Improvement of recombinant cellulase A production by *Streptomyces lividans* TK24: study on the effect of medium composition

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To my parents for giving me the opportunity to study abroad, for all the affection and support while away and for the encouragement in the hardest moments.
Resumo

A *Streptomyces lividans* tem sido utilizada com sucesso na produção heteróloga de várias enzimas. Um factor importante que afecta esta produção é a composição do meio. Dependendo do microorganismo e da biomolécula alvo, o meio óptimo pode mudar significativamente. Estudos anteriores provaram que os aminoácidos têm um papel importante no assunto.

O objectivo deste estudo foi o aumento da produção da celulase A (celA) do termófilo Eubacterium *Rhodothermus marinus* utilizando a *S. lividans* TK24 recombinante como hospedeiro. Foram testados diferentes meios, de composição conhecida e complexos, adequados ao seu cultivo submerso em modo descontínuo usando bioreactores arejados. A celA foi quantificada e a composição do meio em aminoácidos e respectivo perfil de consumo foram determinados para diferentes pontos experimentais. Provou-se que nutrientes prontamente disponíveis, nomeadamente a glucose, aumentavam o crescimento celular, diminuíam o rendimento de celA e resultavam num excesso de ácidos orgânicos. Confirmou-se o uso de aspartato e glutamato na formação de biomassa. O meio que atingiu a maior concentração de celA foi o Duplo "Nutrient Broth" (2NB) rendendo 100mg/L. Ficou provado que o extracto de carne bovina foi o constituinte chave para esta maior produção. A análise de aminoácidos mostrou que uma concentração adequada de glutamina e triptofano no meio é provavelmente essencial para aumentar a produção.

Os conhecimentos obtidos devem ajudar a desenvolver um ainda melhor meio de cultivo para a produção de celA. Uma celulase de qualidade e barata poderá diminuir significativamente o custo de produção do bioetanol tornando-o num combustível alternativo mais competitivo.

**Palavras-chave:** celulase A, *Streptomyces lividans*, produção heteróloga, composição do meio, utilização de aminoácidos, ácidos orgânicos.
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 strength 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.
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<th>Definition</th>
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<tbody>
<tr>
<td>acet</td>
<td>acetate</td>
</tr>
<tr>
<td>akg</td>
<td>α-ketoglutarate</td>
</tr>
<tr>
<td>ALA</td>
<td>alanine</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ASN</td>
<td>asparagine</td>
</tr>
<tr>
<td>ASP</td>
<td>aspartate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>C</td>
<td>measured concentration</td>
</tr>
<tr>
<td>c</td>
<td>vector with the concentration of the intercellular metabolites</td>
</tr>
<tr>
<td>CA</td>
<td>clauvlic acid</td>
</tr>
<tr>
<td>celA</td>
<td>cellulase A</td>
</tr>
<tr>
<td>CE-MS</td>
<td>capillary electrophoresis-mass spectrometry</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CPC-A</td>
<td>weighted-consensus principal component analysis</td>
</tr>
<tr>
<td>DAD</td>
<td>diode array detector</td>
</tr>
<tr>
<td>DCW</td>
<td>dry cell weight</td>
</tr>
<tr>
<td>DCW₀</td>
<td>initial dry cell weight</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DO</td>
<td>dissolved oxygen</td>
</tr>
<tr>
<td>FA</td>
<td>feed flow rate of acid</td>
</tr>
<tr>
<td>FB</td>
<td>feed flow rate of base</td>
</tr>
<tr>
<td>FBA</td>
<td>flux balance analysis</td>
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<tr>
<td>FT-IR</td>
<td>Fourier transform-infrared spectroscopy</td>
</tr>
<tr>
<td>GC-FID</td>
<td>gas chromatography-flame ionization detector</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GLC</td>
<td>glucose</td>
</tr>
<tr>
<td>GLN</td>
<td>glutamine</td>
</tr>
<tr>
<td>GLU</td>
<td>glutamate</td>
</tr>
<tr>
<td>GLY</td>
<td>glycine</td>
</tr>
<tr>
<td>HILIC-MS</td>
<td>hydrophilic interaction liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HIS</td>
<td>histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ILE</td>
<td>isoleucine</td>
</tr>
<tr>
<td>lac</td>
<td>lactate</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LEU</td>
<td>leucine</td>
</tr>
<tr>
<td>ln</td>
<td>natural logarithm</td>
</tr>
<tr>
<td>LYS</td>
<td>lysine</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>MET</td>
<td>methionine</td>
</tr>
<tr>
<td>MFA</td>
<td>metabolic flux analysis</td>
</tr>
<tr>
<td>mTNFα</td>
<td>murine Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate hydrogen</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OTR</td>
<td>oxygen transfer rate</td>
</tr>
<tr>
<td>PCA</td>
<td>principle component analysis</td>
</tr>
<tr>
<td>PHE</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>partial least squares discriminant analysis</td>
</tr>
<tr>
<td>PPP</td>
<td>pentose phosphate pathway</td>
</tr>
<tr>
<td>PRO</td>
<td>proline</td>
</tr>
<tr>
<td>pyr</td>
<td>pyruvate</td>
</tr>
<tr>
<td>RED</td>
<td>undecylprodigiosin</td>
</tr>
<tr>
<td>RID</td>
<td>refractive index detector</td>
</tr>
<tr>
<td>S</td>
<td>stoichiometric matrix</td>
</tr>
<tr>
<td>Sav</td>
<td>streptavidin</td>
</tr>
<tr>
<td>Sav&lt;sup&gt;core&lt;/sup&gt;</td>
<td>streptavidin core region</td>
</tr>
<tr>
<td>Sav&lt;sup&gt;nat&lt;/sup&gt;</td>
<td>streptavidin whole protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SER</td>
<td>serine</td>
</tr>
<tr>
<td>Sm</td>
<td>stoichiometric matrix of measured fluxes</td>
</tr>
<tr>
<td>Su</td>
<td>stoichiometric matrix of unmeasured fluxes</td>
</tr>
<tr>
<td>succ</td>
<td>succinate</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>TCA</td>
<td>trycarboxylic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
</tbody>
</table>
THR – threonine
TRP – tryptophan
TTBS – tris-tween-buffer-saline
TYR – tyrosine
v – flux vector
VAL – valine
VIP – variable importance in projection
v_m – vector of measured fluxes
v_{m,max} – maximum measured flux
v_{m,min} – minimum measured flux
v_{max} – maximum flux values
v_u – vector of unmeasured fluxes
WT – wild type
XCO_2 – carbon dioxide concentration
Y_{celA/X} – cellulase A yield in respect to biomass
\mu – specific growth rate
1. Introduction

1.1 Contextualization

Since the creation of the first DNA recombinant microorganism in 1973 (Junod, 2007) and the production of the first human protein (somatotarcin) in 1977 using Escherichia coli by Genentech (Genentech, n.d.), the field of biotechnology has undergone a great and fast development. Many types of microorganisms (bacteria, yeasts, filamentous fungi, microalgae) and cells (prokaryote or eukaryote) have been used for the production of recombinant proteins. Although the most widely used host is E. coli, it is not efficient in the production of all the proteins the market needs, either for the low yield, wrong protein conformation, formation of inclusion bodies or absence of glycosylation. Here arises the need for the development of new hosts. A promising one is Streptomyces lividans which has proved to be valuable in the production of numerous heterologous proteins and is able to secrete many of them in their native conformation with reasonable yields to the extracellular medium (Anné et al., 2012). S. lividans studies report the heterologous secretion of mammalian and microbial proteins including industrial enzymes and biopharmaceuticals.

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 cheaper, more efficient way to produce high quality cellulases would make bioethanol a more competitive alternative and ecological source of fuel.

Unfortunately, heterologous protein yields from Streptomyces lividans are not always at a level which allows commercialization of the production process (Anné et al. 2012, 2014). There are different approaches that may be considered when the goal is to improve heterologous protein production. Four routes for improvement have been presented in Anné et al. (2014): (i) increase expression/translation efficiency, (ii) increase secretion efficiency, (iii) avoid stress response and (iv) relax metabolic burden. Relaxation of the metabolic burden can be resolved by genome reduction, metabolic engineering, medium optimization or bioprocess optimization. Metabolic engineering (i.e., the targeted genetic engineering) requires knowledge and modelling of the cellular metabolism. Metabolomics and fluxomics analysis are essential to understand the burden caused to the host’s metabolism. Metabolomics focuses on the identification and quantification of intra- and extracellular metabolites, while fluxomics is able to determine the metabolite fluxes inside the cells. The combination of these two approaches enables to discover which metabolic pathways should be manipulated. The knowledge obtained is then essential to construct a genetically modified highly efficient recombinant protein producing microorganism.
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).

The major on-going efforts in optimization of *S. lividans* into a cost-effective industrial producer of heterologous proteins and small molecules is the research project named STREPSYNTH (Grant number 613877) financed by the European Union’s Seventh Programme for Research, Technological Development and Demonstration, coordinated by KU Leuven. The results established in this thesis are part of the STREPSYNTH project.

### 1.2 Research objectives

This Master thesis has as its main focus 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.

### 1.3 Research methodology

To achieve the objectives proposed 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 on the cellulase production.

### 1.4 Outline

This Master thesis is divided in six chapters with the following content:

Chapter 1 Introduction – includes the contextualization of this thesis on the scientific field, research objectives, research methodology, outline and a note.

Chapter 2 Literature review – presents the literature study made on *Streptomyces* as a recombinant host, cellulase A, metabolomics, fluxomics, metabolomics and fluxomics studies on *Streptomyces* and medium composition.

Chapter 3 Materials and methods – describes the materials used together with the techniques applied in this study, including the bacterial strain and media tested, bioreactor experiments conditions and analytical methods.
Chapter 4 Results – shows the results and comparison of the different media tested, including dry cell weight, specific growth rate, dissolved oxygen, outlet air carbon dioxide concentration, oxygen transfer rate, glucose, organic acids and amino acids data and celA concentration.

Chapter 5 Discussion – presents the interpretation and argumentation of the results obtained.

Chapter 6 Conclusion – includes the description of the most important findings obtained with this thesis research jointly with some future suggestions.

1.5 Note

The former thesis title was *Metabolomics and fluxomics analysis of Streptomyces lividans for unravelling the metabolic burden caused by heterologous proteins expression*; however it was not possible to perform experiments with labelled carbon $^{13}\text{C}$ or to use Gas Chromatography-Mass Spectrometry (GC-MS) equipment. Taking this into account the thesis title changed but the Chapter 2 Literature review still includes metabolomics and fluxomics as the major topics.
2. Literature review

The necessity to create a new host for the heterologous protein production leads to the attempt of different types of approaches. Metabolomics and fluxomics are two connected branches of the systems biology that together integrate the metabolites and fluxes data with the aim of predicting the phenotype of a microorganism subjected to a certain environment (Cascante et al., 2014). This knowledge is crucial to the construction of new *Streptomyces* protein and other biomolecules producing highly efficient strains. An important factor that extremely affects the heterologous protein production is the medium composition, which optimization is considered as a means to reduce the metabolic burden caused to the host (Anné et al., 2014).

This literature review will focus on recent publications on *Streptomyces* starting with the description of this genus general characteristics, advantages of its use as a recombinant host and other applications besides protein expression. A short overview on cellulase A is presented followed by two reviews on systems biology metabolomics and fluxomics and a sum up of recent articles on which metabolomics and fluxomics approaches were used for *Streptomyces* study. The last section is dedicated to the importance of medium composition study on the improvement of heterologous protein production.

2.1 *Streptomyces* as a recombinant host

*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., 2015b) even though the most studied one is *Streptomyces coelicolor* (Muhamadali et al, 2015). Although *E. 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).

Anné et al. (2012) publication focuses on the special features that make *Streptomyces* a suitable host for heterologous protein production, *Streptomyces* protein secretion pathways and suggests methods to improve protein secretion. Some of the improvement strategies include: choice and modification of a signal peptide,
overproduction of signal peptidases, metabolomics and fluxomics approaches. This article also enumerates twenty of the heterologous proteins already successfully produced by *S. lividans*. Table 2.1 shows the latest achievements in recombinant protein expression by *S. lividans* a posteriori of this publication.

### Table 2.1 – Recent examples on proteins expression by *S. lividans*.

<table>
<thead>
<tr>
<th><em>S. lividans</em></th>
<th>Vector</th>
<th>Medium</th>
<th>Protein (source)</th>
<th>Size (kDa)</th>
<th>Yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1326</td>
<td>pUC702</td>
<td>TSB</td>
<td>Sphingomyelinase C (<em>Streptomyces griseocarneus</em> NBRC13471)</td>
<td>120</td>
<td>mg/L</td>
<td>Sugimori et al. (2012)</td>
</tr>
<tr>
<td>1326</td>
<td>pUC702</td>
<td>TSB+xylose</td>
<td>Transglutaminase (<em>Streptosporangium cinnamoneum</em>)</td>
<td>530</td>
<td>mg/L</td>
<td>Noda et al. (2012)</td>
</tr>
<tr>
<td>1326</td>
<td>pZRJ362</td>
<td>TSB</td>
<td>Endoglucanase Cel6A (<em>Thermobifida fusca</em>)</td>
<td>~45</td>
<td></td>
<td>Li et al. (2013)</td>
</tr>
<tr>
<td>1326</td>
<td>pUC702 (modified)</td>
<td>TSB</td>
<td>Transglutaminase (<em>Streptomyces cinnamoneus</em>)</td>
<td>7.2</td>
<td>mg/L</td>
<td>Noda et al. (2013)</td>
</tr>
<tr>
<td>1326</td>
<td>pUC702</td>
<td>TSB (modified)</td>
<td>β-glucosidase (<em>Thermobifida fusca</em>)</td>
<td>1.1 g/L</td>
<td></td>
<td>Miyazaki et al. (2013)</td>
</tr>
<tr>
<td>TK24</td>
<td>pUWL201</td>
<td>Mixed sugar</td>
<td>β-agarase DagA (<em>Streptomyces coelicolor</em>)</td>
<td>32</td>
<td>8.7 U/mL</td>
<td>Park et al. (2014)</td>
</tr>
<tr>
<td>1326</td>
<td>pTONA4</td>
<td>TSB</td>
<td>Streptavidin (<em>Streptomyces avidinii</em>)</td>
<td>66</td>
<td>0.056 mg/mL</td>
<td>Noda et al. (2015b)</td>
</tr>
</tbody>
</table>

Some of these achievements and other potential applications of *Streptomyces* beside the production of recombinant proteins are described in the next sections with more detail.

#### 2.1.1 Recombinant protein expression with *Streptomyces*

Park et al. (2014) produced DagA, a β-agarase from *S. coelicolor* using *S. lividans* as the host. In this study different carbon sources were tested concluding that galactose and xylose were the best for DagA production. However the authors decided to use glucose for economic reasons. A screening for the key nutrients was performed and their concentrations were statistically optimized obtaining: 25g/L glucose, 10g/L yeast extract and 5g/L MgCl₂·6H₂O. With this medium the authors predicted to reach a DagA activity of 6.96U/mL and experimentally obtained 7.25U/mL. As *Streptomyces* is known to use sugars originating from microalgae hydrolysate as carbon source, a second study was performed using a mixed sugar medium of 25g/L. They concluded that glucose and galactose should be eliminated from the medium before the start of DagA production because the activity shifted from 4.81U/mL to 8.7U/mL.

Noda et al. (2015b) were able to produce a full-length streptavidin from *S. avidinii* by recombinant expression in *S. lividans*. The authors used the gene encoding for the whole protein (Sav\text{nat}) instead of only the core region (Sav\text{core}), which is usually expressed in *E. coli*. They also tested truncated Sav in the C- or the N-terminal regions which revealed to have lower binding capacity to biotin than Sav\text{nat}. The attempt to produce the Sav\text{core} was of no success using *S. lividans*. To assess the thermostability of Sav\text{nat} it was boiled at 100°C for 60 minutes and...
then a SDS-PAGE was run. The streptavidin produced presented a high thermostability not completely dissociating and still having affinity to biotin after boiling, even though this protein is known for losing this capacity after the process. Also the productivity of Sav with S. lividans was 9.2 times higher than what is achieved when using E. coli.

2.1.2 Antibiotics heterologous production with Streptomyces

Lamichhane et al. (2014) accomplished the production of the antibiotic spectinomycin using recombinant S. venezuelae SM5. While this strain was able to produce 89.2mg/mL in an optimized medium, a S. venezuelae dTDP-actinospectose-deficient strain did not produce any antibiotic. This can support the theory that dTDP-actinospectose, which is formed in the desoamine pathway, is an essential precursor for its production in this organism.

2.1.3 Other compounds

Noda et al. (2015a) used modified S. lividans, which expressed phenolic acid decarboxylase and tyrosine ammonia lyase, in order to produce 4-vinylphenol, which is an important compound that can be transformed to resins or inks. The strain was grown in phosphoric acid swollen cellulose (PASC). This study showed that cellulose should be applied in future production of this compound and also that its saccharification must be performed in Streptomyces.

2.1.4 How to improve enzyme production in Streptomyces lividans

Anné et al. (2014) suggests a wide variety of approaches possible to improve the production of heterologous proteins in S. lividans which include: the choice of promoter and vector, choice of signal peptides, overexpression of signal peptidases, overexpression of Tat translocon, systems biology approaches (transcriptomics, metabolomics and fluxomics) and medium optimization. One of these strategies was already tested by Sevillano et al. (2016) that used the suggested xysA strong promoter from S. halstedii.

Sevillano et al. (2016) studied how to enhance enzyme production in S. lividans. For this the authors used different approaches: promoter choice, modification of secretion signal peptides, gene deletion, gene overexpression. They concluded that from the promoters studied xysAp (xylanase promoter) and pstSp (pstS promoter) were the ones that resulted in a higher production of xylanase. Although the secretion was the same, a 17% increase of xylanase activity was achieved when its signal peptide was replaced by the α-amylase one. The deletion of genes which encode repressors of xysAp led to a 70% increase in protein production. The overexpression of SsgA protein in mutant strains had a considerable impact in xylanase and amylase production (40% and 70% increase respectively) compared to the wild type.

2.2 Cellulase A (celA)

CelA is a cellulase from the thermophilic Eubacterium R. marinus classified as an endo-1,4-β-glucanase (E.C. 3.2.1.4) belonging to the Glycosyl Hydrolase (G.H.) family 12. This type of cellulases are able to hydrolyse the
β1-4 linkages in cellulose and lichenin. The enzyme is composed of 260 amino acids and has a molecular weight of 28.8kDa (Hreggvidsson et al., 1996; Halldoarsdoattir et al., 1998).

Currently the problems associated to the use of enzymes in the industry are related to: instability at high temperatures, the cost of their isolation and consequent purification but also the difficulty to recover then from the reactions where they are employed (Verardi et al., 2012).

Cellulase A is referred as the most thermostable endoglucanase identified so far, which makes it very attractive to the technical enzymes’ market which includes all industrially used enzymes (Hreggvidsson et al., 1996; Okano et al., 2014; Research and Markets, 2016). The native enzyme maintained 50% of its activity when subjected to 100°C for 3.5h and when exposed to 90°C for 16h it kept 80% of the activity (Hreggvidsson et al., 1996). These facts make this enzyme of particular interest for the bioethanol industry. The second step of bioethanol production process includes the depolymerisation of cellulose into individual sugar molecules by cellulosic enzymes (Johnson, 2016). Besides this fact, the cellulase production cost is considered to have a major impact in the total bioethanol producing cost (Johnson, 2016). According to Verardi et al. (2012) thermostable enzymes possess a high specific activity that among other factors allow them to be added to reaction mixtures at lower concentrations and higher temperatures resulting in a reduction of the process time and cost.

So, if one would be able to produce a high quality thermostable cellulase using a cheap methodology this would decrease significantly the bioethanol total producing cost making it into a more competitive alternative source of fuel.

The production of thermostable enzymes by thermophilic microorganisms requires the usage of unconventional medium and usually has problems of low specific growth rate and product inhibition (Verardi et al., 2012). Here arises the need for the production of this cellulase in a microorganism other than the native one. The use of E. coli as the host for this enzyme’s heterologous production led to a low production (Halldoarsdoattir et al., 1998; Anne et al., 2014). Although the results were not yet published, this enzyme’s production was of success when using S. lividans as the host (Anne et al., 2012; Anne et al., 2014).

2.3 Metabolomics

Metabolomics is described as “an omics technique aiming at qualitatively and quantitatively describing a metabolome” (Gao et al., 2015). The metabolomics strategies can be classified as targeted or untargeted depending on its focus. The prior refers to the quantification of a particular set of metabolites that usually belong to specific or related pathways in a cell (Patti et al., 2012). The latter, also often entitled metabolite profiling, aims to identify and quantify all the metabolites of a certain biological sample. However, there is no technique available nowadays that enables the identification of all the metabolites in it (Cascante et al., 2008).

There are two types of samples that can be analysed with a metabolomics approach and they return either the endometabolome or the exometabolome. The first has origin in cell pellets and can also be designated as the metabolic fingerprint of an organism. The second is obtained from the culture medium and can also be named
metabolic footprint. While the latter is considered to be easy to obtain (only a quick filtration or centrifugation step) for the endometabolome extraction there is currently no standard, completely efficient procedure (Gao et al., 2015).

The steps required for metabolic fingerprint analysis include: sampling, quenching, metabolite extraction, instrument analysis and data analysis (Figure 2.1).

![Figure 2.1 – Steps of metabolomics driven-approach (taken from Gao et al., 2015).](image)

The quenching step aims to “arrest” the metabolic activity. Sampling and quenching should be performed simultaneously if possible, in order to stop all the enzymatic activity at the sampling moment. Some metabolites, especially from the central carbon metabolism and energy metabolism are rapidly converted due to enzyme activity. For example, the half-life of ATP is around one to sixty seconds. There are different ways of quenching. For microbial cells, this is usually done by mixing the culture with a large volume of a low temperature organic solvent, by putting the sample in liquid nitrogen, or by heating (Gao et al., 2015).

For metabolite extraction, the main used technique consists of a two phase extraction with water, methanol and chloroform. The top phase dissolves water-soluble metabolites and the bottom phase dissolves non polar or weakly polar metabolites. Sometimes it is also necessary to include mechanical procedures in this step (Gao et al., 2015).

The following analytical techniques can be used to identify the metabolites: gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS), nuclear magnetic resonance (NMR) and Fourier transform-infrared spectroscopy (FT-IR). In Table 2.2 the advantages and disadvantages of different analysis instruments are indicated as stated by Cascante et al. (2008).
Table 2.2 – Advantages and disadvantages of different instruments used in metabolomics analysis (taken from Cascante et al., 2008).

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-MS</td>
<td>• High sensitivity &lt;br&gt; • High chromatographic resolution, ideal to resolve complex biological samples &lt;br&gt; • Enables simultaneous analysis of different classes of metabolites</td>
<td>• Unable to analyse thermolabile metabolites &lt;br&gt; • Non-volatile metabolites must be derivatized before analysis &lt;br&gt; • Difficult to identify unknown compounds after derivatization</td>
</tr>
<tr>
<td>LC-MS</td>
<td>• High sensitivity &lt;br&gt; • Average to high chromatographic resolution &lt;br&gt; • No derivatization required &lt;br&gt; • Enables analysis of thermolabile metabolites</td>
<td>• A few restrictions on LC eluents, desalting may be needed &lt;br&gt; • Limited structural information &lt;br&gt; • Matrix effects</td>
</tr>
<tr>
<td>NMR</td>
<td>• Potential for high throughput metabolic profiling &lt;br&gt; • Rapid analysis &lt;br&gt; • Minimal sample preparation &lt;br&gt; • Non-selective and non-destructive technique &lt;br&gt; • Quantitative</td>
<td>• Low sensitivity: only medium-to-high abundance metabolites will be detected</td>
</tr>
<tr>
<td>FT-IR</td>
<td>• High speed of spectra acquisition &lt;br&gt; • Able to analyse different classes of analytes simultaneously (e.g. carbohydrates, amino acids, fatty acids, lipids, proteins and polysaccharides) &lt;br&gt; • Useful for identification of functional groups</td>
<td>• Low level of detailed molecular identification that can be achieved</td>
</tr>
<tr>
<td>CE-MS</td>
<td>• Useful for complex biological samples &lt;br&gt; • Small volumes of sample &lt;br&gt; • High resolution &lt;br&gt; • Wide range of analytes to be analysed, including polar and thermolabile compounds</td>
<td>• Complex methodology and quantification &lt;br&gt; • Buffer incompatibility &lt;br&gt; • Difficulty in interfacing &lt;br&gt; • Needs further development</td>
</tr>
</tbody>
</table>

The GC-MS is currently widely used for microbes. It has advantages and disadvantages: it has a high resolution and separation reproducibility but it can only analyse volatile compounds if the sample is not subjected to derivatization. Derivatization consists in alkylation or silylation of non-volatile compounds to volatile at high temperature (Gao et al., 2015). Without derivatization it is possible to measure the amounts of for example ketones, aldehydes, alcohols, heterocyclic compounds, isocyanates, isothiocyanates, sulphides and some lipids.
whereas sugars, sugar-phosphates, sugar alcohols, organic acids, amino acids, other lipids, peptides, long-chain alcohols, alkaloids, amines need to be derivatized (Wittman et al., 2007).

GC-MS starts with the injection of the sample and its vaporization before entering a column. This column is usually composed of dimethylsiloxane or a mixture of phenyl and dimethylsiloxane and as the carrier gas it is common to use helium (Wittman et al., 2007). After separation, based on the different interactions between the molecules, the carrier gas and the solid phase, the molecules are ionized. In the case of electron impact ionization, the sample is heated and subjected to an electron beam (from the MS) which results in the loss of one electron from the molecules present. Because of the large amount of energy involved, the molecular ions formed tend to fragment. Identification occurs on the basis of these fragments (Wittman et al., 2007).

The data obtained from chromatography-MS is generally composed of m/z (mass-to-charge ratio) of ion fragments information, corresponding retention times and intensity. It is necessary to align data from different runs to make sure that the fragments which are identical have the same retention times. After this the data should be normalized for further statistical analysis if the aim of the study is only to make a comparative analysis of the sample metabolites. There are different methods of uni- and multivariate analysis such as: t test, ANOVA, artificial neural networks, principal component analysis (PCA) or partial least squares discriminant analysis (PLS-DA) (Gao et al., 2015).

For metabolite identification and quantification open source software programmes like MetaQuant, which are able to perform both simultaneously, are currently available. With this software the metabolites can be identified based on their retention time, characteristic mass spectrum or retention index (retention time normalized to the n-alkanes retention time). It is possible to obtain a calibration curve using the GC-MS spectrum of a mixture of metabolite standards with known concentration. The program integrates the peaks areas of this spectrum and along with the concentration information the user is able to define the calibration curves. For the quantification of sample metabolites, the derivatized metabolite specific peaks are firstly detected on the chromatogram data generated by the GC-MS. Secondly, the integration of these peaks is performed and finally these results are compared to the calibration curves previously constructed enabling the quantification (Bunk et al., 2006).

2.4 Fluxomics

Despite all the information obtained from a metabolomics study, it only provides the data related to the metabolites present at a certain time point inside and outside the cell, which is not enough to predict the phenotype of a cell exposed to a certain environment (Cascante et al., 2014). Here arises the need for another omics: Fluxomics.

Fluxomics is a field of systems biology that “integrates in vivo measurements of metabolic fluxes with stoichiometric network models” of (or part of) the cellular metabolism to allow the determination of flux (Winter et al., 2013).
2.4.1 General equation

In fluxomics, the metabolic network model is defined by a stoichiometric matrix \( (S) \) which contains the stoichiometric coefficients for the metabolites involved in the metabolic reactions (Llaneras et al., 2008). If a coefficient has a negative sign it indicates that the metabolite is consumed in that reaction, otherwise it is being produced (D’Huys et al., 2012). The mass balances of the intercellular metabolites over time \((t)\) are given by the following differential equation:

\[
\frac{dc}{dt} = S \cdot v - \mu \cdot c \quad (1)
\]

where \( \mu \) corresponds to the specific growth rate \((h^{-1})\), \( c \) represents the vector with the concentration of the intercellular metabolites \((\text{mmol gDCW}^{-1})\), and \( v \) represents the flux vector \((\text{mmol gDCW}^{-1} h^{-1})\) containing intracellular fluxes and exchange fluxes. The second term (dilution attributed to biomass growth) of the Eq.1 is usually discarded because its value is typically much lower than the fluxes affecting each metabolite (Llaneras et al., 2008).

During exponential growth or in a stable continuous culture, the intracellular metabolites are in (pseudo) steady-state and Eq. 1 simplifies to:

\[
S \cdot v = 0 \quad (2)
\]

A homogenous system of linear equations is obtained which needs to be solved for the unknown fluxes.

2.4.2 Metabolic flux analysis (MFA)

Metabolic flux analysis uses directly measured fluxes and the general equation in order to determine the remaining unknown fluxes and obtain a unique solution for Eq.2. The use of the measured fluxes decreases the underdeterminancy of the general equation “by considering a partition between measured \((m)\) and unmeasured or unknown fluxes \((u)\)” (Llaneras et al., 2008). This results in Eq. 3:

\[
S_u \cdot v_u = -S_m \cdot v_m \quad (3)
\]

The system of Eq.3 can only be solved when the rank of matrix \( S_u \) is equal to the number of unmeasured/unknown fluxes (Llaneras et al., 2008).

It is necessary to apply constraints in order to decrease the number of possible solutions for Eq.2, and so to determine the organism metabolic behaviour. By doing this it is possible to determine which flux distributions are consistent with the known information. There are different types of constraints that can be applied and these are classified into two categories: adjustable or nonadjustable. The first can vary from cell to cell and the second is invariant, which means it cannot change with time (Llaneras et al., 2008). In Table 2.3 there are some examples of constraints as described by Llaneras et al. (2008).
Table 2.3 – Types of constrains (taken from Llaneras et al., 2008).

<table>
<thead>
<tr>
<th>Constraint</th>
<th>Type</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic stoichiometry</td>
<td>Nonadjustable</td>
<td>$S \cdot v = 0$</td>
</tr>
<tr>
<td>Irreversibility of fluxes</td>
<td>Nonadjustable</td>
<td>$v \geq 0$</td>
</tr>
<tr>
<td>Enzyme/transporters capacity</td>
<td>Nonadjustable</td>
<td>$v \leq v_{\text{max}}$</td>
</tr>
<tr>
<td>Measured fluxes</td>
<td>Adjustable</td>
<td>$v = v_m \text{ or } v_{m,\text{min}} \leq v \leq v_{m,\text{max}}$</td>
</tr>
<tr>
<td>Regulatory constrains</td>
<td>Adjustable</td>
<td>For example: $v_1 = 0 \text{ if } (v_2 \neq 0)$</td>
</tr>
<tr>
<td>Kinetic constraint</td>
<td>Adjustable</td>
<td>$v = k \cdot C$</td>
</tr>
</tbody>
</table>

As constraints are added to the model the space of solution evolves from a hyperplan (only the general equation) to a bounded convex polyhedral cone as it can be observed in Figure 2.2.

![Figure 2.2](image)

2.4.3 Flux balance analysis (FBA)

Flux balance analysis considers all the nonadjustable constraints known about a certain cell metabolism. As inputs flux values, generally substrate uptakes are considered. This does not lead to a unique solution but to a space of solutions. In order to obtain the set of fluxes which describes the observed phenotype of the microorganism, an optimization condition is included (Llaneras et al., 2008). One of the main objectives of cell metabolism is to optimally grow (maximum specific growth rate) so this is the most widely used optimization condition (Eq.4), although others may be considered such as minimizing ATP production (Eq.5), minimizing nutrient uptake (Eq.6) or maximizing a certain metabolite production (Llaneras et al., 2008).

\[
\text{max } v_{\text{biomass}} \quad (4)
\]

\[
\text{min } v_{\text{ATP}} \quad (5)
\]

\[
\text{min } v_{\text{nutrient}} \quad (6)
\]

The FBA goal is to develop a model that has as inputs the conditions or environment in which a microorganism is growing and as output it can predict its flux distribution or phenotype (Llaneras et al., 2008).
2.4.4 $^{13}$C-Metabolic flux analysis ($^{13}$C-MFA)

$^{13}$C-Metabolic flux analysis is nowadays the most advanced MFA technique. It is an extension of MFA which uses intracellular isotope ($^{13}$C) labelling data as another source of measured information opposite to normal MFA that as mentioned above only uses extracellular fluxes (Wiechert et al., 2015). For this type of analysis to take place it is necessary to have: isotopically enriched substrates, mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) equipment to measure the carbon enrichment of the metabolites, isotopomer balancing software and labelling transition information (Winter et al., 2013).

As in the previous described fluxomic approaches, $^{13}$C-MFA assumes that the cell is at (pseudo) steady-state and that there is no isotope effect. The later means that the system is well mixed and the enzymes have no preference for labelled or unlabelled substrates (Krömer et al., 2009).

This approach can be divided into four stages, the first being experimental design. Before starting an experiment it is necessary to make in silico studies in order to conclude what are the best labelled substrates to use and which are the best metabolites to quantify, because not all expressed compounds give vital information on the flux change and the labelled molecules are expensive (Krömer et al., 2009). The price of $^{13}$C labelling is perhaps the key reason for its lower application compared to the other -omics, which makes it difficult to perform large scale studies and not affordable to all research teams (Winter et al., 2013). Currently the most used isotope labelled substrates are $^{13}$C-glucose and $^{13}$C-glutamine (Cascante et al., 2014). This step cannot be performed unless labelling transition is known a priori.

$[1,2-^{13}C_2]$-glucose can offer information on the glycolysis, pentose phosphate pathway and pyruvate oxidation or carboxylation (Cascante et al., 2014). Figure 2.3 shows the label propagation of $[1,2-^{13}C_2]$-glucose in Krebs cycle.

![Figure 2.3](image-url) – Isotope labelled molecules transitions in Krebs cycle. The white circles represent carbon molecules without labelling, while grey circles represent labelled carbons. The arrows show the direction of the fluxes (taken from Cascante et al., 2014).
For example, the $^{13}$C transition from $[1,2^{-13}$C$_2]$-glucose to lactate enables to determine the ratio of fluxes on the pentose phosphate pathway in respect of the carbon coming from the glycolysis. Also the $^{13}$C transition from $[1,2^{-13}$C$_2]$-glucose to glutamate can be used to estimate the contribution of pyruvate carboxylase and pyruvate dehydrogenase to the Krebs cycle (Cascante et al., 2014).

The second step consists of the experiment itself, where it is necessary to ensure the (pseudo) steady-state and also that there are no nutrient limitations (Krömer et al., 2009).

The third step involves the determination of the biomass composition and the quantification of the labelled carbon enrichment in the target metabolites by use of MS or NMR equipment (Krömer et al., 2009).

In the fourth and final stage the fluxes are estimated by using dedicated software. Combining the information of the stoichiometric matrix (S) with the labelling transition information and experimentally obtained information (substrate uptake, metabolites secretion and growth rate) it is possible to estimate the labelling output. This is then compared with the one determined by the MS or NMR analysis and the fluxes are iteratively calculated until the difference is minimized. The sensitivity of the fluxes is also analysed enabling to obtain a confidence interval for each flux calculated (Krömer et al., 2009). There are currently some software available for flux analysis such as $^{13}$C-FLUX, Fiatflux, OpenFLUX, FIA and Isodyn (Cascante et al., 2014).

### 2.5 Metabolomics and fluxomics studies on Streptomyces

In the next sections recent studies in the fields of metabolomics and fluxomics on *Streptomyces* are described.

#### 2.5.1 Metabolomics

Guo et al. (2015) performed a metabolomics driven study to better understand why the avermectin, an antiparasitant agent, production changed from the wild type *S. avermitilis* to a currently used industrial *S. avermitilis* 9-39 mutant which shows higher product yield. To do so the authors inoculated flasks with spore cultures and incubated them at 28°C for 40h and then transferred part of the seed cultures to new medium and incubated then for more 10 days in the same conditions. To extract the intercellular metabolites from both strains quenching was performed using cold methanol followed by centrifugation and disruption of the cells with bead mill containing a chloroform, ethanol and water buffer. The mixture was frozen and thawed five times before being centrifuged, mixed with an internal standard and dried in a vacuum centrifuge dryer. The derivatization step consisted in two stages: the samples were first incubated with methoxamine hydrochloride and then with N-methyl-N-(trimethylsilyl)trifluoroacetamide. After derivatization the authors proceeded to GC-MS analysis. With SIMCA program they were able to perform PCA and PLS-DA analysis. As it can be visualized in Figure 2.4, PCA revealed that the wild type (WT) strain behaved differently in each day (6, 8, 10th days) sampled while the mutant only had a different pattern in one day (6th day). The PLS score plots for each sample day showed different clusterings for the WT and mutant strains which indicates that the pool of metabolites changed between these strains. The PLS loading plots and Variable Importance in Projection (VIP) interpretation allowed to conclude which metabolites were responsible for the clustering difference: D-
cellobiose, D-galactose, D-glucopyranose, D-mannose, D-turanose, glutamine, L-serine and maltose. The major part of these metabolites belongs to glycometabolism which led to the authors’ final conclusion that the improvement of this metabolism was the key factor for higher production of avermectin in the industrial strain.

Figure 2.4 – PCA score plot derived from the metabolites profile of the wild type (WT) and the mutant strain (9-39) (taken from Guo et al., 2015).

Wu et al. (2015) discovered a new isatin antibiotic, 7-prenylsatin, produced by Streptomyces sp.MBT28 using a metabolomics approach. The authors selected streptomycin resistant mutant colonies which were then cultured in agar plates together with Bacillus subtilis in order to obtain organisms with antibacterial action. One mutant with strong activity against B.subtilis was chosen to continue the experiment, along with a low activity one and the wild type for comparison. To prepare metabolome samples the Streptomyces grown in agar plates were extracted with ethyl acetate, dried with reduced pressure at 40°C and redissolved in methanol. Then the methanol was evaporated and the samples were lyophilized in a freeze-dryer. To obtain enrichment in isatin compounds these samples were subjected to partition with methanol and n-hexane in order to remove the lipids. The methanol phase was kept and separated in a silica gel chromatography column. The fractions obtained were next dissolved in d4-methanol. Later the prepared samples from each strain were subjected to 1H nuclear magnetic resonance (1H NMR) which revealed no difference between the WT and low activity strain but showed that the high activity strain had a different peak in the aromatic region of the chromatogram. The next step consisted in the use of multivariate data analysis with SIMCA program. The PLS score plot for the activity against B. subtilis, expressed as the diameter of the inhibition zone in the agar plates, revealed clear difference between the strains showed by 3 distinct clusters and it was also possible to verify variability among the 10 replicates used. To identify the compound that was responsible for the different peaks in the high activity strain, a NMR-guided fractionation was executed that enabled to identify it as 7-prenylisatin, which had never been identified before. Further tests were conducted with purified 7-prenylisation that revealed it had a minimal inhibitory concentration of 25µg/L against B. subtilis.

Qi et al. (2014) studied the effect of cell morphology together with a metabolomics driven-approach to enhance the production of rapamycin, a macrocyclic polyketide with pharmaceutical interest, by S. hygroscopicus. To obtain four different types of pellet morphology, four batch fermentations with different medium were performed using a 7.5L fermenter containing 4 L of medium for 120h at 28°C, 300rpm and 1vvm. The difference in the media consisted only on the concentration of (NH₄)₂SO₄ which had been previously
determined as a key factor on the level of rapamycin production. The authors decided to classify the morphology in three categories: size and shape of pellets, size and shape of pellet core and branching frequency and length of peripheral filaments that arise from pellets. As results it was discovered that the change in medium and fermentation time did not influence greatly the shape of the pellet or pellet core whereas the pellet size was bigger in the conditions that allowed higher rapamycin production. It was also determined that longer filaments and higher branching frequency in the periphery of pellets were characteristics that were connected to higher rapamycin production. In order to understand the intracellular metabolites effect in this, samples had to be prepared for metabolomics analysis. For quenching the samples were first filtered with a vacuum system, washed with NaCl and the filter used was transferred to a tube containing cold methanol (50% v/v). The filters were stored at -80°C until the metabolites were extracted. The metabolites extraction was achieved by freeze-thaw cycles with the methanol (50% v/v). Before injection in the GC-MS the extracts were mixed with the internal standard, lyophilized and derivatized with O-methylhydroxyamine and then with N-methyl-N-(trimethylsilyl) trifluoroacetamide. Afterwards samples were subjected to GC-MS and the data generated was analysed with SIMCA program. The PCA score plot showed that the data from the four different conditions was divided in four different clusters and for each condition the different time samples also formed time distinctive clusters. From the PCA loadings plot it was possible to identify 18 metabolites that had a higher contribution to the separation of the four morphologies. The VIP chart enabled to identify 19 metabolites as the source of the differences and also inferred about each metabolite relative contribution. The major part of the metabolites belonged to the central carbon metabolism and the nucleotide phosphate metabolism. It was assessed that during the logarithmic growth phase, the cells in the two conditions that allowed higher rapamycin production, had higher level of metabolites involved in the central carbon metabolism and also in the nucleotide phosphate metabolism. This high level of metabolites could help the growth and proliferation of peripheral hyphae. As during the stationary phase a problem with oxygen availability started to happen in the core of the pellets, affecting the hyphae growth and so the rapamycin production as concluded in the study, the authors decided to optimize the dissolved oxygen concentration in order to enhance the production. The optimized conditions led to a 63% increase in the production of rapamycin to a concentration of 536mg/L.

Muhamadali et al. (2015) performed a metabolomics study to understand the impact of recombinant production of murine Tumour Necrosis Factor alpha (mTNFα) in S. lividans TK24. Three different strains (wild type, empty plasmid and recombinant protein producing) were grown in minimal medium containing aspartate for four days. After that the growth profile was analysed and revealed no significant difference among the strains. Besides that, the strain producing mTNFα did not achieve higher production levels compared to other experiments reported in the literature, which led to the conclusion that the aspartate was only consumed for growth and had no effect on the protein production. A PCA-discriminant function analysis of metabolites fingerprint data generated by FT-IR spectroscopy revealed that the WT had a different cluster position when compared to the empty plasmid and protein producing strains. Also the empty plasmid and protein producing strains had time clusters very close to each other in the beginning and then started to separate, probably due to the start of mTNFα production. The intracellular and extracellular metabolites were further analysed by GC-
MS. To prepare the samples for GC-MS, quenching was performed by use of cold methanol (60% v/v). For the extraction of intracellular metabolites the samples were subjected to three freeze and thaw cycles with 100% cold methanol (-45°C). Afterwards the samples were centrifuged and 100% cold methanol (-45°C) was added to the supernatant (repeated one more time). Then the internal standard was added to the samples and these were lyophilised using a speed vacuum concentrator and stored at -80°C. Before the GC-MS step the stored samples were derivatized first with methoxyaminehydrochloride in pyridine and secondly with N-methyl-N-(trimethylsilyl)trifluoroacetamide. With the data from GC-MS a weighted-consensus principal component analysis (CPCA-W) score plot was made for each strain and confirmed once more a time dependent distribution of the metabolites profile. With a time blocking CPCA-W score plot it was detected a separation of the clusters of the three strains, being the cluster of the mTNFα producing strain clearly separated from the other two clusters. From the data obtained from GC-MS it was possible to verify that the mTNFα producing strain had a higher rate of glucose 6-phosphate and aspartate consumption, a higher accumulation of pyruvate and 2-ketoglutarate in the medium overlapping the increase of mTNFα production and also a higher level of propanoate and xylitol, mannose and fructose. Concluding this study could evidently reveal the metabolic burden caused by the introduction of a recombinant protein in S. lividans TK24.

2.5.2 Fluxomics

Coze et al. (2013) were able to discover the difference in the carbon metabolism of the S. coelicolor A3(2) actinohodin (antibiotic) producing strain M145 and the non-producing mutant strain M1146 using metabolic flux analyses and 13C labelling. For the constraints necessary for the MFA and to define the metabolic pathways the data was acquired from previous genome, transcriptomics and proteome studies. The labelling compound chosen was a mixture of [1-13]C glucose (20%) and unlabelled glucose (80%). As an instrument of analyses GC-MS was used to measure the levels of carbon enrichment in the amino acids and HPLC to quantify the glucose and extracellular metabolites present in the media. The data generated was analysed by the program OpenFlux. In this study the use of [1-13]C glucose enabled the quantification of the flux ratio between the pentose phosphate pathway (PPP) and the glycolysis. Such was possible because when [1-13]C glucose enters PPP loses its label, while when it enters the glycolysis the label is still present in the amino acids generated. The results revealed that the M1146 had a higher specific growth rate, lower glucose specific uptake rate, produced no antibiotic and released more pyruvate and α-ketoglutarate to the medium. The 13C labelling in M1146 amino acids was lower than the M145 strains which meant that the carbon flux was more directed to the PPP than the glycolysis. It was concluded that the higher flux in PPP in M1146 was due to the higher need for NADPH correlated to the higher growth rate. The authors concluded that there may be a competition between the actinohodin and triacylglycerol pathways for their precursor acetyl-CoA and also that their findings showed that S. coelicolor has chances to become a producing strain of important biological products.

Chen et al. (2013) compared the production of ε-poly-L-lysine, a natural amino acid homo polymer used for food preservation, by Streptomyces sp.M-Z18 using glucose or glycerol as the carbon source. The seed culture was obtained by inoculating shake flasks with spores of the strain in M3G medium and culturing for 24h at 30°C
and 200 rpm. The experiments were performed in a 5L fermenter (containing 3.5L) in batch or fed-batch mode in two media with the same composition, only differing in the carbon source (glucose or glycerol). The culturing conditions were 30°C, agitation between 200 and 900 rpm to ensure minimal dissolved oxygen of 20 to 30% and initial pH 6.8 for both modes. For the fed-batch mode, glucose or glycerol were added when the concentration dropped to 10g/L and \((\text{NH}_4)_2\text{SO}_4\) when ammonium had a concentration lower than 1g/L. ε-poly-L-lysine concentration in samples supernatant was determined by the methyl orange method, glucose and glycerol were quantified by means of HPLC whilst amino acids were first derivatized then subjected to HPLC. The model used for MFA on the ε-poly-L-lysine production included glycolysis, gluconeogenesis, pentose phosphate pathway (PPP), tricarboxylic acid cycle, diaminopimelic acid pathway and the conversion of L-lysine into ε-poly-L-lysine for both substrates. This model assumed (pseudo) steady-state for intermediate intracellular metabolites, that the biomass formation had the same reaction as \textit{E. coli} and that its composition did not change, reactions with no sidesteps were converted to one reaction, in the biomass creation flux the synthesis of unknown metabolites was included. To solve the model Matlab was used. For batch mode the biomass yield, growth rate and profile were similar for both substrates while the highest ε-poly-L-lysine yield and specific production rate was achieved using glycerol with a 44% increase in production. For fed-batch mode glycerol also led to higher ε-poly-L-lysine production, productivity and titer. MFA revealed that in glycerol the fluxes from oxaloacetate to aspartate and lysine towards ε-poly-L-lysine were higher than in glucose and also that the fluxes from glycerol to the PPP were increased. Also in the glycerol medium the fluxes involved in by-products formation were lower. It was demonstrated that glycerol is a good substitute for glucose as the carbon source for ε-poly-L-lysine production.

Sánchez et al. (2014) studied the effect of amino acid feeding to the production of clavulic acid (CA), a β-lactamase inhibitor, by \textit{S. clavuligerus}. For that they used a sensitivity and metabolic flux analysis approach with CellNetAnalyzer. The pre-cultures were first grown in TSB for 36h at 28°C and 220 rpm while the cultures were grown in PM medium in bioreactors at 28°C, 500 rpm and pH 6.8. Three different situations were tested: batch mode and continuous mode (after 36h of batch) at the dilution rates of 0.02 h⁻¹ and 0.03 h⁻¹. HPLC was used for the quantification of glycerol, amino acids and CA from samples supernatant while phosphate was assessed by the molybdenum blue method. The metabolic model used included the central carbon metabolism, urea cycle and the biosynthesis of biomass and CA. The sensitivity analysis was performed by changing the measured fluxes individually and checking its effect on the calculated vector of fluxes. It was discovered that the fluxes that had a higher sensitivity impact were the measured fluxes of isoleucine, phenylalanine and tyrosine while when aspartate, asparagine and glutamate measured fluxes were taken into account these revealed no influence in CA production. For the metabolic flux analysis (MFA) the conclusions obtained from the sensitivity analysis were considered. MFA showed that CA production is enhanced at lower dilution rates which results in lower growth rate and higher fluxes belonging to the glycolysis. Also, the ornithine flux was different depending on the dilution used being limited for the higher one that was related to lower CA production. It was concluded that the fluxes towards glyceraldehyde-3-phosphate, phosphoenolpyruvate, pyruvate, oxaloacetate and ornithine should be high in order to improve CA production. Another possible strategy for this aim would be the addition of ornithine to the medium.
2.6 Medium composition

According to Anné et al. (2014) one of the possible ways to enhance heterologous protein production is by the relaxation of the metabolic burden caused to the host microorganism. This may be done by: (i) bioprocess optimization, (ii) genome reduction, (iii) metabolic engineering and (iv) medium optimization. In order to accomplish the latter the study of the medium composition is required. Only by understanding the type and/or individual nutrients effect/role present in a medium on the host’s growth and protein production it will be possible to design an optimized medium for higher production.

Different types of methodologies have been employed from the test of different complex media, the supplementation of defined media with amino acids, design mixture experiments to amino acids uptake profiles studies. Below are presented some of these studies related to the medium composition influence on the production of heterologous proteins and secondary metabolites by *Streptomyces* and other microorganisms.

Pozidis et al. (2001) tested five different rich media (Luria-Bertani broth, PHAGE medium, NM medium, modified Trypticase Soy broth with and without 2.5g/L dextrose) for the production of recombinant mTNFα by *S. lividans* TK24. From these the medium with highest mTNFα secretion was Trypticase Soy broth. By adding different amounts of dextrose to this medium the authors were able to obtain the highest protein secretion of 200 to 300mg/L when 10g/L of dextrose were supplemented.

Voelker et al. (2001) compared the influence of different nitrogen sources (NH₄Cl, NaNO₃, alanine, glutamate and valine) on the growth and pristinamycin production by *S. pristinaespiralis* using glucose as the carbon source in a synthetic medium. While valine was solely used as nitrogen source, alanine and glutamate were used both as nitrogen and carbon sources.

Corvini et al. (2004) study revealed that glutamate was first totally consumed and only after glucose and arginine were used when *S. pristinaespiralis* was grown in a defined medium containing glucose as the carbon source and glutamate and arginine as carbon/nitrogen sources.

Nowruzi et al. (2008) performed design mixture experiments in order to achieve the optimum minimal medium composition for the production of the recombinant human interleukin-3 by *S. lividans* 66. The authors first conducted starvation trials with 20 amino acids which revealed the minimum amino acids total concentration necessary for the growth and eight potential essential amino acids. By executing screening mixture experiments and distance–based multivariate analysis it was obtained a ranking reduced to four potential essential amino acids (methionine, aspartate, phenylalanine and leucine). Using a simplex lattice design mixture experiment it was concluded that the optimal minimal medium for this protein production should have 0.25g/L amino acids (42% aspartate, 5% methionine, 53% phenylalanine).

Amino acid supplementation to chemically defined media has been used as a strategy to improve heterologous protein production also in other types of microorganisms. Görgens et al. (2005) studied the effect of the
addition of individual amino acids and amino acids mixtures to a defined minimal medium having glucose as the carbon source on the heterologous production of xylanase by *Saccharomyces cerevisiae* in batch cultures. Before the switch from growth on glucose to ethanol, the amino acids were added. This study concluded that the medium supplementation with alanine, arginine, asparagine, glutamate, glutamine and glycine could enhance the protein production.

D’Huys et al. (2011) studied the growth, glucose and ammonium consumption, organic acids excretion and amino acids uptake profiles by *S. lividans* TK24 wild type, empty–plasmid and recombinant mTNFα producing strain. The pre-cultures were grown in PHAGE medium for 48h at 30°C and 250rpm. These cultures were then homogenized and transferred to shake-flasks containing PHAGE medium and cultivated in the same conditions. The bioreactor experiments were carried out in batch mode using 5L bioreactors filled with 3L of modified minimal medium (NMMP) containing 10g/L glucose as the carbon source and casamino acids at concentrations of 5g/L or 15g/L. The culturing conditions were constant 30°C, 400rpm, pH7 and aeration flow of 2L/min. To all the recombinant strain cultures thiostrepton was added for plasmid maintenance. Biomass formation was determined by dry cell weight, glucose by the glucose dehydrogenase test while the organic acids (pyruvic acid, lactic acid, α-ketoglutaric acid and succinic acid) by gas-chromatography and a flame ionization detector, the amino acids were measured by EZ:faast™ GC-MS kit and mTNFα quantified by enzyme-linked immunosorbent assay. This study revealed that aspartate and glutamate were used both as nitrogen and carbon sources being metabolized as the same time as glucose and ammonium in a first exponential growth phase while in the second growth phase characterized by a lower specific growth rate only glucose and ammonium were consumed. The higher growth rate associated with these amino acids consumption did not improve the recombinant protein production. The analysis of casamino acids determined that this compound contains all of the considered as essential amino acids except for asparagine, glutamine and tryptophan. By observing the different cultures on the microscope it was clear that the empty-plasmid and recombinant protein strains formed bigger clumps than the wild-type and besides that they possessed lower growth rates. For all the strains it was detected organic acids excretion to the extracellular medium whereas the amino acids profiles showed an alanine overflow. The organic acids and alanine excreted to the medium were eventually reuptake during the stationary growth phase. Apart from leucine’s higher uptake rate in the second growth phase, in which the protein secretion was higher, and this amino acid’s high content in mTNFα the authors could not find another connection between the amino acids uptake rates and the protein’s production. This study revealed the metabolic burden caused by the plasmid presence in *S. lividans* TK24 and a possible link among some amino acids and their uptake profiles with the mTNFα heterologous protein production.

The latter article is one of the rare publications available on the effect of amino acids on the growth and heterologous protein production.

All of these studies reveal that the medium composition and most importantly the different amino acids composition and presence in the medium play a major role on the growth and heterologous protein production by *Streptomyces* and other microorganisms.
3. Materials and methods

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

3.1 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°C.

3.2 Media for testing celA production

Different types of medium were tested, both defined and complex media. A list of all the media tested along with their composition is presented in Table 3.1.

**Table 3.1 – Composition of all media tested.**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Medium abbreviation</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal Medium</td>
<td>MM</td>
<td>10g/L glucose, 3g/L (NH₄)₂SO₄, 2.6g/L K₂HPO₄, 1.8g/L NaH₂PO₄, 0.6g/L MgSO₄·7H₂O, 25mL/L minor elements solution (40mg/L ZnSO₄·7H₂O, 40mg/L FeSO₄·7H₂O, 40mg/L CaCl₂, 40mg/L MnCl₂·4H₂O)</td>
</tr>
<tr>
<td>Minimal medium supplemented with aspartate</td>
<td>MM+ASP2,5</td>
<td>MM supplemented with 2.5g/L aspartate</td>
</tr>
<tr>
<td>Minimal medium supplemented with glutamate</td>
<td>MM+GLU2,5</td>
<td>MM supplemented with 2.5g/L glutamate</td>
</tr>
<tr>
<td>Minimal medium supplemented with casamino acids</td>
<td>MM+CAS5</td>
<td>MM supplemented with 5g/L Bacto™ casamino acids</td>
</tr>
<tr>
<td>Minimal medium supplemented with casamino acids</td>
<td>MM+CAS15</td>
<td>MM supplemented with 15g/L Bacto™ casamino acids</td>
</tr>
<tr>
<td>Tryptic Soy Broth</td>
<td>TSB</td>
<td>17g/L casein peptone (pancreatic), 5g/L NaCl, 3g/L soya peptone (papain digest), 2.5g/L K₂HPO₄, 2.5g/L glucose</td>
</tr>
<tr>
<td>Bennett medium</td>
<td>Bennett</td>
<td>10g/L glucose, 2g/L LAB M™ tryptone, 1g/L yeast extract, 1g/L beef extract (Atlas, 2010)</td>
</tr>
</tbody>
</table>
Table 3.1 – Composition of all media tested (cont.).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Medium abbreviation</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Broth</td>
<td>NB</td>
<td>5g/L peptic digest of animal tissue and 3g/L beef extract</td>
</tr>
<tr>
<td>Reconstituted nutrient broth</td>
<td>PEP5+BEEF3</td>
<td>5g/L peptone and 3g/L beef extract</td>
</tr>
<tr>
<td>Peptone</td>
<td>PEP5</td>
<td>5g/L peptone</td>
</tr>
<tr>
<td>Beef extract</td>
<td>BEEF3</td>
<td>3g/L beef extract</td>
</tr>
<tr>
<td>Double strenght Nutrient Broth</td>
<td>2NB</td>
<td>10g/L peptic digest of animal tissue and 6g/L beef extract</td>
</tr>
<tr>
<td>Minimal medium with reconstituted nutrient broth</td>
<td>MM+PEP5+BEEF3</td>
<td>MM supplemented with 5g/L peptone and 3g/L beef extract</td>
</tr>
<tr>
<td>Minimal medium with modified reconstituted nutrient broth</td>
<td>MM+PEP5+MEAT3</td>
<td>MM supplemented with 5g/L peptone and 3g/L meat extract</td>
</tr>
</tbody>
</table>

MM is a modification of the minimal liquid medium (NMMP) reported in Kieser et al. (2000).

Double strenght Nutrient Broth (2NB) is the same medium as NB but it was applied the double amount recommended by the supplier.

3.3 Bioreactor experiments

3.3.1 Pre-culture preparation

The pre-culture was prepared by inoculating 100mL of PHAGE medium (Korn et al., 1978) (10 g/L glucose, 5g/L LAB M™ tryptone, 5g/L yeast extract, 5g/L OXOID Lab lemc, 0.74g/L CaCl₂.2H₂O, 0.5g/L MgSO₄.7H₂O), 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.

3.3.2 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.

3.3.3 Medium preparation

Each medium was prepared in the bioreactor vessel and autoclaved for 21min at 121°C. For the media containing glucose, (NH₄)₂SO₄ and MgSO₄.7H₂O, solutions of this compounds were prepared and autoclaved.
separately before being added to the bioreactors while the minor elements solution was sterile filtered over a 0.22µm filter.

For all the media except TSB, NB and 2NB, that are prepared powder media from Sigma-Aldrich®, all the compounds which are part of the composition had to be weighted and/or a solution had to be prepared.

All media and solutions were prepared with ultra-pure water (Arium 611 DI water) apart from TSB, NB and 2NB which were prepared with distilled water as indicated by the supplier.

3.3.4 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 maintained 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 H₂SO₄ or 4M KOH). Calbiochem® thioestrepton (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.

Figure 3.1 – Eppendorf DASGIP® monitoring and control modules. (A) DASGIP® PH4PO4 pH and DO monitoring, (B) DASGIP® GA4 Gas analyser, (C) DASGIP® MIX4 Gas mixer, (D) DASGIP® MP8 Acid and base pumps and (E) DASGIP® TC4SC4 Temperature and agitation control.
3.3.5 Samples

During the experiments 10mL samples were collected at different time points, transferred to 15mL falcons 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 1.5mL reaction tube and concentrated using a LABCONCO CentriVap® concentrator for celA quantification. The remaining filtered supernatant was stored at 8°C in 15mL falcons for further use.

3.4 Analytical methods

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

3.4.1 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.

3.4.2 Organic acids and glucose assay

To determine the amount of some organic acids (α-ketoglutaric acid, pyruvic acid, succinic acid, lactic acid, acetic acid) and glucose in the fermentation broth, the filtered supernatant of the samples collected was
analysed by 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 H₂SO₄ 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. The compound identification and quantification was achieved by comparing the retention time and the area of each peak, present in the chromatograms generated, with calibration curves.

### 3.4.3 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. The kit usage consisted on the solid phase extraction of the amino acids present in the collected samples supernatant using sorbent binding amino acid tips followed by their release into the sample vials, derivatization and liquid-liquid extraction. The organic layer generated on the top of the vial contained the derivatized amino acids sample. 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.

### 3.4.4 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 (Figure 3.3) with a capacity for four gels each run.

![Figure 3.3 – Biocom Direct Omnipage Electrophoresis system (left) and Biocom Direct Power Source (right).](image)
Resolving gel: Each gel prepared had a concentration of 17% in acrylamide and a 10cm x 10cm dimension with 1mm thickness. For each gel a solution containing 5.4mL Arium 611 DI water, 3.75mL 1.5M Tris-HCl pH 8.8, 5.6mL 30% Bio-Rad® acrylamide, 150µL 10% SDS, 75µL 10% Bio-Rad® Ammonium persulfate (APS) and 7.5µL Bio-Rad® Tetramethylethylenedianine (TEMED) was prepared and mixed. The solution was then pipetted between the glass plates of the setup and covered with isopropanol. After the solidification of the gel, which usually lasted 1 hour, the isopropanol overlay was removed.

Stacking gel: For each gel a solution containing 4.5mL Arium 611 DI water, 1.875mL 0.5M Tris-HCl pH 6.8, 1mL 30% Bio-Rad® acrylamide, 75µL 10% SDS, 37.5µL 10% Bio-Rad® APS and 7.5µL Bio-Rad® TEMED was prepared and mixed. The solution was pipetted on top of the resolving gel and the combs were placed. The solidification lasted around 20 min.

Sample preparation: 100 or 200µL of Arium 611 DI water were added to the CentriVap® samples stored at -20°C. The samples were then put in an Eppendorf ThermoMixer® F1.5 for 30min at 37°C and 1500rpm. Calibration samples of 1x, 2x, 4x, 8x and 16x dilution were also prepared using a celA standard stock solution (200.2 or 267.4mg/L). A mixture of 40µL 2-mercaptoethanol and 760 µL Lamelli sample buffer (1.25mL Tris-HCl 0.5M pH6.8, 2.5mL glycerol, 0.2mL 0.5% Bromophenol blue, 2 mL 10% SDS, 3.55mL distilled water) was prepared. The calibration and reactor samples were diluted to a ratio of 1:1 with this mixture. Subsequently all the samples were heated at 95°C for 5min in an Eppendorf ThermoMixer® F1.5.

Running conditions: The setup tank was filled with running buffer (3g Tris, 14.4g glycine, 10g SDS in 1L of distilled water, pH 8.6). 20µL of the prepared samples were appointed in each well (5µL for the Molecular weight marker). The electrodes from the Biocom Direct Power Source were connected and the electrophoresis was carried out for 75min at maximum 200V or 100W.

Western-blot

This wetblotting technique was performed using a Biocom Direct Omnipage Blotting system (Figure 3.4) with a capacity for four transfer cassettes.

Figure 3.4 – Biocom Direct Omnipage Blotting system.
Transfer: For each gel a 0.2µm Amersham Protran Nitrocellulose membrane and six Whatman 3MM Chr paper were cut to appropriate size. The membrane, filter paper and sponges were soaked in transfer buffer (135mL 10xRunning buffer, 270mL methanol, 945mL distilled water). The gel was removed from between the glasses of the electrophoresis setup. A layout as presented in Figure 3.5 was prepared. Each transfer cassette was placed inside the buffer tank and the latter was filled with the transfer buffer. The electrodes from the Biocom Direct Power Source were connected and the protein transfer was carried out for 1 hour at maximum 100V or 400W.

![Figure 3.5 – Layout of the transfer cassette.](image)

Membrane blocking and incubation with antibodies: The membrane was removed from the cassette and washed with a 5% milk powder solution in TTBS buffer (20mM Tris, 8.74g/L NaCl, 0.3% Tween 20, pH7.5) for 15min to block the membrane and reduce background noise. The membrane was washed four times with TTBS buffer for 5min 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 for 15min and twice for 5min 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 six times for 5min with TTBS buffer and kept in this buffer until the detection was performed. All the washing and incubations were performed at 8°C and 240rpm using a shaker.

Detection: The Charged Coupled Device (CCD) camera of the GE Image Quant LAS 4000 was cooled to -25°C. For each membrane blot 2mL of each Thermo Fisher SuperSignal® reagents (luminol and peroxide buffer) were mixed. Each blot was drained and placed on the detection plate. The mixture prepared was poured on the top of the blots. The blots were drained and the image detection started.

Quantification software

In order to quantify the celA present in the samples, the images obtained with the GE Image Quant LAS 4000 were manually processed using paint.net v4.0.9 and ImageJ 1.50g software. Calibration curves were obtained knowing the celA standard stock solution dilutions. With the calculated regression it was possible to estimate the celA concentration at certain time points of the experiments.
4. Results

In this chapter the experimental data obtained according to the Materials and methods procedures is presented and analysed. The results obtained are further discussed in Chapter 5. Media were selected based on a literature review and on prior knowledge within the research group. For each medium tested plots were drawn for dry cell weight, glucose, organic acids and amino acids evolution throughout time. Specific growth rate (µ) and celA production were also calculated. When available dissolved oxygen (DO), outlet air carbon dioxide concentration (XCO₂ Out) and Oxygen Transfer Rate (OTR) plots are included. Assuming pseudo steady state at each measurement time point, the OTR corresponds to the Oxygen Uptake Rate (OUR).

The results are presented in chronological order. Firstly, the data for minimal medium (MM) and MM with the addition of certain amino acids and Bacto™ casamino acids is presented. Secondly the results obtained using well-known complex media are shown followed by the combination of the most promising medium with MM, its reconstruction and use of individual components. The last section includes a comparison of all the media tested.

4.1 Reference experiments

Motivation

With the aim of improving celA secretion by S. lividans TK24, the first experiments included the test of four interconnected media: minimal medium (MM), MM supplemented with 2.5g/L of aspartate (MM+ASP2,5), MM supplemented with 2.5g/L of glutamate (MM+GLU2,5) and MM with the addition of 5g/L of Bacto™ casamino acids (MM+CAS5).

As mentioned in Chapter 3, MM is composed of 10g/L glucose and some salts. It constitutes a modification of the NMMP medium reported in Kieser et al. (2000). The concentration of ammonium salts was increased 1.5 times and no casamino acids were added to obtain a minimal medium in which glucose was the only growth-limiting carbon source. MM was chosen as a reference medium in order to verify the effect of the addition of amino acids in cellular growth and target protein production.

The NMMP medium is referred by Kieser et al. (2000) as a suitable liquid medium for Streptomyces growth. Furthermore, the use of media containing a significant amount of amino acids has previously been used as a strategy to improve the heterologous protein yield in S. lividans (Pozidis et al., 2001). With that in mind Bacto™ casamino acids was used, this constitute a mixture of amino acids originated by the acid hydrolysis of casein and contains all amino acids except for asparagine, glutamine and tryptophan (D’Huys et al., 2011).

D’Huys et al. (2011) notices that glutamate and aspartate are co-metabolized with glucose and act as important carbon sources. Therefore, aspartate and glutamate are the amino acids chosen to individually be supplemented to MM.
Results

All experiments were sampled up to 48 hours. The cultures grown in MM and MM+CASS belonged to the same pre-culture while the same happened for MM+ASP2.5 and MM+GLU2.5. During the experiment samples for dry cell weight (DCW) determination, organic acids and celA quantification were collected. For this first attempt the samples were not subjected to amino acids measurement since the media supplemented with aspartate and glutamate had an amino acids concentration extremely over the EZ:faast™ GC-FID kit detection limit.

The experimental data obtained are presented and analysed below. In Figures 4.1 A to 4.4 A the biomass growth evaluated by the dry cell weight (DCW), and the glucose (GLC) consumption are represented. Figures 4.1 B to 4.4 B show the progress of the dissolved oxygen and the CO₂ concentration variation in the outlet air throughout the experiment time while Figures 4.1 D to 4.3 D represent the evolution of the Oxygen Transfer Rate (OTR). In Figures 4.1 C to 4.4 C is represented the production and consumption of some organic acids.

![Figure 4.1](image1)

**Figure 4.1** – Experimental data for recombinant celA secreting *S. lividans* TK24 grown on MM. Plot A: (●) dry cell weight and (▲) glucose. Plot B: (—) dissolved oxygen and (—) outlet air carbon dioxide concentration. Plot C: (●) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (X) lactate and (X) acetate. Plot D: (—) oxygen transfer rate.

In Figure 4.1 A, it can be seen that the lag phase took approximately 15 hours (corresponding to the start of CO₂ increase in Figure 4.1 B), the exponential phase lasted around 28 hours (15 to 43h) and the stationary phase continued until the end of the experiment. Glucose consumption seems to coincide with the start of the exponential phase and glucose depletion coincides with the start of the stationary phase.
Comparing Figures 4.1 A, B and D it is clear that the OTR and CO$_2$ concentration were very low in the beginning corresponding to the lag phase, started to increase with cells growth in the exponential phase and dropped abruptly with the end of this phase. The OTR did not drop to zero but to a small OTR which was due to the consumption of organic acids. The dissolved oxygen values also accompanied cell growth, dropping during the exponential phase and culminating with a sudden increase at the end of this phase.

The organic acids concentration (Figure 4.1 C) increased with cell growth reaching its highest concentration at the end of the exponential phase. Acetic acid reached the highest concentration followed by pyruvic acid and lactic acid. The concentration of all organic acids decreased due to their consumption which can be verified by the switch of the addition of base to acid for pH control around 48h (Figure 4.5) generating the small peak of XCO$_2$ Out at the same time (Figure 4.1 B).

In Figure 4.2 A it is clear that after 48h the cells in MM+ASP2,5 were still growing. Comparing Figures 4.2 A and 4.2 B is possible to determine that the lag phase took approximately 14.5h that coincide with the start of the increase of CO$_2$ concentration in the outlet air due to cellular growth. The exponential phase started at 14.5h and extended until the end of the experiment, occurring no detection of stationary phase. Glucose started to be consumed with the beginning of the exponential phase, at 33h almost half had already been spent and after 44h glucose was no longer detected.
Figures 4.2 B and D show that OTR and CO$_2$ had a low concentration at the beginning (lag phase), started to increase at 14.5h with the exponential phase and dropped after 43h matching the total consumption of glucose. Then surged a second peak related to the cell switch to growth on another substrate, which was likely the remaining aspartate. The dissolved oxygen follows cellular growth, dropping continuously until 43h when a sudden increase occurred followed by a more or less constant value which also confirms the change in metabolism.

In Figure 4.2 C the organic acids concentration (except for acetic acid) as a function of time is plotted. α-Ketoglutaric, succinic and lactic acid increased in concentration between 33 and 44hrs and were then consumed (decrease of concentration between the last two time points and second peak in XCO$_2$ plot). Pyruvic acid is the one that reached the highest concentration, however there is no data for the last two time points making it impossible to confirm also its consumption.

Figure 4.3 – Experimental data for recombinant celA secreting S. lividans TK24 grown on MM+GLU2,5. Plot A: (▲) dry cell weight and (△) glucose. Plot B: (—) dissolved oxygen and (—) outlet air carbon dioxide concentration. Plot C: (◊) α-ketoglutarate, (▲) pyruvate, (△) succinate, (X) lactate and (X) acetate. Plot D: (—) oxygen transfer rate.

By the comparison of Figures 4.3 A and 4.3 B a lag phase of around 14.5h is estimate which matches the start of the increase of outlet air CO$_2$ concentration for MM+GLU2,5. Then the exponential phase lasted until approximately 43h followed by the stationary phase until the end of the experiment. Glucose started to decrease with the start of the exponential phase, and like with MM+ASP2,5 at 33h almost half had been consumed and after 44h there was no glucose in the medium.
Figures 4.3 A, B and D have a similar profile to the respective MM+ASP2,5 plots. The OTR and CO₂ concentration were small during the lag phase, started to increase at 14.5h with the exponential phase and dropped after 43h matching the end of this phase and glucose depletion. After that, due to the organic acids consumption these two parameters values started to increase once more. The dissolved oxygen dropped until around 43h matching more or less the end of the exponential phase when it increased and stabilized until the end of the experiment.

In Figure 4.3 C is possible to observe that pyruvic, succinic, lactic and acetic acid concentration increased throughout the experiment time achieving their highest value at around 43h (end of exponential/start of stationary phase) while α-ketoglutaric acid reached the highest concentration at approximately 33h. It is also clear that between 44 and 48h there was organic acids consumption confirmed by their concentration decrease and an outlet air XCO₂ and OTR increase.

Comparing Figures 4.4 A and B, it is possible to infer that for MM+CASS the lag phase lasted until 9h corresponding to the start of the outlet air CO₂ concentration increase, then the exponential phase took approximately 19h (9h to 28h). The stationary phase started after 28h. Glucose was consumed and was totally depleted at the end of the exponential growth phase.

In Figure 4.4 B the CO₂ concentration plot presents two distinct peaks. The first corresponding to the start of exponential phase and glucose consumption (the peak end matches the glucose depletion on Figure 4.4 A)
while the second is related to the organic acids consumption. The dissolved oxygen dropped until 20h matching the XCO₂ first peak, started to rise and stabilized with the second XCO₂ peak. At around 30.5h the DO started to rise because cell growth was stopped.

The organic acids concentration tended to increase during the exponential phase (Figure 4.4 C). α-Ketoglutaric, pyruvic and succinic acid reached their highest values at around 25h being then consumed in the stationary phase. Acetic acid concentration also rose until 25h, being the organic acid that achieved the highest concentration, and then it dropped and had a small increase at the end of the experiment. Lactic acid increased until 33h, decreasing then its value.

![Figure 4.5 – Feed flow rate of acid (FA) and base (FB) for recombinant celA secreting S. lividans TK24 grown on MM.](image)

![Figure 4.6 – Natural logarithm of dry cell weight divided by initial dry cell weight (ln(DCW/DCW₀)) versus time (t). Plot A: MM. Plot B: MM+ASP2.5. Plot C: MM+GLU2.5. Plot D: MM+CASS.](image)

The duration of the lag phase, exponential phase and the start of the stationary phase can only be determined exactly from a ln(DCW/DCW₀) plot versus time (Figure 4.6). However since there is not enough data regarding

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the lag and exponential phases no more information can be derived from these plots when compared to Figures 4.1 A to 4.4 A.

Table 4.1 displays celA concentrations obtained with the Western-blot technique for the final two time points of each experiment, and also the celA yield with respect to biomass ($Y_{celA/X}$). After 48h, the medium with the highest celA production was MM+CAS5 while the medium with the highest yield was MM+GLU2.5. It is also clear that the medium with the lowest production was MM+ASP2.5. The celA production increase in MM+GLU2.5 and MM+CAS5 compared to MM was 16.75% and 29.58%, respectively. However, the amount of celA produced per gram of biomass was not increased in MM+CAS5, but only in MM+GLU2.5. Taking that into account it was clear other media had to be tested in order to increase celA production. On the next experiment different media rich in amino acids were tested.

Table 4.1 – CelA concentration and respective yield ($Y_{celA/X}$).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>44</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>MM</td>
<td>MM+ASP2,5</td>
</tr>
<tr>
<td>celA (mg/L)</td>
<td>6.54</td>
<td>5.59</td>
</tr>
<tr>
<td>$Y_{celA/X}$ (mg/g)</td>
<td>2.13</td>
<td>2.12</td>
</tr>
</tbody>
</table>

* relative to maximum DCW value.

4.2 Testing other media rich in amino acids

Motivation

With the same objective of increasing celA heterologous production by *S. lividans* TK24 three other different media were tested: MM with the addition of 15g/L of Bacto™ casamino acids (MM+CAS15), Tryptic Soy Broth (TSB) and Nutrient Broth (NB).

Even though a medium containing Bacto™ casamino acids had already been tried but with a three times lower concentration, it was decided to check the effect of this increase in concentration with the expectation that it could also triple the amount of protein produced.

Tryptic Soy Broth or a modification of this medium has been widely used in recent publications for recombinant protein expression in *Streptomyces* (see Table 2.1 Chapter 2), taking this into account it was chosen for test.

Nutrient Broth was another complex medium referred by Kieser et al. (2000) as a suitable liquid medium for *Streptomyces* growth.
Results

Experiments were sampled for 35hrs. The cultures grown in TSB and NB belonged to the same pre-culture while the culture grown in MM+CAS15 belonged to another. It is important to mention that between 23.3h and 35h the NB culture changed from the usual yellow colour to pink. During this experiment samples for dry cell weight (DCW) determination, organic acids, celA and amino acids quantification were collected.

The experimental data obtained is presented and analysed below. Plots containing the data are drawn for each medium in the following order: MM+CAS15, TSB, NB. In the end of this section, a Table with the celA yield for all these media is presented.

![Experimental data for recombinant celA secreting S. lividans TK24 grown on MM+CAS15. Plot A: (●) dry cell weight and (▲) glucose. Plot B: (——) dissolved oxygen and (-----) outlet air carbon dioxide concentration. Plot C: (◇) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (◆) lactate and (▲) acetate. Plot D: (——) oxygen transfer rate.](image)

By the comparison of DCW in Figure 4.7 A and XCO₂ in Figure 4.7 B it is possible to determine that the lag phase for MM+CAS15 took 9h, the exponential phase followed and should have lasted until around 24.5h (end of glucose consumption – first XCO₂ peak). From 24.5h to the end of the experiment, the stationary phase occurred. Glucose started to be consumed with the beginning of the exponential phase and was depleted at its end.

The first and second peak in the XCO₂ plot (Figure 4.7 B) correspond respectively to the glucose and organic acids consumption, which have two different CO₂ production rates. The dissolved oxygen dropped until around
24.5h matching more or less the XCO\textsubscript{2} first peak, started to rise and had an inverted peak corresponding to the second XCO\textsubscript{2} peak. CO\textsubscript{2} production dropped when the organic acids were depleted.

In Figure 4.7 C is possible to observe the organic acids evolution with time. \(\alpha\)-Ketoglutaric, pyruvic and succinic acid rose during the exponential phase, while lactic acid only had its highest concentration at the end of the experiment. Pyruvic acid was the organic acid that reached the highest concentration. The organic acids were again consumed in the stationary phase.

**Figure 4.8** – Experimental data for recombinant celA secreting *S. lividans* TK24 grown on TSB. Plot A: (◊) dry cell weight and (▲) glucose. Plot B: (---) dissolved oxygen and (——) outlet air carbon dioxide concentration. Plot C: (◊) \(\alpha\)-ketoglutarate, (▲) pyruvate, (▲) succinate, (X) lactate and (▲) acetate. Plot D: (—) oxygen transfer rate.

Figure 4.8 A shows the biomass growth and glucose consumption during the experiment for TSB. TSB contains 1.5 g/L glucose as initial concentration, which was fully consumed in approximately 18hrs. The lag phase took approximately 9 hours, the exponential phase lasted 8 hours (9 to 17h) and then the stationary phase continued until the end of the experiment (35h).

Comparing Figures 4.8 A, B and D it is clear that the OTR and CO\textsubscript{2} concentration were very low during the lag phase, started to increase in the exponential phase and dropped with two phases in the stationary phase (oxygen consumption was continued due to the metabolization of amino acids and organic acids). The dissolved oxygen values decreased with cell growth and then remained constant until 21h when a slight increase occurred.
Figure 4.8 C shows the production and consumption of some organic acids throughout the experiment time (lactic acid and acetic acid could not be detected). TSB contains already a reasonable concentration of pyruvic acid and a small concentration of succinic acid. All detected organic acids concentration increased with cellular growth. The highest concentration was achieved close to the end of the exponential phase. Pyruvic acid is the organic acid which achieved the highest concentration followed by succinic acid and α-ketoglutaric acid. The decrease of all organic acids concentration (see Figure 4.8 C) due to their consumption also occurred in this medium which can be corroborated by the peak of XCO$_2$ Out around 19h in Figure 4.8 B.

![Figure 4.8](image)

Figure 4.9 – Experimental data for recombinant celA secreting S. lividans TK24 grown on NB. Plot A: (○) dry cell weight and (▲) glucose. Plot B: (---) dissolved oxygen and (---) outlet air carbon dioxide concentration. Plot C: (●) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (X) lactate and (X) acetate. Plot D: (---) oxygen transfer rate.

In Figure 4.9 A the evolution of dry cell weight is represented for NB. Considering the latter figure it is not possible to determine exactly how long the lag phase lasted, but taking into account that the CO$_2$ concentration in Figure 4.9 B only started to increase after approximately 6 hours, that was probably its length. The exponential phase lasted then 18 hours (6 to 24h) and the stationary phase followed until the end of the experiment (35h).

The CO$_2$ concentration (Figure 4.9 B) started to increase with the start of the exponential phase, reaching its highest concentration at around 12h when it began to decrease until the end of the experiment. The OTR (Figure 4.9 D) increased until 12h, it had a slight decrease and at around 24h had an increase peak and dropped once more. The dissolved oxygen decreased until 12h and remained constant until around 24h having a slight drop matching the OTR peak.
In Figure 4.9 it is possible to observe the production of all the organic acids measured except lactic acid (not possible to detect). α-Ketoglutaric, pyruvic and succinic acid had more or less constant values until 19h when they suffered a slight increase as it may be observed and remained constant until the end. On the other hand, acetic acid dropped throughout all the experiment.

In Figure 4.10 is shown the amino acids concentration obtained with the the EZ:faast™ GC-FID kit for some experimental time points of NB. Alanine, aspartate, asparagine and most likely glutamate were mainly uptake during the early exponential phase while the other amino acids were only consumed in the late exponential or stationary phase. After 13h there was an increase in glycine, proline, tryptophan, histidine and glutamine. An increase in lysine, aspartate and glutamate concentration occurred between 17h and 21.1 h.

![Graph A: Amino acids concentration](image1)

![Graph B: Amino acids concentration](image2)

![Graph C: Amino acids concentration](image3)

![Graph D: Amino acids concentration](image4)

**Figure 4.10** – Amino acids concentration for recombinant celA secreting *S. lividans* TK24 grown on NB 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.

Table 4.2 displays the celA concentration and celA yield in respect to biomass ($Y_{celA/X}$) for some time points of MM+CAS15, TSB, and NB. In this table it is possible to verify that even though TSB has been used in many recent publications for heterologous protein production it had the lowest production so far. The cellular growth with MM+CAS15 reached a DCW of 5.60g/L but only 15.68mg/L of celA after 35h. Surprisingly NB produced 23.13mg/L of celA with a cellular concentration of just 1.07g/L (DCW). The latter was until this point the medium with simultaneously the highest production and yield.
Table 4.2 – CelA concentration and respective yield \((Y_{\text{celA} / X})\).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>19</th>
<th>21.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>MM+CAS15</td>
<td>TSB</td>
</tr>
<tr>
<td>CelA (mg/L)</td>
<td>2.83</td>
<td>0.46</td>
</tr>
<tr>
<td>(Y_{\text{celA} / X}) (mg/g)</td>
<td>0.96</td>
<td>0.18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>23.3</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>MM+CAS15</td>
<td>TSB</td>
</tr>
<tr>
<td>CelA (mg/L)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Y_{\text{celA} / X}) (mg/g)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{(1)}\) DCW value not available.

4.3 Testing other medium with beef extract

Motivation

Since Nutrient Broth was so far the medium that generated the highest secretion of celA and besides peptone it is composed of beef extract, it was decided to try another medium with this compound. The medium selected was Bennett medium which is referred by Atlas (2000) as a suitable medium for Streptomyces growth.

Results

Figure 4.11 – Photos of the bioreactor and DCW filters at different experiment times for recombinant celA secreting S. lividans TK24 grown on Bennett medium.
The experiment was sampled for 67.2 hrs, but the stationary phase was not yet reached at the end of the sampling. Samples were collected for DCW, glucose, organic acids and celA determination. During the experiment after around 20.5h small red agglomerates started to form which were visible in the DCW filter (Figure 4.11), at 24.5h the culture had already obtained a pink colour, by 41.5h it was almost red and at the end of the experiment it still kept the same colour and the agglomerates. The XCO₂, DO and OTR plots are not available for this experiment.

Figure 4.12 – Experimental data for recombinant celA secreting *S. lividans* TK24 grown on Bennett medium. Plot A: (○) dry cell weight and (▲) glucose. Plot B: (●) α-ketoglutarate, (▲) pyruvate, (△) succinate, (×) lactate and (□) acetate.

In Figure 4.12 A is possible to observe that even after 67.2h the cells were still growing and glucose was not yet depleted.

Figure 4.12 B shows that all organic acids, except for the lactic acid (not measured), increased their concentration throughout the experiment. Acetic acid was the one with the highest concentration during all the time followed by pyruvic, α-ketoglutaric and succinic acid.

Table 4.3 displays celA concentration and celA yield in respect to biomass (Y<sub>celA/X</sub>) for some time points of the Bennett medium. Even though this medium contained beef extract in its composition as NB, the celA concentration and yield obtained were the lowest of all the media tested.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>24.5</th>
<th>44.5</th>
<th>65.5</th>
<th>67.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>celA (mg/L)</td>
<td>0.015</td>
<td>0.55</td>
<td>1.92</td>
<td>1.43</td>
</tr>
<tr>
<td>Y&lt;sub&gt;celA/X&lt;/sub&gt; (mg/g)</td>
<td>0.013</td>
<td>0.31</td>
<td>(1)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

<sup>(1)</sup> DCW value not available.

### 4.4 Testing the effect of glucose addition and beef substitution by meat extract

**Motivation**

In order to test if the addition of glucose to a medium containing beef extract and peptone (NB reconstituted) would increase celA production and not only cellular growth (all media with glucose had achieved so far a
higher DCW than NB), MM+PEP5+BEEF3 was created. This medium is also important for the comparison with other MM supplemented media.

Since it was not clear the difference between beef and meat extract from Sigma-Aldrich®, it was decided to also test the same but with meat extract instead, creating the medium MM+PEP5+MEAT3.

**Results**

The experiment was sampled for 67.2hrs. The cultures grown on MM+PEP5+BEEF3 and MM+PEP5+MEAT3 belonged to the same pre-culture. During the experiment samples for DCW, glucose, organic acids, amino acids and celA quantification were collected.

Figure 4.13 shows the evolution of the cultures throughout the experiment time. Opposite to NB, these cultures did not change their colour, however at 67h it was possible to detect in DCW filters small red agglomerates similar to the ones formed when grown in Bennett medium.

![Figure 4.13](image)

**Figure 4.13** – Photos of the bioreactors and DCW filters at different experiment times for recombinant celA secreting *S. lividans* TK24 grown on MM+PEP5+BEEF3 and MM+PEP5+MEAT3.

Below the experimental data are presented relative to these two new media tested. DO, XCO₂ and OTR plots are missing the data for the first hours due to a system software failure.

By the comparison of Figures 4.14 A and B is determined that the lag phase for MM+PEP5+BEEF3 lasted approximately 8h (outlet air CO₂ concentration increase). The exponential phase lasted for 18h (8h to 26h),
then the stationary phase occurred between around 26h and 49 to 67h and subsequently cell death began which can be confirmed by the DCW decrease. Glucose was consumed during the exponential phase and was depleted when this phase ended.

In Figure 4.14 B the XCO₂ plot presents two peaks. The first peak relates to the start of exponential phase and glucose consumption whilst the second is again due to organic acids consumption. The dissolved oxygen matched the XCO₂ peaks inversely and rose at the end due to cell death.

The organic acid that achieved the highest amount was acetic acid followed by pyruvic, succinic and α-ketoglutaric acid (Figure 4.14 C). The concentrations increased during the exponential phase lowering then due to their consumption at approximately the end of this phase: α-ketoglutaric acid 24h, pyruvic acid 20h, succinic acid after 24h and acetic acid 24h.

![Figure 4.14 – Experimental data for recombinant celA secreting S. lividans TK24 grown on MM+PEP5+BEEF3. Plot A: (●) dry cell weight and (▲) glucose. Plot B: (---) dissolved oxygen and (—) outlet air carbon dioxide concentration. Plot C: (●) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (✘) lactate and (✘) acetate.](image)

Figure 4.15 shows that all amino acids were depleted within the total duration of the experiment. Some amino acids were fully consumed when the end of the exponential growth phase was reached (approximately 26 hrs). Examples are aspartate, glutamate and tyrosine. The remaining amino acids were consumed during the exponential growth phase as well as during the stationary phase (until depleted after less than 67 hrs).
Figure 4.15 – Amino acids concentration for recombinant celA secreting S. lividans TK24 grown on MM+PEP5+BEEF3 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.

Comparing Figures 4.16 A and B is possible to determine that the lag phase for MM+PEP5+MEAT3 lasted until around 9h (XCO₂ increase), the exponential phase followed for 17h (9h to 26h) starting then the stationary phase that was prolonged until between 49h and 67h when cell death occurred. Glucose was again consumed in the exponential phase.

The XCO₂ plot (Figure 4.16 B) presents two peaks corresponding to the glucose and organic acids consumption respectively. The dissolved oxygen decreased with cell growth matching the XCO₂ and then started to rise when cell growth stabilized and later due to cell death. The OTR plot matches the XCO₂ profile.

The organic acid that achieved the highest concentration was acetic acid followed by succinic, pyruvic and α-ketoglutaric acid (Figure 4.16 C). Their concentrations rose during the exponential phase being then consumed at approximately the end of it: α-ketoglutaric acid 24h, pyruvic acid 22h, succinic and acetic acid after 26h.

Figure 4.17 shows that until 20h coinciding the early exponential phase leucine, serine, histidine, glutamate, aspartate and asparagine were highly consumed and an increase in glycine occurred whereas the remaining amino acids were uptake mostly in the late exponential or stationary phase. After 20h and 24h there was an increase in alanine and glutamine respectively.
Figure 4.16 – Experimental data for recombinant celA secreting \textit{S. lividans} TK24 grown on MM+PEP5+MEAT3. Plot A: (●) dry cell weight and (▲) glucose. Plot B: (-----) dissolved oxygen and (-----) outlet air carbon dioxide concentration. Plot C: (●) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (×) lactate and (×) acetate. Plot D: (-----) oxygen transfer rate.

Figure 4.17 – Amino acids concentration for recombinant celA secreting \textit{S. lividans} TK24 grown on MM+PEP5+MEAT3 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.
Table 4.4 shows the celA concentration and celA yield in respect to biomass \(Y_{\text{celA/x}}\) for some time points of this experiment.

In Table 4.4 is possible to observe that MM+PEP5+MEAT3 had the highest celA production so far, but only a 13.7% increase when compared to NB. Also, this concentration was present at 67.2h whereas for NB 23.13mg/L were achieved only after 35h and with a DCW 5.14 times lower. Based on these data, it can be seen that the celA production continued during the stationary (where no growth takes place).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>26</th>
<th>43.1</th>
<th>47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>MM+PEP5+BEEF3</td>
<td>MM+PEP5+MEAT3</td>
<td>MM+PEP5+BEEF3</td>
</tr>
<tr>
<td>celA (mg/L)</td>
<td>1.53</td>
<td>1.07</td>
<td>6.49</td>
</tr>
<tr>
<td>(Y_{\text{celA/x}}) (mg/g)</td>
<td>0.30</td>
<td>0.21</td>
<td>1.29</td>
</tr>
</tbody>
</table>

\(Y_{\text{celA/x}}\) relative to maximum DCW value.

### 4.5 Researching Nutrient Broth

#### Motivation

Since Nutrient Broth (NB) seemed to be the most promising medium it was obvious that it required a deeper study. So it was clear that to discover why this medium led to a higher production and yield its individual components had to be studied separately. Taking this into account \textit{S. lividans} TK24 was grown in media containing only peptone (PEP5) or beef extract (BEEF3) in the same proportion as present in NB. To understand if it was possible to recreate the already prepared sold NB by joining its constituents the medium PEP5+BEEF3 was generated. This medium was also important to compare the effect of the MM (especially glucose) in MM+PEP5+BEEF3. In order to check the effect on cellular growth and celA production due to the use of the double medium amount compared to NB, Double strength Nutrient Broth (2NB) was also prepared.

#### Results

The experiment was sampled for 67.2hrs. The cultures grown on PEP5+BEEF3 and 2NB belonged to the same pre-culture while PEP5 and BEEF3 belonged to another. During this experiment samples for DCW, glucose, organic acids, amino acids and celA quantification were collected. Figure 4.18 shows the evolution of the cultures throughout the experiment time. Like NB medium, 2NB also changed its colour to pink between 24.5h and 42h while the other media changed to grey with a slight pink shade. It was detected that in PEP5+BEEF3 red cell agglomerates had been formed after 42h.
The experimental data obtained is presented and analysed below.

![Figure 4.18](image1)

**Figure 4.18** – Photos of the bioreactors and DCW filters at different experiment times for recombinant celA secreting *S. lividans* TK24 grown on PEP5+BEEF3, PEP5, BEEF3 and 2NB.

From Figure 4.19 A it is not clear how long the lag phase for PEP5+BEEF3 lasted but taking into account that the XCO$_2$ in the outlet air (Figure 4.19 B) only started to rise after 6h this should have been its length. The exponential phase followed from 6h to between 24.5h and 42h when the cells were in the stationary phase. At the end of the experiment cell death was already happening. The XCO$_2$ plot (Figure 4.19 B) presents two peaks related to carbon sources consumption. This time these peaks did not correspond to glucose (not present) consumption with the first peak possibly associated to aspartate and glutamate (see Figure 4.20). The OTR (Figure 4.19 C) increased until around 37h being then more or less stable. The DO decreased until 12h, stayed stable until 24h when it started to rise and remained constant from 48h to the end of the experiment because cell growth was stopped.

Figure 4.19 C shows that the organic acids that reached higher concentrations were succinic and acetic acid which increased until 24h and were then partially consumed. On the other hand pyruvic acid decreased its concentration and then stabilized. For α-ketoglutaric acid there is only data for the last time points when it was stable.

In Figure 4.20 the amino acids profile for PEP5+BEEF3 reveals that asparagine, aspartate and glutamate were uptake in the exponential phase while the remaining amino acids in the exponential as well as the stationary phase. It was noticed an increase in glycine concentration after 19h.
Figure 4.19 – Experimental data for recombinant celA secreting *S. lividans* TK24 grown on PEP5+BEEF3. Plot A: (▲) dry cell weight and (●) glucose. Plot B: (---) dissolved oxygen and (——) outlet air carbon dioxide concentration. Plot C: (▲) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (X) lactate and (X) acetate. Plot D: (---) oxygen transfer rate.

Figure 4.20 – Amino acids concentration for recombinant celA secreting *S. lividans* TK24 grown PEP5+BEEF3 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 4.21 – Experimental data for recombinant celA secreting *S. lividans* TK24 grown on PEP5. Plot A: (●) dry cell weight and (▲) glucose. Plot B: (●) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (×) lactate and (X) acetate.

For PEP5 the DCW plot (Figure 4.21 A) only reveals that the cellular growth reached the highest value of 0.65 g/L at around 48h and that at 67h cellular death was already occurring. The XCO₂, DO and OTR plots are not available for this experiment.

The organic acids plot (Figure 4.21 B) shows that α-ketoglutaric acid had a slight increase until 24.5h while pyruvic and succinic acid remained constant. After 42h all organic acids had suffered a concentration decrease. Acetic and lactic acid were not possible to detect.

Figure 4.22 – Amino acids concentration for recombinant celA secreting *S. lividans* TK24 grown PEP5 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 4.22 shows that for PEP5 after 19h there was an increase of leucine, alanine, isoleucine, proline, glutamine and threonine concentrations. All the amino acids were slowly uptake and were not even totally depleted from the medium except for glutamate and aspartate. The latter were already almost depleted after 19h.

Figure 4.23 – Experimental data for recombinant celA secreting S. lividans TK24 grown on BEEF3. Plot A: (●) dry cell weight and (▲) glucose. Plot B: (●) α-ketoglutarate, (▲) pyruvate, (▲) succinate, (▲) lactate and (▲) acetate.

Figure 4.24 – Amino acids concentration for recombinant celA secreting S. lividans TK24 grown BEEF3 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.

For BEEF3 as for PEP5, the DCW plot (Figure 4.23 A) does not reveal much: the stationary phase should have started at around 24h and after 67h cell death was already occurring. The XCO₂, DO and OTR plots are not available for this experiment.
From the organic acids plot (Figure 4.23 B) it is possible to observe that α-ketoglutaric and pyruvic acid had a more or less constant concentration (taking into account the values magnitude) and succinic acid dropped throughout the experiment time. Acetic and lactic acid were not possible to measure.

For BEEF3 Figure 4.24 reveals that the amino acids that were uptake in the exponential phase were alanine, leucine, valine, lysine, isoleucine, methionine, histidine, glutamate aspartate and asparagine. The other amino acids were uptake in the exponential as well as in the stationary phase. It is also verified that after 19h there was an increase in glutamine concentration.

In Figure 4.25 A the evolution of dry cell weight is represented for 2NB. Considering the latter figure it is not possible to determine exactly how long the lag phase lasted, but taking into account that the CO\textsubscript{2} concentration in Figure 4.25 B only started to increase after approximately 7.5 hours, that was most probably its length. The exponential phase lasted then 22 hours (7.5 to 29.5h) matching XCO\textsubscript{2} decrease. The stationary phase followed and cell death occurred which can be confirmed by the decrease of DCW at the end of the experiment.

The CO\textsubscript{2} concentration (Figure 4.25 B) started to increase with the start of the exponential phase, reached its highest concentration at around 15.5h, having a more or less stable value until 29.5h when it began to decrease until the end of the experiment. Like the CO\textsubscript{2} concentration, the OTR increased until 15.5h, then it also stabilized from 15.5 to 29.5h and started to drop. The dissolved oxygen decreased until 15.5h and remained with a slight oscillation until around 29.5h, increasing then until the end (cell growth stopped).
In Figure 4.25 C it is possible to observe the production of some of the organic acids measured. Pyruvic and succinic acid had an increase until 42h when their concentration stabilized. α-ketoglutaric acid values remained more or less constant whereas acetic acid dropped throughout all the experiment.

In Figure 4.26 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.

![Figure 4.26 – Amino acids concentration 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.](image_url)

Table 4.5 displays the celA concentration obtained with the Western-blot technique for some time points and also the celA yield in respect to biomass \(Y_{\text{celA} / X}\) for the last four media tested.

Considering the data from Table 4.5 it is clear that PEP5+BEEF3, BEEF3 and 2NB are from all the media tested the ones that achieved higher celA production. Even though the highest production is achieved with 2NB, the highest yield in respect to biomass belongs to BEEF3.
### Table 4.5 – CelA concentration and respective yield ($Y_{celA/X}$).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>24.5</th>
<th>42.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEPS+BEEF3</td>
<td>PEPS</td>
<td>BEEF3</td>
</tr>
<tr>
<td>celA (mg/L)</td>
<td>-</td>
<td>5.32</td>
</tr>
<tr>
<td>$Y_{celA/X}$ (mg/g)</td>
<td>-</td>
<td>7.52</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>48.0</th>
<th>67.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEPS+BEEF3</td>
<td>PEPS</td>
<td>BEEF3</td>
</tr>
<tr>
<td>celA (mg/L)</td>
<td>25.91</td>
<td>0.73</td>
</tr>
<tr>
<td>$Y_{celA/X}$ (mg/g)</td>
<td>16.80</td>
<td>1.13</td>
</tr>
</tbody>
</table>

(*) relative to maximum DCW value.

### 4.6 Media Comparison

In order to have a better understanding of the medium composition effect on *S. lividans* TK24 growth and celA production, each medium analysed above individually is compared with all the media tested in this section. DCW, organic acids production, amino acids composition, specific growth rate ($\mu$) and celA production/yield are compared.

![Figure 4.27 – DCW throughout time for all the media tested.](image)

Figure 4.27 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 3.00g/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 a way 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 Figure 4.29). 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 Figure 4.29), it is normal that the DCW of MM+PEP5+MEAT3 also did not differ a lot (5.18g/L).

Table 4.6 – Minimum DO, Maximum XCO2 and specific growth rate (µ) for all the media tested.

<table>
<thead>
<tr>
<th>Medium</th>
<th>MM+CAS15</th>
<th>MM+PEP5+BEEF3</th>
<th>MM+PEP5+MEAT3</th>
<th>MM+CAS5</th>
<th>MM+ASP2,5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min DO (%)</td>
<td>13.85</td>
<td>15.99</td>
<td>17.33</td>
<td>22.42</td>
<td>28.74</td>
</tr>
<tr>
<td>Max XCO2 (%)</td>
<td>0.65</td>
<td>0.67</td>
<td>0.64</td>
<td>0.62</td>
<td>0.45</td>
</tr>
<tr>
<td>µ (h⁻¹)</td>
<td>-</td>
<td>0.058 ± 0.007</td>
<td>0.060 ± 0.005</td>
<td>0.078 ± 0.002</td>
<td>0.136 ± 0.014</td>
</tr>
<tr>
<td>µ₁ (h⁻¹)</td>
<td>0.387 ± 0.005</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>µ₂ (h⁻¹)</td>
<td>0.085 ± 0.007</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.6 – Minimum DO, Maximum XCO₂ and specific growth rate (µ) for all the media tested (cont.).

<table>
<thead>
<tr>
<th>Medium</th>
<th>MM</th>
<th>MM+GLU2,5</th>
<th>TSB</th>
<th>2NB</th>
<th>PEP5+BEEF3</th>
<th>NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min DO (%)</td>
<td>40.47</td>
<td>49.02</td>
<td>7.62</td>
<td>60.15</td>
<td>9.55</td>
<td>68.55</td>
</tr>
<tr>
<td>Max XCO2 (%)</td>
<td>0.45</td>
<td>0.48</td>
<td>0.72</td>
<td>0.29</td>
<td>0.23</td>
<td>0.16</td>
</tr>
<tr>
<td>µ (h⁻¹)</td>
<td>0.181 ± 0.009</td>
<td>0.134 ± 0.014</td>
<td>0.323 ± 0.062</td>
<td>0.044 ± 0.002</td>
<td>-</td>
<td>0.079 ± 0.013</td>
</tr>
</tbody>
</table>

Table 4.6 confirms that the media that reached the highest DCW also achieved a lower percentage of DO in the medium (except for TSB and PEP5+BEEF3) and a higher CO₂ concentration on the outlet air. This was expected since cellular growth is associated in aerobic microorganisms to oxygen consumption and CO₂ production. The type of substrate (glucose, organic acids or amino acids) will determined the required amount of O₂ or CO₂ produced per gram of substrate consumed. For Bennett, BEEF3 and PEP5 media there is unfortunately no data regarding DO or XCO₂.

The specific growth rate (µ), which corresponds to the slope of the exponential growth phase in a semi-logarithmic plot of DCW versus time, is also displayed in Table 4.6. No specific growth rate is reported when not sufficient biomass data are available. MM+CAS15 was the only media with enough time points in the exponential phase that made possible to separate the specific growth rate into two growth phases using the two-phase linear model presented in D’Huys et al. (2012), being the first reportedly associated with aspartate,
glutamate, glucose and ammonium uptake ($\mu_1$) while the latter with glucose and ammonium consumption ($\mu_2$). Since there is no amino acids profile available for MM+CAS15 and ammonium was not quantified it was not possible to confirm this fact. The remaining media have only one specific growth rate calculated, using the linear regression in Microsoft Office Excel 2010, due to the lack of enough data in the exponential phase. The data used for $\mu$ calculation in MM+CAS5, MM+PEP5+BEEF3, MM+PEP5+MEAT3 and 2NB belong only to the late exponential phase which makes it not correct to compare with the other media. Regarding the remaining media it is clear why TSB is broadly used for S. lividans study since it had a higher $\mu$ and reached earlier the stationary phase. The supplementation of aspartate and glutamate to MM reduced slightly the specific growth rate.

![Graphs showing organic acids concentrations](image)

**Figure 4.28** – Organic acids concentrations in function of time for all the media tested. Plot A: ($Akg$) $\alpha$-ketoglutarate, Plot B: (Pyr) Pyruvate, Plot C: (Succ) Succinate, Plot D: (Acet){Acetate.

Figure 4.28 shows the concentration profiles of organic acids for all the media (except lactic acid – not enough information). $\alpha$-Ketoglutaric acid (Figure 4.28 A) presented values lower than 10mg/L except for Bennett (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 4.28 B) also achieved high values for Bennett (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 4.28 C) had the highest excretion in MM+PEP5+MEAT3 and in MM+PEP5+BEEF3 already 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 4.28 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 (1229.41mg/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.

Figure 4.29 – 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 4.29 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) became 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 asparagine or glutamine in its composition, these 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.

![Figure 4.30 – 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.

Analysing the amount of each single amino acid for some of the media (Figure 4.30), it is clear that the media with casamino acids did not contain tryptophan as expected. MM+CAS15 and MM+CASS 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 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 4.31](image)

**Figure 4.31** – CelA throughout time for all the media tested.

The celA concentration for some experimental time points of all the media tested is displayed in Figure 4.31. 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 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 4.27), 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.

Table 4.7 shows the celA and DCW maximum concentrations achieved for all the media tested together with glucose and amino acids initial concentration in the medium and peptide sources. 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 included beef extract in their composition but no glucose.
Table 4.7 – Overview of the maximum concentration of celA and DCW obtained in each tested medium. The corresponding elapsed fermentation time is given together with glucose and amino acids initial concentration and peptide sources.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Maximum DCW (g/L)</th>
<th>Elapsed fermentation time (h)</th>
<th>Maximum celA (mg/L)</th>
<th>Elapsed fermentation time (h)</th>
<th>CelA yield (mg/g DCW)</th>
<th>Initial glucose concentration (g/L)</th>
<th>Total initial concentration of amino acids (mM)</th>
<th>Peptide sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bennett</td>
<td>2.25</td>
<td>67.2</td>
<td>1.92</td>
<td>65.5</td>
<td>0.85</td>
<td>10</td>
<td></td>
<td>Lab M™ tryptone Yeast extract Beef extract</td>
</tr>
<tr>
<td>TSB</td>
<td>2.55</td>
<td>23.3</td>
<td>2.77</td>
<td>35.0</td>
<td>1.09</td>
<td>2.5</td>
<td></td>
<td>Casein peptone Soya peptone</td>
</tr>
<tr>
<td>MM+ASP2,5</td>
<td>3.23</td>
<td>48.0</td>
<td>7.51</td>
<td>48.0</td>
<td>2.33</td>
<td>10</td>
<td>18.78 (2)</td>
<td></td>
</tr>
<tr>
<td>PEP5</td>
<td>0.65</td>
<td>48.0</td>
<td>8.97</td>
<td>67.0</td>
<td>13.80</td>
<td>-</td>
<td>15.11</td>
<td>Peptone</td>
</tr>
<tr>
<td>MM</td>
<td>3.08</td>
<td>44.0</td>
<td>11.08</td>
<td>48.0</td>
<td>3.60</td>
<td>10</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MM+GLU2,5</td>
<td>3.02</td>
<td>48.0</td>
<td>12.93</td>
<td>48.0</td>
<td>4.28</td>
<td>10</td>
<td>16.99 (2)</td>
<td></td>
</tr>
<tr>
<td>MM+CAS5</td>
<td>4.54</td>
<td>28.0</td>
<td>14.36</td>
<td>48.0</td>
<td>3.16</td>
<td>10</td>
<td>13.24 (*)</td>
<td>Bacto™ casamino acids</td>
</tr>
<tr>
<td>MM+CAS15</td>
<td>5.60</td>
<td>35.0</td>
<td>15.68</td>
<td>35.0</td>
<td>2.80</td>
<td>10</td>
<td>39.73</td>
<td>Bacto™ casamino acids</td>
</tr>
<tr>
<td>MM+PEP5+BEEF3</td>
<td>5.19</td>
<td>26.0</td>
<td>18.09</td>
<td>67.2</td>
<td>3.49</td>
<td>10</td>
<td>18.67</td>
<td>Peptone Beef extract</td>
</tr>
<tr>
<td>NB</td>
<td>1.01</td>
<td>35.0</td>
<td>23.13</td>
<td>35.0</td>
<td>22.90</td>
<td>-</td>
<td>6.99</td>
<td>Peptone Beef extract</td>
</tr>
<tr>
<td>MM+PEP5+MEAT3</td>
<td>5.18</td>
<td>45.0</td>
<td>26.31</td>
<td>67.2</td>
<td>5.08</td>
<td>10</td>
<td>18.63</td>
<td>Peptone Meat extract</td>
</tr>
<tr>
<td>BEEF3</td>
<td>0.72</td>
<td>48.0</td>
<td>33.87</td>
<td>48.0</td>
<td>47.04</td>
<td>-</td>
<td>4.53</td>
<td>Beef extract</td>
</tr>
<tr>
<td>PEP5+BEEF3</td>
<td>1.54</td>
<td>48.0</td>
<td>41.97</td>
<td>67.0</td>
<td>27.25</td>
<td>-</td>
<td>18.05</td>
<td>Peptone Beef extract</td>
</tr>
<tr>
<td>2NB</td>
<td>2.41</td>
<td>48.0</td>
<td>100.54</td>
<td>67.0</td>
<td>41.72</td>
<td>-</td>
<td>12.24</td>
<td>Peptone Beef extract</td>
</tr>
</tbody>
</table>

(1) Amino acids concentration not measured.  
(2) Calculated values.  
(*) Not analysed value based on MM+CAS15.
5. Discussion

As shown in the Results (Chapter 4), noticeable differences in the final celA concentration and the celA yield can be observed amongst the various media (see Table 4.7). Eventually, the test of different defined and complex media led to a reasonable heterologous production of cellulase A by *S. lividans* TK24. Since we were interested in understanding the background of the impact of medium composition on the growth and cellulase A production, growth curves and concentration profiles of substrates and extracellular metabolites were gathered. As explained in the Literature review (Chapter 2), many factors affect heterologous production but medium composition can be considered as an important one. Although medium composition affects heterologous production a screening, as established in this thesis, is seldom reported. In this chapter, the results summarized in Chapter 4 are discussed in more detail.

*Glucose addition mainly promotes biomass growth*

Taking into account the data shown in the Results (Chapter 4), 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+CASS and MM+CAS15) achieved some of the highest final biomass concentration as compared to the other media (see Table 4.7). 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 CO₂ production was due to the metabolization of organic acids (e.g., Figure 4.4). 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).

*Influence of organic acids secretion*

CelA production was negatively influenced by the glucose presence in the media (see Table 4.7), 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 (Figure 4.12 (acetic acid concentration reached up to 2.9 g/L) and Table 4.3 and 4.7).
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 leads 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 (e.g., Figure 4.14 versus Figure 4.5). 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 (see Table 4.7).

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 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 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 and 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.
As mentioned before 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, 2NB and BEEF3 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, 2NB and BEEF3, 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.

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 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.

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 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 purification step included medium supernatant ultrafiltration, precipitation with ammonium sulphate, centrifugation, size exclusion chromatography, ion exchange chromatography and a final Western blot to confirm the purified protein presence. 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.
6. Conclusion

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 strength Nutrient Broth, it was possible to improve celA heterologous production in *S. lividans* TK24 to 100.54mg/L (or 41.63 mg/g DCW). A comparison of reconstituted Nutrient Broth 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 strength 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


Noda, S., Kawai, Y., Tanaka, T., Kondo, A., 2015a. 4-Vinylphenol biosynthesis from cellulose as the sole carbon source using phenolic acid decarboxylase- and tyrosine ammonia lyase-expressing *Streptomyces lividans*. Bioresource Technology 180, 59–65.


Research and Markets, 2016. Technical Enzymes Market by Type (Cellulases, Amylases, Proteases, Lipases, Other Enzymes), Application (Bioethanol, Paper & Pulp, Textile & Leather, Starch Processing, Other


APPENDICES

Western-blot Images

**Figure I** – Western-blot image obtained with the GE Image Quant LAS 4000 for the reference experiments. Lanes 1 to 5 celA standards (1x, 2x, 4x, 8x, 16x). Lanes 6 to 9 bioreactor samples at 44h (MM, MM+CASS, MM+ASP2,5, MM+GLU2,5).

**Figure II** – Western-blot image obtained with the GE Image Quant LAS 4000 for the reference experiments. Lanes 1 to 4 bioreactor samples at 48h (MM+GLU2,5, MM+ASP2,5, MM+CASS, MM). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).
Figure III – Western-blot image obtained with the GE Image Quant LAS 4000. Lanes 1 to 4 bioreactor samples at 19h (NB, TSB, MM+CAS15, MM). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).

Figure IV – Western-blot image obtained with the GE Image Quant LAS 4000. Lanes 1 to 5 celA standards (1x, 2x, 4x, 8x, 16x). Lanes 6 to 9 bioreactor samples at 21.1h (MM, MM+CAS15, TSB, NB).
**Figure V** – Western-blot image obtained with the GE Image Quant LAS 4000. Lanes 1 to 4 bioreactor samples at 23,3h (NB, TSB, MM+CAS15, MM). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).

**Figure VI** – Western-blot image obtained with the GE Image Quant LAS 4000. Lanes 1 to 4 bioreactor samples at 35h (NB, TSB, MM+CAS15, MM). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).
Figure VII – Western-blot image of blot 1 obtained with the GE Image Quant LAS 4000 for Bennett medium. Lanes 1 to 5 celA standards (1x, 2x, 4x, 8x, 16x). Lanes 6 to 9 bioreactor samples (24.5h, 44.5h, 65.5h, 67.2h).

Figure VIII – Western-blot image of blot 2 obtained with the GE Image Quant LAS 4000 for Bennett medium. Lanes 1 to 4 bioreactor samples (67.2h, 65.5h, 44.5h, 24.5h). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).
**Figure IX** – Western-blot image of blot 3 obtained with the GE Image Quant LAS 4000 for Bennett medium. Lanes 1 to 4 bioreactor samples (67.2h, 65.5h, 44.5h, 24.5h). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).

**Figure X** – Western-blot image of blot 4 obtained with the GE Image Quant LAS 4000 for Bennett medium. Lanes 1 to 5 celA standards (1x, 2x, 4x, 8x, 16x). Lanes 6 to 9 bioreactor samples (24.5h, 44.5h, 65.5h, 67.2h).
**Figure XI** – Western-blot image obtained with the GE Image Quant LAS 4000 for MM+PEPS+BEEF3. Lanes 1 to 4 celA standards (2x, 4x, 8x, 16x). Lanes 5 to 9 bioreactor samples (26h, 43,1h, 47h, 49h, 67,2h).

**Figure XII** – Western-blot image obtained with the GE Image Quant LAS 4000 for MM+PEPS+MEAT3. Lanes 1 to 4 celA standards (2x, 4x, 8x, 16x). Lanes 5 to 9 bioreactor samples (26h, 43,1h, 47h, 49h, 67,2h).
Figure XIII – Western-blot image obtained with the GE Image Quant LAS 4000 for Nutrient Broth research. Lanes 1 to 4 bioreactor samples at 24,5h (PEP5+BEEF3, PEP5, BEEF3, 2NB). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).

Figure XIV – Western-blot image obtained with the GE Image Quant LAS 4000 for Nutrient Broth research. Lanes 1 to 4 bioreactor samples at 42h (PEP5+BEEF3, PEP5, BEEF3, 2NB). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).
**Figure XV** – Western-blot image obtained with the GE Image Quant LAS 4000 for Nutrient Broth research. Lanes 1 to 4 bioreactor samples at 48h (PEP5+BEEF3, PEP5, BEEF3, 2NB). Lanes 5 to 9 celA standards (16x, 8x, 4x, 2x, 1x).

**Figure XVI** – Western-blot image obtained with the GE Image Quant LAS 4000 for Nutrient Broth research. Lanes 1 to 5 celA standards (1x, 2x, 4x, 8x, 16x). Lanes 6 to 9 bioreactor samples at 67h (2NB, BEEF3, PEP5, PEP5+BEEF3).