IMPROVEMENT OF RECOMBINANT CELLULASE A PRODUCTION BY *STREPTOMYCES LIVIDANS* TK24: STUDY ON THE EFFECT OF MEDIUM COMPOSITION

Joana Basílio

Instituto Superior Técnico, University of Lisbon, Lisbon, Portugal

Abstract

Streptomyces lividans has been previously successfully used for the heterologous production of many enzymes. An important factor that extremely affects this production is the medium composition. Depending on the microorganism and target biomolecule the optimal medium may change significantly. Previous studies proved that amino acids play a major role in the matter.

The aim of this study was to improve cellulase A (celA) production from the thermophilic Eubacterium *Rhodothermus marinus* using recombinant *S. lividans* TK24 as the host. Different defined and complex media suitable for this species submerged cultivation were tested using aerated bioreactors in batch mode. CelA was quantified at different experimental points and amino acids medium composition and uptake profile were determined. Readily available nutrients namely glucose proved to increase cellular growth but led to a lower celA yield and resulted in organic acids overflow. Aspartate and glutamate were confirmed to be used for biomass formation. The medium which achieved the highest celA concentration was "Double" Nutrient Broth (2NB) yielding 100mg/L. It was proved that beef extract was the key constituent to this higher cellulase production. Amino acids analysis showed that glutamine and tryptophan adequate concentration in the medium is most likely essential to enhance celA production.

This study revealed once more the specificity of medium composition on heterologous production. The findings obtained should help to develop an even better cultivation medium for celA production. A high quality and cheap cellulase could significantly decrease the bioethanol producing cost and make it a more competitive alternative fuel.

Key words: cellulase A, *Streptomyces lividans*, heterologous production, medium composition, amino acids use, organic acids.

1. Introduction

Streptomyces are gram-positive bacteria which belong to the *Actinobacteria* phylum (*Muhamadali et al*., 2015). These filamentous and spore-producing bacteria can be found in soil and decaying vegetation (*Anné et al*., 2012). *Streptomyces* have a great importance in the agricultural, environmental and pharmaceutical industries since they synthesize and secrete many antibiotics and they are even considered as the largest microbial producer of antibiotics (*D'Huys et al*., 2011; *Muhamadali et al*., 2015). When grown in liquid media this genus tends to form a hyphae network (*Anné et al*. 2012). Not only antibiotics can be produced but also other secondary metabolites and recombinant proteins (e.g. enzymes) with economic value that are secreted to the fermentation medium at high concentrations (*Anné et al*. 2012).

Streptomyces lividans is considered the "most versatile" species for the recombinant production of proteins of this genus (*Noda et al*., 2015) even though the most studied one is *Streptomyces coelicolor* (*Muhamadali et al*., 2015). Although *Escherichia coli* has been widely used for heterologous production, the frequent formation of inclusion bodies (amongst other problems) still complicates the downstream of the production process (*Sevillano et al.*, 2016). *S. lividans* on the contrary does not usually form inclusion bodies, is adequate for the expression of GC-rich genes (*Sevillano et al*., 2016), secretes biologically active proteins (even eukaryotic) at high concentrations that are easy to recover from the cultivation medium, has low protease activity and is biologically safe (*D'Huys et al*., 2011).

An important factor that affects not only cellular growth but also protein production is the composition of the medium used. A medium that is capable of achieving a high biomass production does not mean that it will also lead to a high protein production. So, it is necessary to test different types of media either defined or complex in order to achieve the goal of increasing the yield. A previously used approach was to study the amino acids uptake of a medium containing casein amino acids with the aim of discovering the individual role of each amino acid in the heterologous production of murine Tumor Necrosis Factor Alpha (mTNFα) by *S. lividans* TK24 (*D'Huys et al*., 2011).

According to ResearchandMarkets.com, the technical enzymes' market which covers all types of industrially used enzymes with varied applications from paper, detergents to textile, is expected to have a compound annual growth rate (CAGR) of 3.4% between 2016 and 2021 achieving a size of 1.27 billion USD in 2021. The application that is stated as the "fastest-growing" in this market is bioethanol (*Research and Markets*, 2016). Bioethanol is obtained by the fermentation of sugars originated from the depolymerisation of cellulose or hemi-cellulose polymers by cellulosic enzymes. Published studies reveal that the cellulase production has a major role in the total bioethanol producing cost (*Johnson*, 2016). This means that finding a cheapest, more efficient way to produce high quality cellulases would make bioethanol a more competitive alternative and ecological source of fuel.

This study focused on the improvement of the cellulase A (celA) from the thermophilic Eubacterium *Rhodothermus marinus* (*Hreggvidsson et al*., 1996; *Halldoársdoáttir et al*., 1998) heterologous production using *S. lividans* TK24 as the host microorganism. To achieve so different types of medium were tested from defined media containing glucose and amino acids to well-known complex media. Amino acids uptake data was obtained for some of the media tested in order to understand which ones had a major impact in the cellulase production.

2. Materials and methods

All reagents were purchased from Sigma-Aldrich® unless stated otherwise.

2.1 Bacterial strain

The bacterial strain used in this study was *Streptomyces lividans* TK24 pIJ486 *vsi* (2)/celA secreting celA (*Hreggvidsson et al*., 1996; *Halldoársdoáttir et al*., 1998). The plasmid contains a thiostrepton resistance marker and the *celA* gene cloned behind the *Streptomyces venezuelae* subtilisin inhibitor (vsi) promoter (*Van Mellaert et al*., 1998). The strain was gently provided by Ólafur Friðjónsson from Matís, Iceland. The stock was stored in 20% (v/v) glycerol at - 80^{o}C.

2.2 Media

Different types of medium were tested both defined and complex media: Minimal medium (MM) (10g/L glucose, 3g/L $(NH_4)_2SO_4$, 2.6g/L K_2HPO_4, 1.8g/L NaH_2PO_4, 0.6g/L $MgSO_4.7H_2O$, 25mL/L minor elements solution (40mg/L $ZnSO_4.7H_2O$, 40mg/L $FeSO_4.7H_2O$, 40mg/L $CaCl_2$, 40mg/L $MnCl_2.4H_2O$)), MM+ASP2,5 (MM supplemented with 2.5g/L aspartate), MM+GLU2,5 (MM supplemented with 2.5g/L glutamate), MM+CAS5 (MM supplemented with 5g/L Bacto™ casamino acids), MM+CAS15 (MM supplemented with 15g/L Bacto™ casamino acids), Tryptic Soy Broth (TSB) (30g/L), Bennett (*Atlas et al*., 2010) (10g/L glucose, 2g/L LAB M™ tryptone, 1g/L yeast extract, 1g/L beef extract), Nutrient Broth (NB) (8g/L), PEP5+BEE3 (5g/L peptone and 3g/L beef extract), PEP5 (5g/L peptone), BEEF3 (3g/L beef extract), MM+PEP5+BEEF3 (MM supplemented with 5g/L peptone and 3g/L beef extract), MM+PEP5+MEAT3 (MM supplemented with 5g/L peptone and 3g/L meat extract) and "Double" Nutrient Broth (2NB) (16g/L). MM is a modification of the minimal liquid medium (NMMP) reported in Kieser et al. (2000).

2.3 Bioreactor experiments

2.3.1 Pre-culture preparation

The pre-culture was prepared by inoculating 100mL of PHAGE medium (*Korn et al*.,1978) (10g/L glucose, 5g/L LAB M™ tryptone, 5g/L yeast extract, 5g/L OXOID Lab lemco, 0.74g/L $CaCl_2.2H_2O$, 0.5g/L $MgSO_4.7H_2O$), containing 10µg/L of Calbiochem® thiostrepton (50µg/mL in DMSO) for plasmid maintenance, and incubating it for 72h at 30°C and 500rpm using a Heidolph stirrer. 25mL of the pre-culture were centrifuged for 20min at 20°C and 3200rcf. The pellet obtained was resuspended in 100mL of PHAGE medium and added to a shake-flask containing 10µg/L of Calbiochem® thiostrepton (50µg/mL in DMSO). This culture was incubated for 24h at 30°C and 500rpm.

2.3.2 Inoculum preparation

The pre-culture was centrifuged four times, the first for 20min at 20°C and 3200rcf. The subsequent were performed for 15min in the same conditions. Each time the pellet was kept and washed with minimal medium without glucose. 2x 37.5mL were taken from the pre-culture washed and resuspended in 9mL with 3 mL being added to each reactor.

2.3.3 Cultivation conditions

The experiments were carried out in batch mode using Eppendorf DASGIP® bioreactors with a 2L capacity, coupled with pH and dissolved oxygen (DO) probes, and a temperature sensor. Agitation was executed by 2 Rushton impellers with 6 blades each, aeration was provided by a L-sparger and the temperature was mantained by the DASGIP® Bioblock . For the experiments the reactors were filled with 1L of the tested medium. The culturing was performed at a constant temperature of 30°C, 500 rpm and pH 6.8 (controlled by the automated addition of 2M H_2SO_4 or 4M KOH). Calbiochem® thiostrepton (50µg/mL in DMSO) to a concentration of 10µg/L and 500µL of antifoam Y-30 emulsion were added to each reactor at the beginning of each experiment. The foam was controlled by adding antifoam when necessary. Carbon dioxide and oxygen concentrations were monitored using the Eppendorf DASGIP® GA4 gas analyser at the outlet air of each bioreactor.

2.3.4 Samples

During the experiments 10mL samples were collected at different time points and centrifuged for 15min at 20°C and 3200rcf. The pellet was kept for dry cell weight (DCW) quantification. The supernatant was filtered using a Sarstedt Filtropur 0,2µm PES filter and 1mL of each sample was transferred to a reaction tube and concentrated using a LABCONCO CentriVap® concentrator for celA quantification. The remaining filtered supernatant was stored at 8°C.

2.4 Analytical methods

The samples collected throughout the experiments were analysed in order to determine biomass, organic acids, glucose, amino acids and celA concentration.

2.4.1 Dry cell weight quantification

In order to quantify the dry cell weight (DCW) during the experiment, the pellet from the samples collected was resuspended in ultra-pure water (Arium 611 DI water) and filtered with vacuum using a 0,2mm pore size MN PORAFIL® CM predried and preweighted filter. The filter was once more dried (overnight 12-24h in an oven at 105°C) and weighted for DCW determination.

2.4.2 Organic acids and glucose assay

To determine the amount of some organic acids (α-ketoglutaric acid, pyruvic acid, succinic acid, latic acid, acetic acid) and glucose in the fermentation broth, the filtered supernatant of the samples collected was subjected to High Performance Liquid Chromatography (HPLC) using a BIO-RAD HPX-87H (300 mm x 7.8 mm) column. The samples were kept at 4°C and 20µL were injected into the column. The separation was performed at 40°C for 30min using a solution of 5mM H_2SO_4 as the elution buffer with a flow rate of 0.6mL/min. The compounds were detected by the use of a Refractive Index Detector (RID) at 35°C and Diodide Array Detector (DAD) at 210nm.

2.4.3 Amino acids measurements

The concentration of some free physiological amino acids present in the media tested and in the fermentation broth throughout the experiments time was determined using the Phenomenex EZ:faast™ GC-FID kit. 2µL of the derivatized sample were injected into a Zebron ZB-AAA (10m x 0.25mm) GC column with a 1:15 split injection ratio at an inlet temperature of 250°C. The initial oven temperature was 110°C which increased 32°C/min to the final target temperature of 320°C. The carrier gas used was helium with a constant pressure of 60kPa. The Flame Ionization Detector (FID) was at 320°C.

2.4.4 celA quantification

For the quantification of the celA present in the fermentation broth, at certain time points of the experiments, Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) followed by Western-blot was performed.

SDS-PAGE

This technique was performed using a Biocom Direct Omnipage Electrophoresis system. The resolving gel had a

concentration of 17% in acrylamide. CelA samples preparation included the addition of Arium 611 DI water and heating/mixing in an Eppendorf ThermoMixer® F1.5 for 30min at 37ºC and 1500rpm. These and calibration samples were diluted to a ratio of 1:1 with a mixture of 40µL 2-mercaptoethanol and 760 µL Lamelli sample buffer. Subsequently all the samples were heated at 95ºC for 5min in an Eppendorf ThermoMixer® F1.5. 20µL of the prepared samples were appointed in each gel well. The electrophoresis was carried out for 75min at maximum 200V or 100W.

Western-blot

This wetblotting technique was executed using a Biocom Direct Omnipage Blotting system. The transfer was performed using a 0,2µm Amersham Protran Nitrocelulose membrane. The protein transfer was carried out for 1 hour at maximum 100V or 400W. The membrane was removed from the cassette and washed with a 5% milk powder solution in TTBS buffer to block the membrane and reduce background noise. The membrane was washed again with TTBS buffer and incubated overnight with a 1% milk powder solution in TTBS buffer containing the primary antibody (rabbit-derived specific to celA provided by Dr.Lily Karamanou from Rega Institute, KU Leuven, BE). The membrane was washed with TTBS buffer. The next step was the incubation with a 1% milk powder solution in TTBS buffer containing the secondary antibody (anti-rabbit IgG-peroxidase) for 1 hour. Subsequently the membrane was washed with TTBS buffer and kept in this buffer until the detection was performed. All the washing and incubations were performed at 8ºC and 240rpm using a shaker. For the detection the Charged Coupled Divice (CCD) camera of the GE Image Quant LAS 4000 was cooled to -25ºC and the blot was drained with a mixture of Thermo Fisher SuperSignal® reagents. For quantification paint.net v4.0.9 and ImageJ 1.50g software were used.

3. Results

The experimental data obtained from the experiments performed is shown in figures and analysed in the different sections of the results on DCW, glucose, organic acids, amino acids composition and profiles and celA production.

The first media tested were MM, MM+ASP2,5, MM+GLU2,5, and MM+CAS5 since the use of media containing significant amounts of amino acids has previously been used as a strategy to improve the heterologous protein yield in *S. lividans* (*Pozidis et al*., 2001) and aspartate and glutamate were previously determined as favourably uptake by *S. lividans* TK24 (*D'Huys et al*., 2011). As there was no significant increase in celA production with supplements compared to MM, the media MM+CAS15, TSB which has been widely used in recent publications for recombinant protein expression in *Streptomyces* (*Sugimori et al*., 2012; *Li et al,*. 2013; *Noda et al*. 2015) and NB another complex medium referred by Kieser et al. (2000) as a suitable liquid medium for *Streptomyces* growth were tested. NB revealed a higher production, so another medium with beef extract in its composition was tested (Bennett). In order to assess the effect of the addition of glucose to a medium containing beef extract and peptone (NB recreation) in celA production MM+PEP5+BEEF3 and MM+PEP5+MEAT were also used (it was not clear the difference between beef and meat extract from Sigma-Aldrich®). Finally to discover why NB led to a higher production its individual components were studied separately (PEP5 and BEEF3), to understand if it was possible to recreate the already prepared sold NB by joining its constituents the medium PEP5+BEEF3 was tested and to check the effect on cellular growth and celA production due to the use of the double medium amount compared to NB, 2NB was also tested.

3.1 Dry cell weight

Figure 4.1 gives some ideas about the medium impact on cellular growth. The addition of aspartate (MM+ASP2,5) and glutamate (MM+GLU2,5) to MM seem to have almost no effect on DCW with all the media achieving around 3g/L (to be noticed that cells grown on MM+ASP2,5 were not yet stationary). The addition of casamino acids (MM+CAS5) increased biomass creation (4.54g/L) when compared to MM, while the addition of the triple amount of this mixture (MM+CAS15) had not even a double effect (5.60g/L but not yet stationary). TSB although having a high amount of casein peptone in its composition (17g/L) which implicates high quantities of amino acids, did not have such a high DCW as MM+CAS15 probably due to having only a quarter of glucose amount compared to the latter. Bennett also possesses the same quantity of glucose as MM and achieved 2.55g/L, however the cells grown in this medium had a very different behaviour from the rest, that makes it not possible to infer anything about it. The PEP5 and BEEF3 medium reached respectively 0.72g/L and 0.65g/L of DCW, so it might had been expected that PEP5+BEEF3 would generate the sum of these values. PEP5+BEEF3 did not attain a DCW very far away from the predicted reaching 1.54g/L, however it is not a perfect recreation of NB that reached only 1.07g/L which may be justified by the amino acids different composition (see Figures 3 and 4). 2NB produced more than the double biomass of NB with a DCW of 2.41g/L. The combination of MM and PEP5+BEEF3 (MM+PEP5+BEEF3) resulted in a DCW of 5.19g/L, revealing the impact of glucose on biomass production. Since the amino acids composition of MM+PEP5+BEEF3 and MM+PEP5+MEAT3 turned out to be very similar (see Figures 3 and 4), it is normal that the DCW of MM+PEP5+MEAT3 also did not differ a lot (5.18g/L)

3.2 Glucose

For all the tested media containing glucose in its composition (except for Bennett) it was observed that its depletion from the medium coincided with the end of the exponential growth phase and beginning of the stationary phase (data not shown).

3.3 Organic acids

Figure 2 shows the concentration profiles of organic acids for all the media (except lactic acid – not enough information). α-Ketoglutaric acid (Figure 2 A) presented values lower than 10mg/L except for BENNNET (73.69mg/L), MM+CAS5 (48.36mg/L), MM (23.59mg/L) and MM+CAS15 (23.13mg/L). It is possible that MM and MM+CAS15 reached higher vales since there is a time gap near the phase when values were increasing.

Pyruvic acid (Figure 2 B) also achieved high values for BENNET (1088.49mg/L). TSB reached a considerably high amount (522.68mg/L), however considering the initial value only around 150mg/L were excreted. For the minimal medium and MM supplemented with individual and casamino acids, pyruvic acid achieved values between 300 to 400 mg/L. On the other hand MM+PEP5+BEEF3 and MM+PEP5+MEAT3 only got to approximately 100mg/L. All the media without glucose in its composition attained very low values of this organic acid in the extracellular media, being 2NB the one that had the highest value of only 24.86mg/L. Succinic acid (Figure 2 C) had the highest excretion in MM+PEP5+MEAT3 and in MM+PEP5+BEEF3 already

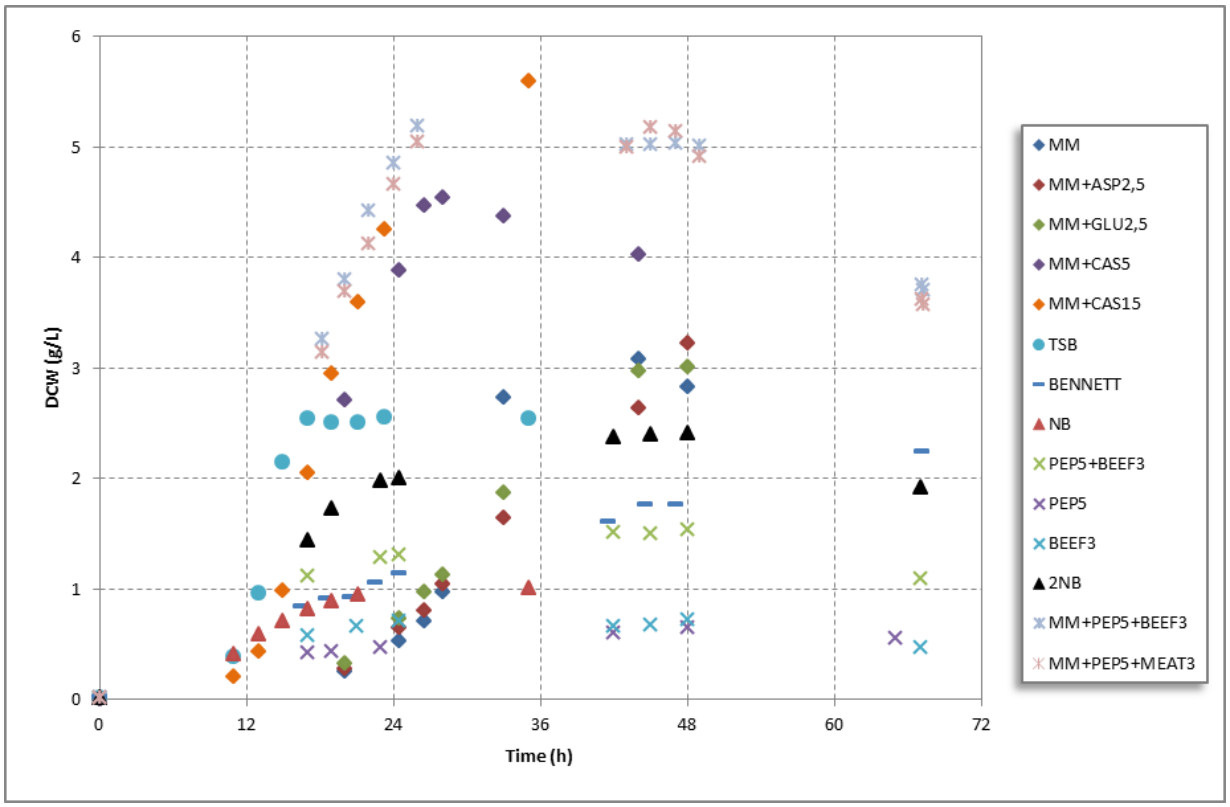

Figure 1 – DCW throughout time for all the media tested.

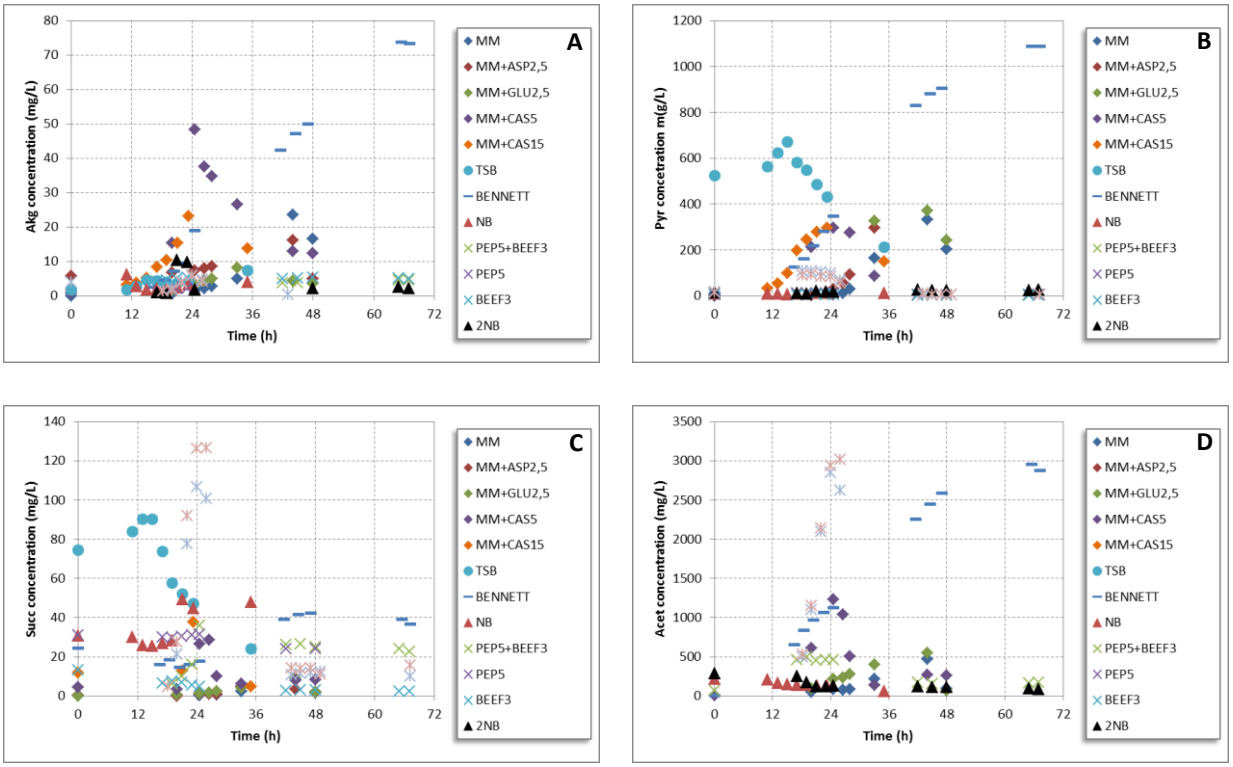

Figure 2 – Organic acids concentrations in function of time for all the media tested. Plot A: (Akg) α-ketoglutarate, Plot B: (Pyr) Pyruvate, Plot C: (Succ) Succinate, Plot D: (Acet) Acetate.

considering the initial values. MM+CAS5, MM+CAS15, TSB, Bennett and PEP5+BEEF3 achieved less than 40mg/L. MM, MM+ASP2,5 and MM+GLU2,5 attained less than 10mg/L while PEP5 and BEEF3 seemed to have no excretion throughout time. On the other hand NB reached around 50mg/L of this organic acid.
Acetic acid (Figure 2 D) achieved values higher than 500 mg/L in MM+PEP5+MEAT3 (3015.91 mg/L), Bennett (2950.72mg/L), MM+PEP5+BEEF3 (2849.27mg/L) and MM+CAS5 (1229.41 mg/L). Apart from Bennett these belong to the media that achieved higher DCW. NB and 2NB seem to have no excretion of this organic acid. For the rest of the media it was not possible to quantify the acetic acid presence.

3.4 Amino acids composition

Figure 3 shows the relative composition of the quantified amino acids in the media tested. Not all the media were subjected to amino acids analysis, only the media with higher cellulase production plus MM+CAS15 (includes a rich amino acids mixture) and PEP5. The inclusion of these media in the analysis is important to try to understand why some media had a higher production than others. It is possible to observe that the medium tested with the aim of recreating NB (PEP5+BEEF3) did not have the same amino acids composition, probably because the peptone and beef extract used (although also from Sigma-Aldrich® like NB) become from different sources. As expected the relative composition of NB and 2NB as well as PEP5+BEEF3 and MM+PEP5+BEEF3 had only slight discrepancies most likely related to sample handling. Even though MM+CAS5 was not analysed it is possible to include it in this comparison since it should have the same relative composition as MM+CAS15. Although Bacto™ casamino acids reportedly do not contain free tryptophan, asparagine or glutamine in its composition (*D'Huys et al*., 2011), the last two were detected using the EZ:faast™ GC-FID kit in low concentrations, most likely due to this samples freeze and thaw that made them release from proteins.

Some facts which should be important for discussion are: the relative composition in alanine and asparagine is significantly higher in NB, 2NB and BEEF3 when compared to the media with casamino acids; MM+CAS15 and consequently MM+CAS5 contain a very superior percentage of aspartate, glutamate and proline; NB and 2NB possess a glycine composition considerably higher than the remaining media and tryptophan is only present in media containing peptone, beef or meat extract. Also, NB/2NB, PEP5+BEEF3 and BEEF3 have a similar relative composition of tryptophan and asparagine.

Analysing the amount of each single amino acid for some of the media (Figure 4), it is clear that the media with casamino acids did not contain tryptophan as expected. MM+CAS15 and MM+CAS5 contained a higher amount for all the amino acids quantified except for asparagine, glutamine, glycine and tryptophan when compared to NB and 2NB. Comparing MM+CAS5 and NB, the former a media that was already in the late stationary phase at 44h and the latter a medium that had produced more celA only after 35h, is important to mention that MM+CAS5 has a noticeably higher amount of aspartate, glutamate, leucine, lysine, methionine, phenylalanine, proline, threonine and valine. The sum of the amounts of the media PEP5 and BEEF3 does not result in the values of PEP5+BEEF3 for almost all the amino acids, this may have been due to PEP5 sample freeze and thaw.

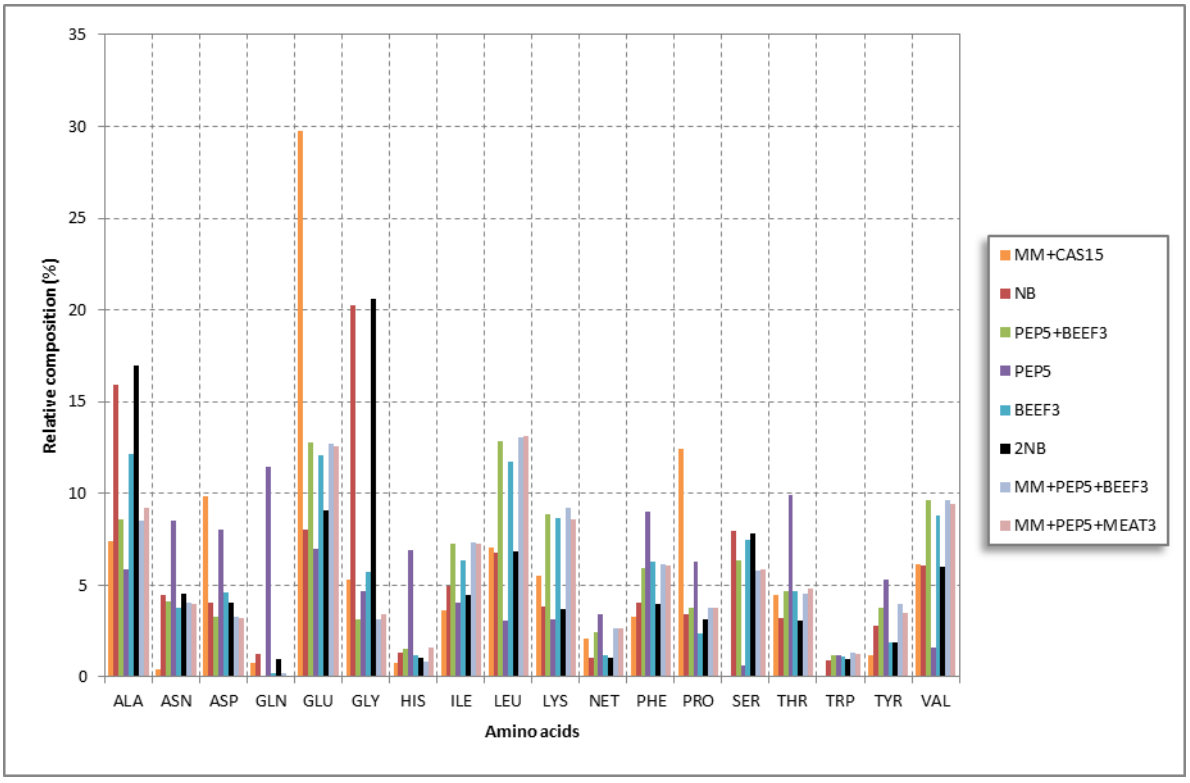

Figure 3 – Relative composition in amino acids for some of the media tested. (ALA) alanine, (ASN) asparagine, (ASP) aspartate, (GLN) glutamine, (GLU) glutamate, (GLY) glycine, (HIS) histidine, (ILE) isoleucine, (LEU) leucine, (LYS) lysine, (MET) methionine, (PHE) phenylalanine, (PRO) proline, (SER) serine, (THR) threonine, (TRP) tryptophan, (TYR) tyrosine and (VAL) valine.

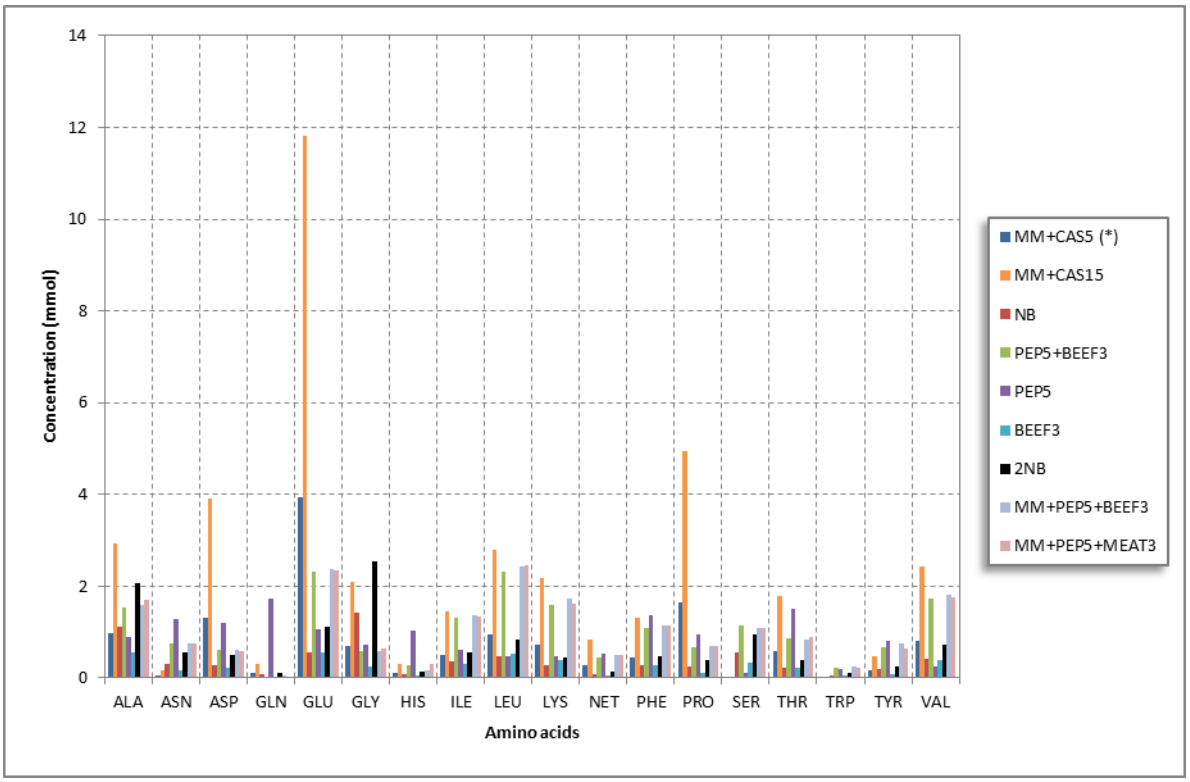

Figure 4 – Amino acids initial amount for some of the media tested. (ALA) alanine, (ASN) asparagine, (ASP) aspartate, (GLN) glutamine, (GLU) glutamate, (GLY) glycine, (HIS) histidine, (ILE) isoleucine, (LEU) leucine, (LYS) lysine, (MET) methionine, (PHE) phenylalanine, (PRO) proline, (SER) serine, (THR) threonine, (TRP) tryptophan, (TYR) tyrosine and (VAL) valine. (*) Not analysed values based on MM+CAS15. Note: glutamine was not possible to quantify for PEP5+BEEF3 and MM+PEP5+BEEF3 whereas serine for MM+CAS15.

3.5 Amino acids profile

The amino acids uptake profile was drawn using samples of some experimental time points for NB, 2NB, PEP5+BEEF3, PEP5, BEEF3, MM+PEP5+BEEF3 and MM+PEP5+MEAT3. Only the profile for 2NB is shown in Figure 6.

In Figure 6 is possible to check that alanine, leucine, serine, valine, glutamate, aspartate and asparagine were uptake during the exponential phase, while the remaining amino acids were consumed during the exponential and stationary phases. It is also clear that some amino acids were produced in the beginning (higher values at 19h): glycine, phenylalanine, lysine, threonine, proline, tryptophan, methionine, glutamine and tyrosine.

3.6 CelA production

The celA concentration for some experimental time points of all the media tested is displayed in Figure 7. It is clear that until 24h of cultivation there was no significant celA production. For NB after 35h 23.13mg/L had already been secreted. The later, TSB and MM+CAS15 have no more data beyond this period. After 48h from all the other media tested only PEP5+BEEF3, BEEF3 and 2NB achieved concentrations above 20mg/L, with 2NB even attaining 59.07mg/L. BEEF3 had at this point more than a half of this concentration whereas PEP5 only 0.73mg/L, which may implicate the beef extract importance in celA production. At around 67h cells grown on 2NB, PEP5+BEEF3, BEEF3, MM+PEP5+MEAT3 and MM+PEP5+BEEF3 were already in the cell death phase (see Figure 1), which might implicate that the high increase in celA concentration from 48h to this point could have been due to cellulase accumulation inside the cells and released due to cell lysis (except for BEEF3 – approximately the same concentration). Also MM+PEP5+BEEF3 had a lower celA concentration at 67h than PEP5+BEEF3 which may suggest the negative impact of glucose presence in the medium. It is interesting that the sum of the final celA concentration of PEP5 and BEEF3 results more or less on the amount produced by PEP5+BEEF3.

Figure 5 shows the celA and DCW maximum concentrations achieved for all the media tested. By looking at this data, it is possible to understand that higher DCW is not synonym of higher celA production. Also, the three higher producing media include beef extract in their composition but no glucose.

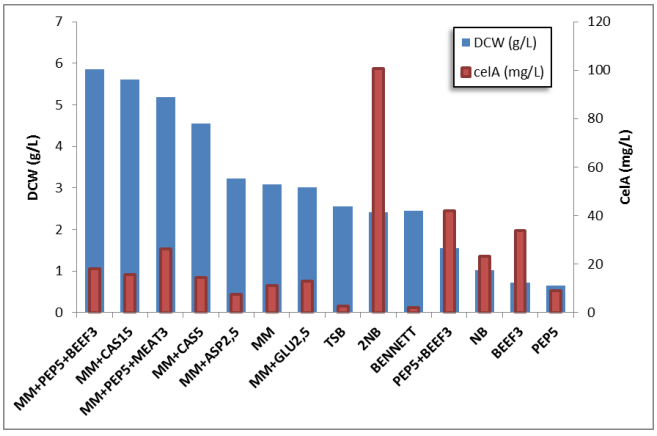

Figure 5 – Maximum DCW and celA concentration achieved for all the media tested.

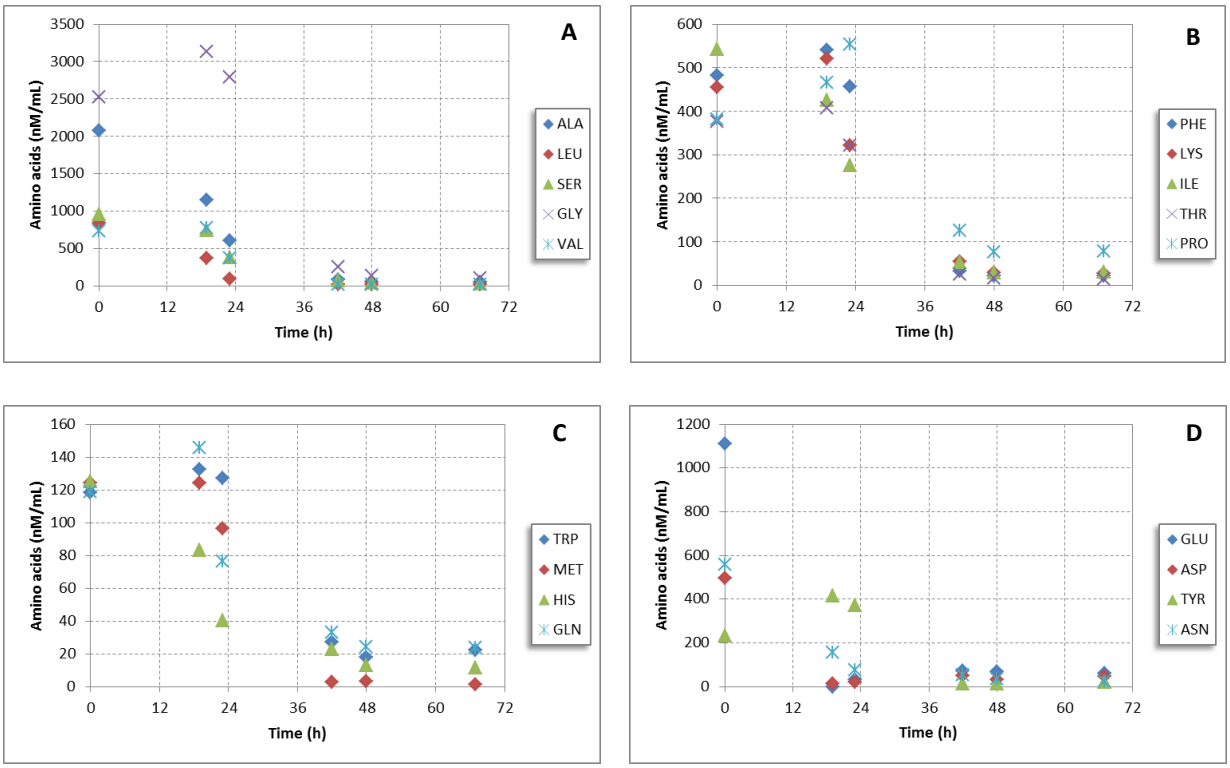

Figure 6 – Amino acids profile for recombinant celA secreting *S. lividans* TK24 grown on 2NB obtained with the EZ:faast™ GC-FID kit. Plot A: (ALA) alanine, (LEU) leucine, (SER) serine, (GLY) glycine and (VAL) valine. Plot B: (PHE) phenylalanine, (LYS) lysine, (ILE) isoleucine, (THR) threonine and (PRO) proline. Plot C: (TRP) tryptophan, (MET) methionine, (HIS) histidine and (GLN) glutamine. Plot D: (GLU) glutamate, (ASP) aspartate, (TYR) tyrosine and (ASN) asparagine.

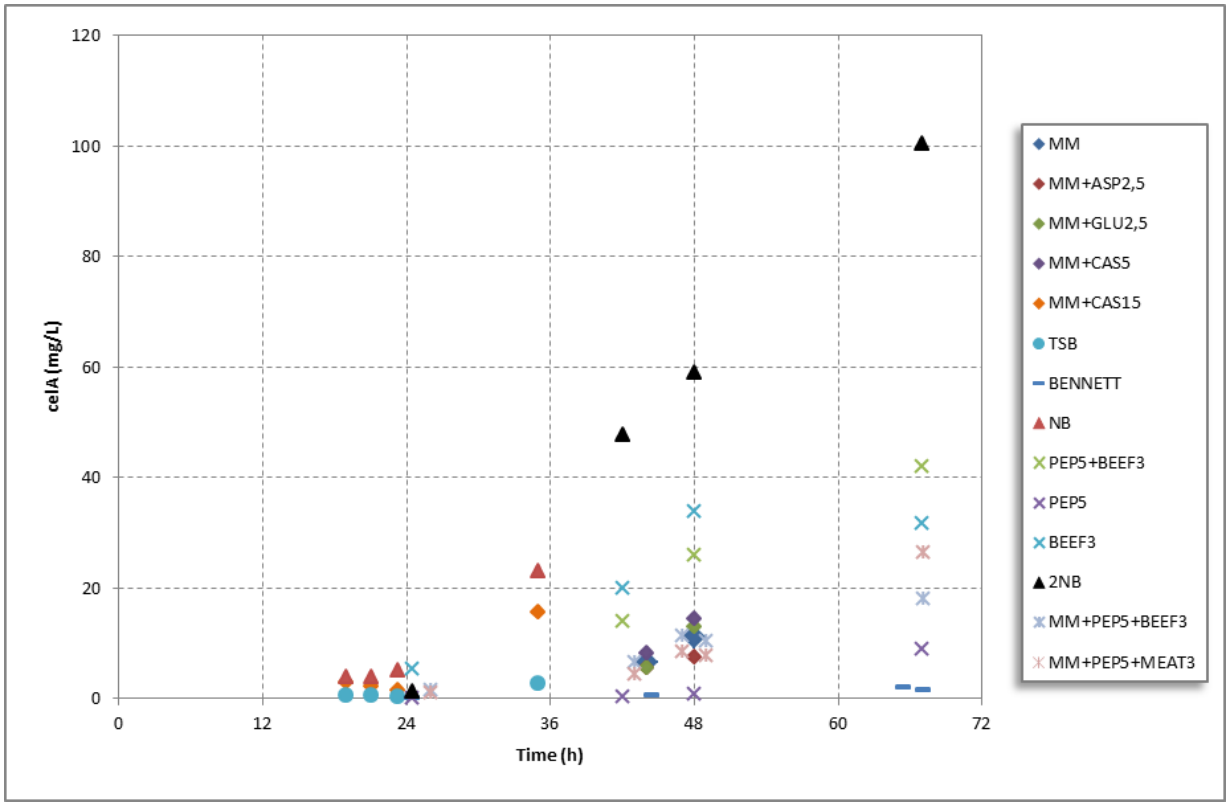

Figure 7 – CelA throughout time for all the media tested.

4. Discussion

4.1 Glucose addition mainly promotes biomass growth

Taking into account the data shown in the Results, it is clear that the presence of glucose in the medium effects on biomass growth, organic acids excretion and celA production. The media containing 10g/L glucose and supplemented with individual or amino acids mixtures (i.e., MM, MM+ASP2,5, MM+GLU2,5, MM+CAS5 and MM+CAS15) achieved some of the highest final biomass concentration as compared to the other media (see Figure 5). Glucose is an easily metabolized carbon source and growth on glucose goes along with organic acids secretion. Amongst the measured organic acids, acetic acid and pyruvic acid excretion were most abundant. Pyruvate is known to have a high excretion in *Streptomyces* species cultured in fast assimilated carbon sources like glucose (*Madden et al*., 1996) resulting from an imbalance among the glycolysis and the Tricarboxylic Acid (TCA) cycle, creating an extracellular carbon reserve to be used later (*Colombié et al*., 2005). Uptake of organic acids was observed when glucose was depleted. Since the total amount of organic acids was (in most cases) relatively low, the uptake did not generated observable growth. The re-uptake thus occurred in the stationary phase which was also confirmed by the acid addition to keep the pH constant as well as the CO_2 production was due to the metabolization of organic acids (data not shown). In almost all experiments, depletion of glucose led to the transition to the stationary phase. This matching between glucose depletion from the medium and the start of the stationary phase had already been identified in other recombinant *S. lividans* strains (*Nowruzi et al*., 2011).

4.2 Influence of organic acids secretion

CelA production was negatively influenced by the glucose presence in the media, or in other words, higher biomass yield was not a synonym for higher celA production. Furthermore it has already been proved that organic acids excretion, depending on the type of medium, could disturb the recombinant proteins production using *S. lividans* as the host (*Madden et al*., 1996). In our study, this is noticeably in the production of an excess of organic acids as observed in the Bennett medium slowed down biomass growth and resulted in a low celA yield (Figures 2 and 5).

Regarding the nature of the organic acids, acetate can be identified as a product of glucose excess in other *Streptomyces* species (*Colombié et al*., 2005), while lactate presence may be due to the fact that when grown on liquid medium these tend to form a hyphae network (*Anné et al*., 2012) which led to the occurrence of pellets and subsequently to insufficient oxygen transfer to its centre (*Vrancken et al*., 2009). Succinate excretion might as well be a consequence of the oxygen deficiency or caused by the imbalance among the glycolysis and the TCA cycle (*D'Huys et al.*, 2011).

Media composed solely of peptone and/or beef extract had low or no excretion of organic acids when compared to media that possess glucose. Typically, some organic acids were already present in the medium constituents. In combination with glucose, organic acids production was less as compared to MM with casamino acids. This may be one of the reasons why these media reached a higher celA yield. The comparison between the celA secretion obtained in media with only beef extract and/or peptone suggests that the former may be a key compound associated to celA production.

4.3 Role of amino acids in the medium

In order to understand why some media had higher cellulase production than others a factor that is important to consider is the nitrogen source (*D'Huys et al.*, 2011). For that, the free amino acids quantification of the media composition and utilization profiles obtained should help to solve this matter.

The media supplemented with Bacto™ casamino acids have simultaneously a high relative amino acids composition and amount of aspartate (9.81%), glutamate (29.75%) and proline (12.43%) when compared to the remaining media. Aspartate and glutamate are associated with *Streptomyces* growth. Aspartate is used as an energy source entering directly in the TCA cycle after deamination (*Madden et al.*, 1996; *Nowruzi et al*., 2008; *Nowruzi et al.*, 2011). The glutamate skeleton also enters in the TCA cycle, can be used both as a carbon or nitrogen source and donates nitrogen for the synthesis of 85% of nitrogen composed cell compounds (*Madden et al.*, 1996; *Voelker et al*., 2001; *Nowruzi et al.*, 2011). Aspartate and glutamate had been previously identified as preferential uptake by *Streptomyces* but reportedly had no effect in heterologous protein production (*D'Huys et al.*, 2011). These facts were once more confirmed in this study since these two amino acids were taken up in the early exponential phase (biomass formation) for all the media analysed with the EZ:faast™ GC-FID kit (data not shown) and the supplementation of media with Bacto™ casamino acids (contains a high amount of these amino acids) or aspartate (MM+ASP2,5) and glutamate (MM+GLU2,5) did not enhance celA production.

The PEP5 amino acids profile (data not shown) confirms the aspartate and glutamate contribute to growth in *S. lividans* since these are the only amino acids that were totally uptake in the exponential phase but also in the whole experiment for this media.

The media containing beef or meat extract have asparagine, glutamine and tryptophan in their compositions which are not present in Bacto™ casamino acids . It is interesting that the four media with higher celA production (2NB, PEP5+BEEF3, BEEF3 and NB) have a very similar asparagine and tryptophan relative composition.

CelA is composed of 260 amino acids (*Hreggvidsson et al*., 1996; *Halldoársdoáttir et al*., 1998) of these the most represented is alanine (31) followed by glycine (27). Asparagine (14), glutamine (2) and tryptophan (14) are also present (*National Center for Biotechnology Information*, 1999). Tryptophan besides being present in celA composition is also an amino acid with a known role in protein synthesis (*Hodgson*, 2000). Asparagine may be transformed into aspartate via asparaginase (*Hodgson*, 2000). While asparagine was mainly taken up in the early exponential phase, glutamine and tryptophan were only consumed in the late exponential or stationary phase when celA started to be detected in the extracellular media. Glutamine profiles of NB, BEEF3, (data not shown) and 2NB even reveal an overflow in the late exponential growth phase. These observations reveal that glutamine and tryptophan presence and/or relative composition must play an important role in celA production being probably uptake from the media for this purpose.

Alanine relative composition in NB, 2NB and BEEF3 is higher than in casamino acids containing media. Alanine is known for being a carbon and nitrogen source entering the TCA cycle by deamination to pyruvate (*Madden et al*., 1996; *Hodgson*, 2000; *Voelker et al*., 2001) and is the highest represented amino acid in celA. Although it was mainly taken up in the early exponential phase for NB, BEEF3 (data not shown) and 2NB it was not almost depleted as aspartate or glutamate which suggests that it may also

have a role in celA synthesis. If this can be proved, it may imply that also the components relative composition is a key factor.

The estimation of MM+CAS5 composition showed that it possesses a considerably higher amount of several amino acids (aspartate, glutamate, leucine, lysine, methionine, phenylalanine, proline, threonine and valine) when compared to NB. This in combination with glucose yielded more biomass but less celA which confirms once more that the high availability of nutrients (either carbon and/or nitrogen sources) inhibits this enzyme's production.

4.4 Secretion of endogenous antibiotics

The pink/red colour obtained in some of the cultures (NB, 2NB and Bennett) is most likely due to undecylprodigiosin (RED) production. This antibiotic associated with red pigmentation is typically produced by *S. lividans* TK24 (*Le Maréchal et al.*, 2013) in late growth usually in the stationary phase. Antibiotics that are produced in *Streptomyces* are either related to carbon, nitrogen or phosphorous source deficit (*Chouayekh et al.,* 2002). RED production is connected to a decrease in flux in the pentose phosphate pathway (*Avignone Rossa et al*., 2002). The lack of phosphate in the media composition is most probably the cause for its production since some of the amino acids present in the media where it occurred can act as both carbon and nitrogen sources but there was no phosphate addition. RED was visually not detected in any MM named media, even though it could be produced but in lower concentration not possible to detect with naked eye, due to the presence of ammonium that acts as a repressor and the great availability of nutrients that favours more biomass formation than secondary metabolites production (*Avignone Rossa et al*., 2002). RED precursors are proline, serine, glycine and acetyl-CoA (*Avignone Rossa et al*., 2002; *Chouayekh et al.,* 2002). It is possible to observe in the NB (data not shown) and 2NB amino acid profiles charts that glycine and proline suffered an increase in concentration and were only uptake in the late exponential or stationary phase which is probably related to precursors synthesis overflow for RED production. These two media simultaneously have a higher composition and total amount of glycine (except for MM+CAS15), a fact that may have influenced the production as well, since in other media with no phosphate addition the colour associated to RED was not observed.

4.5 Established maximum cellulase A concentration

Although the 2NB medium led to a significant concentration of celA production (~ 100mg/L) and the Western-blot technique whereby it was quantified suggests that the enzyme is most likely functional (*Ayadi et al*., 2007) a larger scale fermentation should be performed. Doing so it would be possible to design a purification method which would then allow to assess celA structure and biological function. Also, since all the blots included lower bands than the 28.8kDa celA (data not shown) probably due to proteases activity, a protease inhibitor should be added to the samples. The purification strategy used by Pozidis et al. (2001) for recombinant mTNFα expressed also with *S. lividans* TK24 could be adapted and applied to celA. The purified cellulase should then be subjected to structure analysis and its effect on cellulose degradation must be determined.

In order to check whether the significant increase in celA concentration between 48h and 67h for some media was a result of its secretion or intracellular release due to cell death which implicates cell lysis, these experiments should be repeated. More samples should be collected during this period and besides the enzyme quantification for the samples supernatant, cell lysates might also be prepared from the resulting pellet and analysed for celA content.

5. Conclusions

In this study, heterologous production of cellulase A by *S. lividans* TK24 was tested in several media in order to find a medium with a reasonable celA yield. Moreover, medium compositions and metabolic profiles were determined to obtain some understanding in the background of the observed yield differences amongst the media.

In a reference experiment in minimal medium (with glucose as a sole carbon source), a maximum celA concentration of 11.08mg/L and a celA yield of 3.60mg/g DCW was obtained. Addition of amino acids increased the biomass growth and the celA production, but the final celA concentration as well as the celA yield were strongly dependent on the source of amino acids. Casamino acids were less effective as compared to animal-derived amino acid mixtures (beef/meat extract). By applying "Double" Nutrient Broth, it was possible to improve celA heterologous production in *S. lividans* TK24 to 100.54mg/L (or 41.63mg/g DCW). A comparison of Nutrient Broth recreation with and without glucose showed that glucose addition increased biomass growth but not the cellulase A secretion.

The medium composition thus showed to be a crucial factor affecting the enzyme production. Recurring to analytical techniques, one can determine that the glucose presence in the medium and high availability of other nutrient sources (amino acids) results in organic acids overflow which has been previously reported to inhibit recombinant protein production in *S. lividans*. Aspartate and glutamate like in other studies were taken up by the bacteria for biomass formation and did not enhance the enzyme production. Higher biomass yield is not a synonym of higher celA yield, on the contrary. Beef extract revealed to have a positive contribution to the enzyme production with "Double" Nutrient Broth being from all the media tested the most suitable. The media composition analysis in amino acids showed that one important difference as compared to the other amino acids sources, i.e., glutamine and tryptophan are present in the medium, and are most likely necessary to enhance celA production.

Further studies should include celA quantification in media containing higher amounts of beef extract aiming to prove its importance in this enzyme production. By adding glucose to NB or 2NB in the same concentration as present in MM it would be possible to confirm its negative effect in celA yield. To prove that the basal minimal medium (all components except glucose) have no negative effect on the enzyme production this should be added and tested in all non-MM media. Since MM+PEP5+MEAT3 produces more celA than the equivalent medium using beef extract, the media MEAT3, PEP5+MEAT3 and media with higher meat extract concentration should also be tested. Considering the results obtained, media that produce overflow of organic acids to the extracellular media should be avoided.

A purification, structural and functional analysis protocol should be developed in order to evaluate this cellulase potential for larger scale production. Legislation about animal-derived medium constituents in enzymes production forbids its use in the case of the pharmaceutical or food industry (*Zhang et al.*, 1999). Although the celA potential use is not included in this type of industries, belonging to the technical enzymes, it is anyway recommended to avoid animal derived products like beef or meat extract. So taking into account the amino acids analysis, a chemical

defined medium with the same/or relative amino acids amounts of 2NB should be tested. Also considering the findings of this study it is recommended to test also minimal medium supplemented with glutamine, tryptophan and a combination of both. Another strategy could be to develop a design mixture study like the one performed by Nowruzi et al. (2008), in this case it would be possible to confirm if not identify which total amino acids amounts and relative amounts of each are essential to celA production.

Other possible approaches include metabolomics and fluxomics. By extending the extracellular metabolome study to other metabolites and quantifying intracellular metabolites and fluxes in celA secreting *S. lividans* TK24 together with the empty plasmid and wild type strain it should help to understand which metabolic pathways may be manipulated to generate a high efficient celA producing strain.

References

Anné, J., Maldonado, B., Van Impe, J., Van Mellaert, L., Bernaerts, K., 2012. Recombinant protein production and streptomycetes. Journal of Biotechnology 158, 159–167.

Atlas, R.M., 2010. Handbook of Microbial Media, Fourth Edition, CRC Press, ISBN: 978-1-4398-0408-7.

Avignone Rossa, C., White, J., Kuiper, A., Postma, P.W., Bibb, M., Teixeira de Mattos, M.J., 2002. Carbon flux distribution in antibiotic-producing chemostat cultures of *Streptomyces lividans*. Metabol. Eng. 4, 138–150.

Ayadi, D.Z., Chouayekh, H., Mhiri, S., Zerria, K., Fathallah, D.M., Bejar, S., 2007. Expression by *Streptomyces lividans* of the rat α integrin CD11b A-domain as a secreted and soluble recombinant protein. Journal of Biomedicine and Biotechnology, 6, Article ID 54327.

Chouayekh, H., Virolle, M-J., 2002. The polyphosphate kinase plays a negative role in the control of antibiotic production in *Streptomyces lividans*. Molecular Microbiology 43(4), 919–930.

Colombié, V., Bideaux, C., Goma, G., Uribelarrea, J.L., 2005. Effects of glucose limitation on biomass and spiramycin production by *Streptomyces ambofaciens*. Bioprocess Biosyst. Eng. 28, 55–61.

D'Huys, P-J., Lule, I., Van Hove, S., Vercammen, D., Wouters, C., Bernaerts, K., Anné, J., Van Impe, J.F.M., 2011. Amino acid uptake profiling of wild type and recombinant *Streptomyces lividans* TK24 batch fermentations. Journal of Biotechnology 152, 132–143.

Halldórsdóttir, S., Thórólfsdóttir, E.T., Spilliaert, R., Johansson, M., Thorbjarnardóttir, S.H., Palsdottir, A., Hreggvidsson, G.Ó., Kristjánsson, J.K., Holst, O., Eggertsson, G., 1998. Cloning, sequencing and overexpression of a *Rhodothermus marinus* gene encoding a thermostable cellulase of glycosyl hydrolase family 12. Appl. Microbiol. Biotechnol. 49, 277–284.

Hodgson, D.A., 2000. Primary metabolism and its control in Streptomycetes: a most unusual group of bacteria. In: Pools, R.K. (Ed.), Advances in Microbial Physiology, vol. 42. Academic Press, pp. 47–238, ISBN: 0-12-027742-5.

Hreggvidsson, G.Ó., Kaiste, E., Holst, O., Eggertsson, G., Palsdottir, A., Kristjánsson, J.K., 1996. An extremely thermostable cellulase from the thermophilic Eubacterium *Rhodothermus marinus*. Appl. Environ. Microbiol. 62(8), 3047–3049.

Johnson, E., 2016. Integrated enzyme production lowers the cost of cellulosic ethanol. Biofuels, Bioproducts & Biorefining 10(2), 164–174.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Practical *Streptomyces* Genetics. John Innes Center, Norwich.

Korn, F., Weingärtner, B., Kutzner, H.J., 1978. A study of twenty actinophages: morphology, serological relationship and host range. In: Freerksen, E., Tarnak, I.,Thumin, J.H. (Eds.), Genetics of Actinomycetales. Fisher G, Stuttgart/New York, pp. 251–270.

Le Maréchal, P., Decottignies, P., Marchand, C.H., Degrouard, J., Jaillard, D., Dulermo, T., Froissard, M., Smirnov, A., Chapuis, V., Virollec, M-J., 2013. Comparative proteomic analysis of *Streptomyces lividans* wild-type and ppk mutant strains reveals the importance of storage lipids for antibiotic biosynthesis. Applied and Environmental Microbiology 79 (19), 5907– 5917.

Li, J-X., Zhao, L-M., Wu, R-J., Zheng, Z-J., Zhang, R-J., 2013. High-level overproduction of *Thermobifida* enzyme in *Streptomyces lividans* using a novel expression vector. Int. J. Mol. Sci. 14, 18629–18639.

Madden, T., Ward, J.M., Ison, A.P., 1996. Organic acid excretion by *Streptomyces lividans* TK24 during growth on defined carbon and nitrogen sources. Microbiology 142, 3181–3185.

Muhamadali, H., Xu, Y., Ellis, D.I., Trivedi, D.K., Rattray, N.J.W., Bernaerts, K., Goodacre, R., 2015. Metabolomics investigation of recombinant mTNFα production in *Streptomyces lividans*. Microbial Cell Factories 14(157).

National Center for Biotechnology Information 1999, U.S. National Library of Medicine, accessed 10 October 2016, <https://www.ncbi.nlm.nih.gov/protein/AAB65594.1>.

Noda, S., Matsumoto, T., Tanaka, T., Kondo, A., 2015. Secretory production of tetrameric native full-length streptavidin with thermostability using *Streptomyces lividans* as a host. Microbial Cell Factories 14(5).

Nowruzi, K., Elkamel, A., Scharer, J.M., Cossar, D., Moo-Young, M., 2008. Development of a minimal defined medium for recombinant human interleukin-3 production by *Streptomyces lividans* 66. Biotechnology and Bioengineering 99 (1), 214–222.

Nowruzi, K., Elkamel, A., Scharer, J.M., Cossar, D., Moo-Young, M., 2011. Metabolic flux-based optimization of recombinant human interleukin-3 expression by *Streptomyces lividans* 66. The Canadian Journal of Chemical Engineering 9999, 1–10.

Pozidis, C., Lammertyn, E., Politou, A.S., Anné, J., Tsiftsoglou, A.S., Sianidis, G., Economou, A., 2001. Protein secretion biotechnology using *Streptomyces lividans*: large-ccale production of functional trimeric Tumor Necrosis Factor α. Biotechnology and Bioengineering 72 (6), 611–619.

Research and Markets, 2016. Technical Enzymes Market by Type (Cellulases, Amylases, Proteases, Lipases, Other Enzymes), Application (Bioethanol, Paper & Pulp, Textile & Leather, Starch Processing, Other Applications), & by Region - Global Forecasts to 2021, accessed 3 October 2016,<http://www.researchandmarkets.com/research/k56lv3/technical_enzymes>.

Sevillano, L., Vijgenboom, E., van Wezel, G.P., Díaz, M., Santamaría, R., 2016. New approaches to achieve high level enzyme production in *Streptomyces lividans*. Microbial Cell Factories 15(28).

Sugimori, D., Matsumoto, Y., Tomita, Y., Murayama, K., Ogino, C., 2012. Kinetic characterization and Mg2+ enhancement of *Streptomyces griseocarneus* sphingomyelinase C produced by recombinant Streptomyces lividans. Protein Expression and Purification 81, 151–156.

Van Mellaert, L., Lammertyn, E., Schacht, S., Proost, P., Van Damme, J, Wroblowski, B., Anné, J., Scarcez, T., Sablon, E., Raeymaeckers, J., Van Broekhoven, A., 1998. Molecular characterization of a novel subtilisin inhibitor protein produced by *Streptomyces venezuelae* CBS762.70. DNA Seq. 9(1), 19–30.

Voelker, F., Altaba, S., 2001. Nitrogen source governs the patterns of growth and pristinamycin production in *Streptomyces pristinaespiralis*. Microbiology 147, 2447–2459.

Vrancken, K., Anné, J., 2009. Secretory production of recombinant proteins by *Streptomyces*. Future Microbiol. 4 (2), 181–188.

Zhang, J., Greasham, R., 1999. Chemically defined media for commercial fermentations. Appl. Microbiol. Biotechnol. 51, 407–421.