Effect of cultivation conditions on antimicrobial production in *Lactococcus lactis*

Marta Alexandra Freire Alves
Instituto Superior Técnico

The awareness of the adverse effects of chemical preservatives has been increasing over the years and so, the demand to improve current methods for food preservation has become higher, together with the request for more "natural" food. Therefore, more attention is being brought to the antimicrobial compounds produced by Lactic acid bacteria: gram-positive, facultative anaerobic bacteria with a fermentative metabolism that are safe to consume and widely used in industry. The aim of this project was to determine the effect of cultivation conditions on antimicrobial production in *Lactococcus lactis*. Fermentation experiments in 0.5L fermenters confirmed that antimicrobial production follows primary metabolite kinetics and that the end of the exponential phase is the best time to harvest the broth containing the antimicrobial. In several experiments, the effect of varying the substrate concentration, the pH, the medium composition and other fermentation conditions was established. This work resulted in the design of optimal cultivation conditions with respect to fermentation time and medium costs with a maximized yield of antimicrobial compound production.

Keywords: fermentation, antimicrobials, yield optimization, lactic acid bacteria

Introduction

Lactic acid bacteria (LAB) are industrially important organisms recognized as safe to consume, with health and nutritional benefits. The antimicrobial activities of LAB have long been known and traditionally applied, playing an important role in food fermentation and food preservation, due to their beneficial influence on nutritional, organoleptic (such as taste and texture), and shelf-life characteristics. [1]

In this sense, LAB display a wide range of antimicrobial activities, causing rapid acidification of the raw material through the production of organic acids such as lactic acid (the most important one) and acetic acid. However, certain strains of LAB are further known to produce bioactive molecules like ethanol, carbon dioxide, flavor compounds (e.g. diacetyl, acetoin and acetaldehyde), formic acid, fatty acids, hydrogen peroxide, some enzymes and also antimicrobial proteins called bacteriocins. [2]

The bacteriocins produced by LAB are very useful in food protection for several reasons. In the first place, they are small ribosomally synthesized antimicrobial peptides or proteins that can be modified by genetic engineering. Secondly, by being natural substances they are biodegradable and finally, because each different bacteriocin has its own unique and rather narrow killing spectrum, thus allowing manipulation of food microbial ecosystems. [2]

The antibacterial spectrum frequently includes closely related Gram-positive spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. Besides their antimicrobial action towards undesirable bacteria, bacteriocins are believed to contribute also to the competitiveness of the producer cells. On the other hand, the activity against Gram-negative bacteria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example, after osmotic shock, low pH treatment or in the presence of a detergent or chelating agent.[2]

Compound A is the most extensively studied and characterized antimicrobial protein produced by lactic acid bacteria. It contains 34 amino acids and 4 unusual amino acids (dehydroalanine, dehydrobutyrine, lanthionine and b-methyllanthionine) that should be responsible for the functionally important properties of the bacteriocin molecule, i.e. thermo stability and bactericidal action. [3]

This protein is heat stable and insoluble in nonpolar solvents. Its solubility in polar solvents is pH dependent (12% at pH 2.5, 4% at pH 5), meaning it increases when the pH is lowered. Its isoelectric point is on the alkaline side. Compound A is most stable at low pH and can even stand sterilization (121 ^{o}C for 20 min) when at pH 2.5. However, in a pH above 7, the molecules undergo irreversible changes with loss of activity.[3]

The biosynthesis of compound A occurs in two steps: a precursor peptide is synthesized ribosomally followed by post-translational enzymatic modifications converting the inactive precursor into a mature bioactive peptide. These modifications include dehydration of specific prepeptide serine and threonine residues, with subsequent addition of neighboring cysteine sulphur to the double bonds of the didehydroamino acids resulting in the formation of thioether bridges. [4]

Nowadays, compound A is commercially produced exclusively by homolactic fermentation of some *Lactococcus lactis* strains that are mesophilic and homo fermentative, which mean they grow optimally at temperatures around 30°C and they produce predominantly L-lactic acid from pyruvate via the NAD-dependent lactate dehydrogenase (LDH). Overall, one molecule of glucose (or any six-carbon sugar) is converted to two molecules of lactic acid.

As the lactic acid bacteria are fastidious microorganisms they require a complex medium to grow and to produce the bacteriocin, making the purification of the latter rather difficult. Furthermore, many bacteriocin molecules tend to associate with other molecular substances present in the culture medium, display a high degree of hydrophobicity and form protein aggregates. This fact makes the downstream processing more difficult, requiring the design of specific purification protocols for each bacteriocin. Therefore, the impact of different parameters such as the producer strain, composition of medium, temperature, pH and aeration have to be studied in order to formulate an effective process design for the production of compound A, with a medium composition as simple and cheap as possible. [4]

As a natural food preservative, this bacteriocin is mostly used in processed cheese and cheese spreads, in canned vegetables and fruits, in sterilized milk, milk drinks and, nowadays, in meat. With this vast food applications, it is considered to be one of the most promising and important group of natural

inhibitors, with a highly economic added value.[5]

This study aims to efficiently produce a compound A ferment to be used as a natural preservative in food, testing the effects of varying substrate concentration, pH and medium composition on the growth and biomass production of *Lactococcus lactis* and, consequently, on compound A activity. These results were used to design the optimal medium composition for a high antimicrobial production.

Materials & Methods

Bacterial strain. Strain 1 was improved and adapted to grow in the presence of 15000 $IU.g^{-1}$, resulting in a strain with a higher compound A production. This strain was grown in shake flasks with 100 mL of the following medium: 2% w/w sucrose, 1% w/w Yeast Extract Paste (YEP) (50% ds.) and 1% w/w $CaCO_3$ that constituted the stock culture to be used in this work. 1.5 mL of the stationary phase culture together with 0.5 mL of 60% v/v kosher glycerol were put in aliquots, labeled and then stored at -80°C.

Inoculum. Before every experiment, a 40 mL pre-culture with 2% w/w sucrose, 1% w/w YEP (50% ds.) and 1% w/w $CaCO_3$ was inoculated with 0.3 mL of the -80°C stock cultures. The flask was then incubated for approximately 10 to 15h, at 30°C, with some agitation to prevent the precipitation of $CaCO_3$. By measuring the optical density at 600 nm it was possible to determine the growth of the bacteria.

Medium and Fermentation conditions. The fermentations were run in 0.5 L Multifors fermenters (Infors HT, Switzerland). The standard medium composition being used before this study was: 4% w/w sucrose, 2% w/w YEP (50% ds.) from Biospringer and 0,5 $g.L^{-1}$ $CaCl_2.2H_2O$ from Acros. The fermentations were performed at 30°C, with a stirring speed of 250 rpm and a controlled pH of 5.5, measured by a pH electrode (Mettler Toledo, USA) and maintained by the addition of a titrant: 20% NaOH. To test the effect of the medium composition, a Complex Medium (CM) was used and had the following composition: 3% w/w sucrose, 1% w/w YEP (50% ds.) from Biospringer, 1% w/w peptone, 1% w/w KH_2PO_4, 2 $g.L^{-1}$ NaCl and 0.2 $g.L^{-1}$ $MgSO_4.7H_2O$. The fermentations were performed as batch fermentations operated with no aeration and the reactors were inoculated with 2.5% w/w of the strain 1 pre-culture inoculum. Before starting any fermentation, the reactors were sterilized at 121°C for 20 minutes. Regular samples were taken and analyzed for sucrose and lactate concentration, optical density and compound A activity.

Analytical methods. The optical density of the cells was determined using a spectrophotometer at 600 nm. The samples were diluted with and EDTA solution (pH8, 50mM), depending on the expected density, and measured against the fermentation medium without cells, as a blank. To determine the concentration of sucrose and lactate in the fermentation broth, a biochemical analyzer (SalmenKipp YSI 2700: Glucose and Lactate analyzer) was used. 1 mL of the fermentation broth was centrifuged at 12500 rpm for 2.5 minutes and the supernatant was diluted with demineralized water so the values were within the detection limits of the YSI (0 – 0.5 $g.L^{-1}$ for lactate and below 2.5 $g.L^{-1}$ for glucose).

Therefore, to measure the lactate concentration and sucrose concentration, a 1:100 (v/v) dilution and a 1:10 (v/v) dilution were made, respectively. Since the YSI only measures glucose, the sucrose solution had to be converted, incubating it for 30 minutes at 55°C in the presence of 20µL of the enzyme invertase.

Compound A activity bioassay. A Sigma Standard Compound A with 1000000 $IU.g^{-1}$ was used to prepare the bacteriocin standard solutions, ranging in activity from 30 to 600 $IU.g^{-1}$ in order to set a calibration curve for the bacteriocin activity. The fermentation samples of interest were diluted with 0.02M HCl to fit into the standard range and a few drops of 5M HCl were used to adjust their pH between 2 - 2.5. The standards and the fermentation-diluted samples were boiled for 15 minutes in a water bath at 100ºC, in order to be extracted, and the BHI/agar plates were prepared and inoculated with *Lactococcus lactis* subsp. *cremoris* and some wells were crafted and properly marked. The samples were loaded in the wells and incubated overnight at 30°C. The inhibition of growth was determined measuring the diameter of the circular halo, using a digital caliper, and the bacteriocin concentration of the samples was determined using the standard calibration curve and the dilution factors.

Results and Discussion

Production of compound A during growth

Compound A production kinetics of *Lactococcus lactis* was examined with the standard fermentation conditions described.

The batch fermentation profile of microbial growth and compound A production at a controlled pH is presented in Figure 1.1. The exponential growth phase took place during a period of about 5-6h at a maximum specific growth rate of 0.41 h^{-1} and a doubling time of 1.69 h. The maximum cell density was reached after 7-8h and compound A production levels appear to follow a parallel tendency: the higher titres were reached at the end of the exponential phase and corresponded to the maximal biomass. Consequently, it shows that the bacteriocin was produced in the active growth phase, confirming the literature results that compound A production follows primary metabolic kinetics.

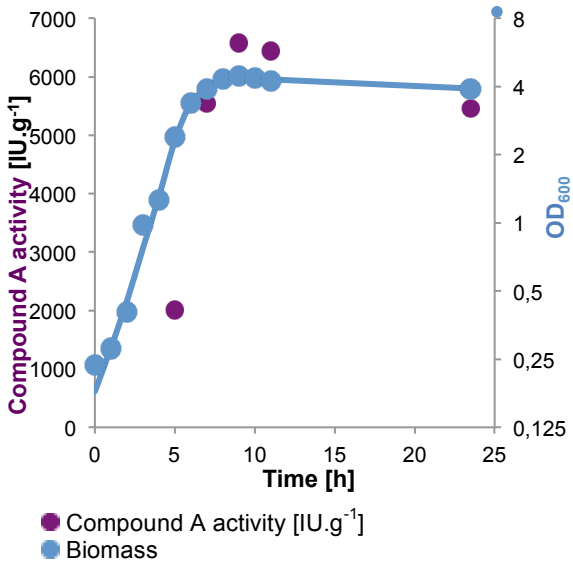

Figure 1.1 – Compound A activity and biomass values of the standard 3% sucrose fermentations of strain 1.

After reaching peak values, sometimes the bacteriocin levels and biomass decreased with a prolonged fermentation time. The decrease of the compound A titre after 8h of fermentation implies that the fermentation process would need to be stopped by then, making this the best time to harvest the broth,

in order to achieve a maximum compound A activity.

A dramatic decrease in compound A level after reaching the peak value is suspected to be a result of proteolytic degradation and/or adsorption of the bacteriocin by producer cells, as stated in literature. In this sense the best method to decrease the loss of compound A is to recover the product during fermentation and protect it at low pH as soon as possible.

Effects of varying sucrose concentration

To determine the effect of varying the carbon source concentration, batch experiments in 0.5L Multifors fermenters were performed with 1%, 2%, 3% and 4% of sucrose, using strain 1. The rest of the conditions were kept the same, as in the standard fermentation ($30^{o}C$; pH 5.5; 250 rpm; 2%YEP, 0,5 $g.L^{-1}$ $CaCl_2$; base: NaOH).

Table 1.1 comprises the biomass values, sucrose and lactate concentration and compound A activity values for each C-source concentration. These values represent an average of the fermentations performed with each sucrose concentration and concern the end of the fermentation time.

Table 1.1 - Average of the results (end of fermentation time) of the anaerobic fermentations performed with the standard conditions, varying the substrate concentration in 1, 2, 3 and 4% of sucrose.

	1% sucrose	**2% sucrose**	**3% sucrose**	**4% sucrose**
Biomass (OD_{600})	3.11	3.07	3.76	4.09
Sucrose ($g.L^{-1}$)	0	0.26	0.63	4.9
Lactate ($g.L^{-1}$)	8.7	18.8	27	33.5
Compound A activity ($IU.g^{-1}$)	2900	4080	6042	6450

In theory, if we provide a higher concentration of substrate, the cells should also show a higher growth, since there is a bigger amount of the carbon source to be converted. However, as it is possible to observe by Table 1.1, biomass has a very similar growth in all four sucrose concentrations, indicating that probably the ATP generated in the higher sucrose concentrations is being used for maintenance, instead of cell growth, or that something else is contributing to the high OD_{600} values that are shown with 1% and 2% of sucrose. It is shown in literature that the carbon source provides energy for the logarithmic-phase growth and that amino acids such as arginine, for example, provide energy after carbohydrate exhaustion, increasing the survival time, the cell numbers and ATP generation. This fact may explain the higher biomass values observed in fermentations with low sucrose concentrations, in which the carbon source is rapidly depleted.[6]

As expected, compound A activity values and lactate values are higher according to the increase of the carbon source, since this bacteriocin is a primary metabolite and its concentration is dependent on the growth performance of the bacteria. Therefore, the higher compound A activity levels can be found when 3% and 4% of sucrose are used: 6042 $IU.g^{-1}$ and 6450 $IU.g^{-1}$, respectively.

The fermentation profiles presented in figures 1.2 and 1.3 concern the average of all the experiments performed with 3% and 4% of the carbon source and are useful to better compare these two sucrose concentrations.

The Qv ($g.L^{-1}.h^{-1}$) value is a measure of volumetric productivity that is automatically calculated by the fermenter's program with the amount of base that is being added during the fermentation time. The fermentation time is

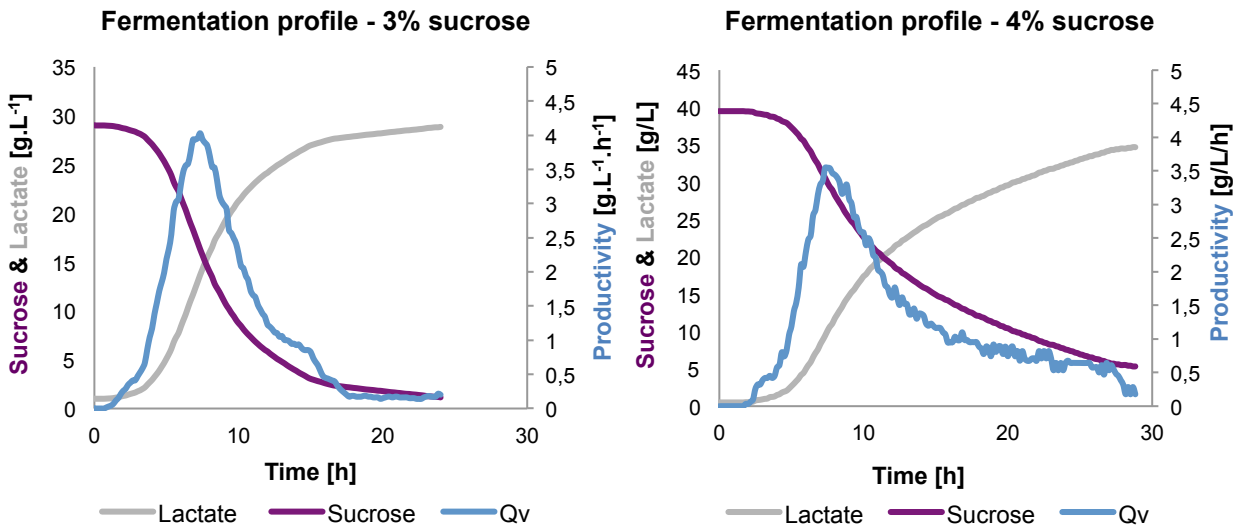

Figure 1.2, 1.3 - Average of the fermentation profiles of all experiments performed with the standard conditions with 3% and 4% of sucrose, respectively.

then considered to be the time before all substrate concentration is converted and it can be visible when the Qv reaches zero, which means that no more substrate is being transformed into lactate, excluding the need to add more base to neutralize the fermentation.

Although the productivity values are very similar with both carbon source concentrations, it is possible to observe that the fermentation time is much lower, when using a 3% sucrose concentration. It undergoes a reduction from around 30h (4% sucrose) to a fermentation that runs between 15h and 20h (3% sucrose).

The tailing effect is an effect that occurs when the substrate is not completely consumed but at the same time it is also being converted to lactate at a very slow rate, prolonging the fermentation time. It can be described as a slow decrease in productivity (Qv) and is very visible in the fermentations with 4% sucrose, making it another disadvantage of using higher concentrations of substrate.

Moreover, using more quantity of sucrose means an increase in the medium costs and, since the fermentations are longer with higher concentrations, the operational costs will be also higher.

For all of the previous reasons, the sucrose concentration that better fits this type of anaerobic fermentations was found to be 3% of sucrose and so this was the substrate concentration taken as a reference for the following experiments performed in this study.

Effects of varying the pH

It is stated in literature that the optimal growth of *L. lactis* is carried out with a controlled constant pH between pH 5.5 – 6.8, depending on the producer strain. However, some studies were made testing different pH profiles, in addition to the constant controlled pH fermentations, that showed an enhanced compound A production.

It has been shown that at pH 6.8 (controlled fermentation) more than 80% of compound A synthetized was bound to the cells, whereas at pH below 6, more than 80% of this bacteriocin was in the culture fluid. Another study reported that the optimal pH for the bacteriocin adsorption to the cells ranges

from pH 5.5 to 6.5. Therefore, it was proved that the increase of the pH from 5.5 to 6.8 enhances the adsorption of compound A to the cells but decreases drastically its solubility and stability. [7]

To confirm these results, some experiments were set with the reference conditions but changing the control of the pH to 5, 6 and 7, by the addition of 20% NaOH. The respective biomass formation (measured by the optical density at 600 nm) during the fermentation time is shown in figure 1.4. The adsorption of compound A to the cells was not tested in this work but some conclusions can be taken concerning cell growth and compound A production.

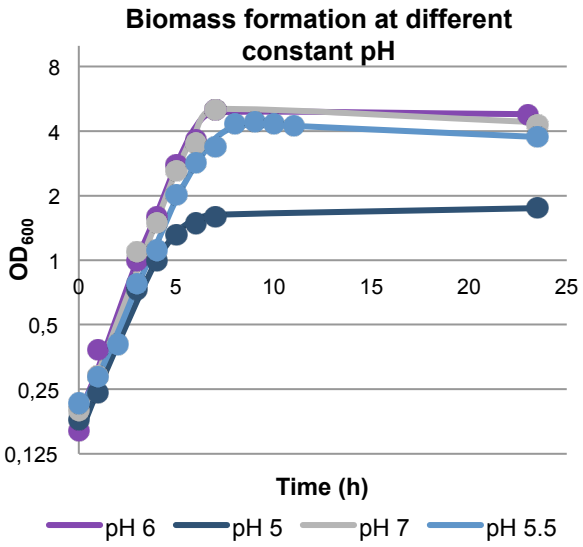

Figure 1.4 - Growth curve of strain Strain 1 with the reference fermentation conditions (3% sucrose), performed with a controlled pH of 5, 5.5, 6 and 7.

Observing the graph, it is clear that the cell growth at pH 5 is significantly lower comparing to biomass formation in higher pH fermentations. This may be due to the fact that LAB growth can be inhibited at low pH.

According to the previous results, the cellular growth is very similar when using pH 5.5 (reference), 6 and 7. However, since compound A titres seem to be proportionally related to the biomass yield, especially under pH-controlled conditions, the bacteriocin activity values have also to be taken into consideration and can be found in table 1.2. There was an error with the compound A activity analysis and there are no values after 5h and 7h of the fermentation time, at pH 6.

Table 1.2 – Compound A activity values, determined by the standard compound A bioassay protocol, of the anaerobic fermentations performed with the reference conditions with a controlled pH of 5, 5.5, 6 and 7, after 5h and 7h of the fermentation time and at its end (23.5h).

	Compound A activity ($IU.g^{-1}$)			
Time (h)	**pH 5**	**pH 5.5**	**pH 6**	**pH 7**
5	710	1623	-	1570
7	1370	4907	-	5340
23,5	920	6043	6600	560

The low compound A activity values at pH 5 can confirm the statement that the bacteriocin production is dependent on the biomass formation, showing a primary metabolite behavior.

At pH 7 the compound A activity values are also low, and they significantly decrease in the end of the fermentation (23.5h) what can confirm the literature statements: at a higher pH more than 80% of the compound A is bound to the cells. Therefore, the bacteriocin concentration on the fermentation broth is lower as confirmed by the compound A activity bioassay values.

It is possible to compare the value of the calculated biomass according to the amount of base added, the biomass formation measured by optical density at 600 nm, compound A

activity values and the lactate concentration, in figure 1.5. All of the values concern the end of the fermentation time.

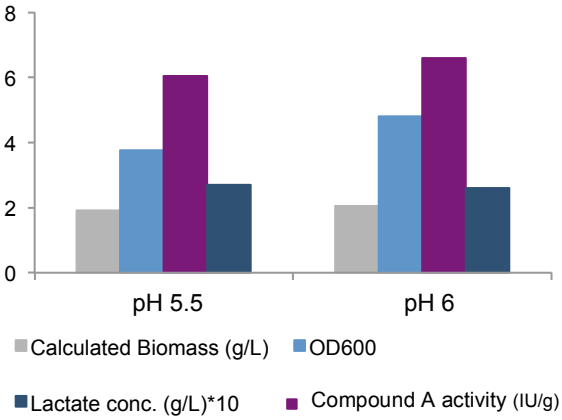

Figure 1.5 - Comparison of the biomass (measured and calculated), compound A activity and lactate concentration results (end of fermentation time) on the anaerobic fermentations performed with the standard conditions and at a controlled pH of 5.5 and 6.

Since the results are very similar, it is safe to say that for this *L. lactis* strain (strain 1) the optimal constant pH would be between 5.5 and 6. Nonetheless, further experiments in this study were taken keeping the pH at 5.5.

Effects of varying the medium composition

The reference medium used so far is a simple medium that provides a good growth of this *L.lactis* strain and consequently, good compound A titres. However, the nitrogen source used, the Yeast extract Paste, is the compound that has the bigger contribute to the medium costs. [8]

In order to try to reduce the costs, some experiments were made with the purpose of reducing the amount of YEP used in the reference fermentations. A Complex medium (CM) was used and experiments were performed in 0.5L Multifors by Infors HT, maintaining the other reference fermentation conditions.

To better understand the role of YEP in the medium, another experiment was done with the same CM but without peptone. This last compound is an enzymatic digest of animal protein whose nutritive value is largely dependent on the amino acid content that supplies essential nitrogen.[9]

The biomass formation, sucrose and lactate concentration and compound A activity values comparing the two CM fermentations (with and without peptone) with the reference fermentation are presented in table 1.3.

Table 1.3 - Biomass, sucrose, lactate and compound A activity results (end of fermentation time) from the anaerobic fermentations performed with the reference conditions, varying the medium composition: Reference medium; Complex medium; Complex Medium without peptone.

	Reference Medium	**Complex Medium**	**Complex Medium (no peptone)**
Biomass (OD_{600})	3.76	2.49	2.48
Sucrose ($g.L^{-1}$)	0.63	2.48	5.1
Lactate ($g.L^{-1}$)	27	28.8	27.9
Compound A activity ($IU.g^{-1}$)	6042	6320	5590

It appears that peptone is not needed as a nitrogen source, since compound A production doesn't suffer significant changes. Moreover, although the biomass values are shown to be slightly lower, the reduction of YEP concentration may be feasible to maintain the same compound A activity.

These experiments were performed only once and so it would be interesting to continue this studies to confirm this data. Other experiments should also be made, reducing

the amount of YEP in the reference medium so the results could be better compared.

Conclusions and Recommendations

Several experiments were performed to test the effect of cultivation conditions on *Lactococcus lactis* for the production of the bacteriocin in study. Its antimicrobial activity plays an important role in food preservation and so it is of best interest to achieve the best conditions for compound A production.

All anaerobic fermentations performed appeared to confirm the literature statements that compound A is produced as a primary metabolite, since it is directly related to cell growth. The highest compound A activity results were almost always obtained at the end of the exponential phase, and the bacteriocin production frequently stopped when the cells entered the stationary growth phase. In this sense, it can be concluded that the end of the exponential phase would be the best time to harvest the broth, in order to achieve maximum specific compound A activity.

The effect of the carbon source concentration in anaerobic fermentations demonstrated that low sucrose concentrations (1%, 2%) result in lower compound A activity values, as expected, because of the fast depletion of the C-source. On the other hand, concentrations higher than 4% of sucrose appear not to increase the bacteriocin's activity, possibly due to substrate inhibition. According to the experiments performed, 3% of sucrose seems to be the most effective concentration of substrate, since it appears to deliver shorter fermentation times with a reduced tailing effect an with unchanged compound A activity and, consequently, fewer costs associated.

However, all of these results should be confirmed with more experimental data to obtain more reliable conclusions and it would be interesting to test a different range of sucrose concentrations (e.g. 1.5, 2, 2.5, 3, 3.5%) to define the optimal concentration for this type of fermentation.

Varying the pH in anaerobic fermentations showed that at pH 5 the cell growth is inhibited and compound A production is lower, as expected. On the other hand, at pH 7, the bacteriocin's activity results were also low, possibly demonstrating the attachment of compound A to the cells. However, some in house studies were made previously to this work and adsorption to biomass could not be reproduced, nor its relationship to pH. Therefore, this effect should be tested again, in order to better understand what happens when the pH is higher than 6 and its influence on compound A production.

In the end, no big difference was found between pH controlled anaerobic fermentations at pH 5.5 and 6, meaning that this range constitutes the adequate one for higher compound A production.

The medium composition is one parameter of great importance on compound A production since it influences greatly the biomass formation and the purpose is to find the best recipe (supplying the carbon and nitrogen sources and all the nutrients necessary for cell growth) that is cost effective. In this sense, some experiments were done reducing the YEP concentration in the medium, since it's the compound with largest influence on the medium costs. Comparing the results of using a complex medium (CM) with

half of the YEP concentration and the same medium without peptone (the other nitrogen source besides YEP), it appears that compound A activity values were very similar. This fact may indicate that no other nitrogen source is needed, besides YEP, and that the reduction of its concentration may be feasible, with similar levels of the bacteriocin.

As mentioned above, these conclusions were made regarding the complex medium. It is, then, recommended that the same study is performed with the reference medium used in the other anaerobic fermentations, to confirm if the reduction of YEP is feasible.

Another recommendation, concerning the medium composition, would be to perform further investigation on the influence of reducing the amount of $CaCl_2$ added. Previous studies showed the importance of adding this compound, since it reduces the tailing effect during fermentation and it appears to slightly improve compound A production, in some *L. lactis* strains. However, it was also shown to affect negatively the meat processing and therefore no/or minimal amount of $CaCl_2$ is desired in compound A end product.

Several other parameters may also influence the biomass formation and could not be performed in this study, like the type of base added to control the pH (or the addition of a mix of different bases) and the type of substrate (e.g. glucose), for example. It would also be interesting to test its effect on compound A production in order to obtain the best, cost effective, recipe.

References

[1] Yang, Z. (2000). Antimicrobial compounds and extracellular polysaccharides produced by lactic acid bacteria: structures and properties. Academic Dissertation. Department of Food Technology, University of Helsinki.

[2] De Vuyst, L., Leroy, F. (2007). Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications. *Journal of Molecular Microbiology and Biotechnology*, 13: 194-199

[3] Neubauer, A., (2010). Intermediate TTR CMX. Corbion.

[4] De Vuyst, L., Callewaert, R., Crabbé, K., (1996). Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology*, 142: 817-827.

[5] De Vuyst, L., Vandamme, E. J., (1994). Bacteriocins of Lactic Acid Bacteria.

[6] Stuart, M. R., Chou, L. S.,Weimer, B. C., (1999). Influence of Carbohydrate Starvation and Arginine on Culturability and Amino Acid Utilization of *Lactococcus lactis* subsp. *Lactis*. *Applied and Environmental Microbiology*, 65(2): 665-673.

[7] Yang, R., Johnson, M. C., Ray, B., (1992). Novel Method to Extract Large Amounts of Bacteriocins from Lactic Acid Bacteria. *Applied and Environmental Microbiology*, 58: 3355-3359.

[8] Wikipedia, “Yeast Extract”, http://en.wikipedia.org/wiki/Yeast_extract

[9] BD Biosciences, “Bacto-Peptone”, https://www.bdbiosciences.com/documents/Bacto_Peptone.pdf