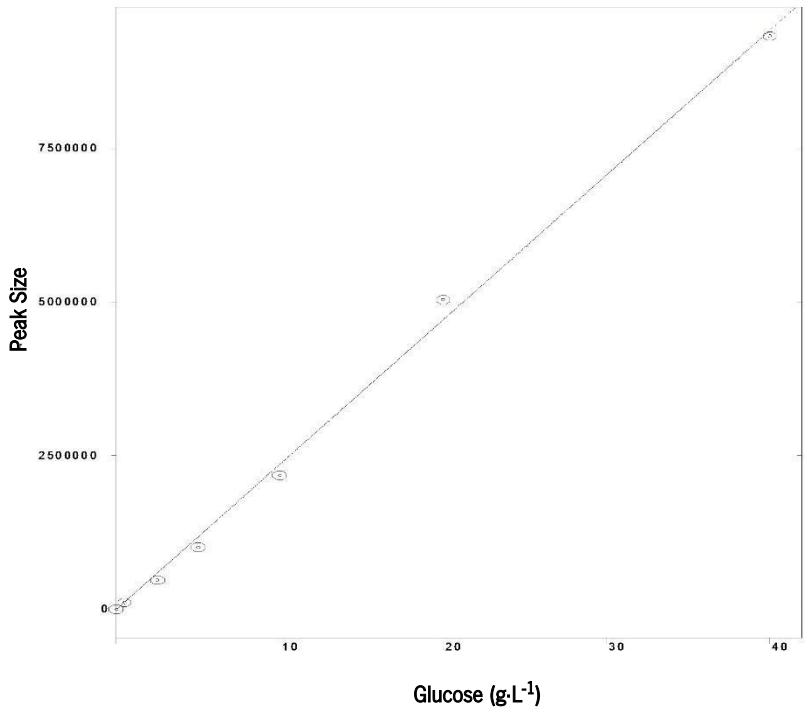

Figure A.2 - Calibration curve of glucose obtained from Star Workstation chromatography data system. Peak size (mVolts) versus glucose concentration ($g \cdot L^{-1}$).

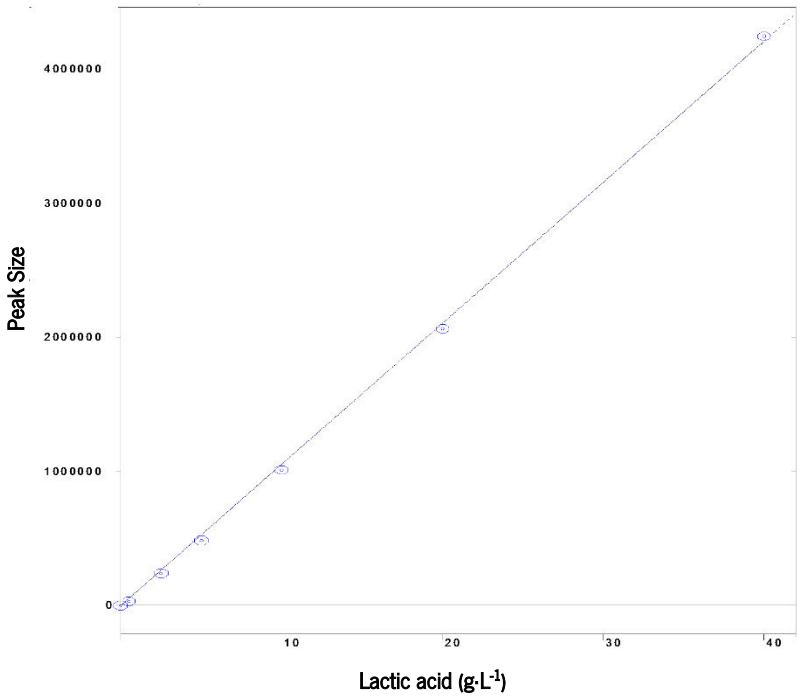

Figure A.3 - Calibration curve of lactic acid obtained from Star Workstation chromatography data system. Peak size (mVolts) versus lactic acid concentration ($g \cdot L^{-1}$).