
**Ana Rita da Silva Bernardino**
Degree in Biochemistry

# A biotechnological approach for vanillin production

Dissertation for the Degree of Master in Biotechnology

Supervisor: Doctor Cristiana Andreia Vieira Torres, Post-doctoral researcher, FCT-UNL
Co-supervisor: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis, Full professor, FCT-UNL

Jury:

President: Prof. Dr. Carlos Alberto Gomes Salgueiro
Examiner: Prof. Dr. Rui Manuel Freitas Oliveira


**September, 2017**

**Ana Rita da Silva Bernardino**
Degree in Biochemistry

# A biotechnological approach for vanillin production

Dissertation for the Degree of Master in Biotechnology

Supervisor: Doctor Cristiana Andreia Vieira Torres, Post-doctoral researcher, FCT-UNL
Co-supervisor: Professor Maria da Ascensão Carvalho Fernandes Miranda Reis, Full professor, FCT-UNL

**A biotechnological approach for vanillin production**

# Agradecimentos

Ao longo deste ano de trabalho desafiante são muitas as pessoas às quais quero deixar uma palavra de agradecimento.

Queria, em primeiro lugar, agradecer à minha orientadora, Cristiana Torres, por toda a ajuda e apoio prestado, todas as discussões e o conhecimento que partilhou comigo e me ajudou a adquirir. Obrigada pelos conselhos, mesmo nos meus momentos de mais insegurança e preocupação.

À minha co-orientadora, a Professora Maria Ascensão Reis, um muito obrigada pela oportunidade de trabalhar e aprender tanto nos laboratórios do BIOENG, por todo o entusiasmo no meu trabalho, todas as discussões, sugestões e conhecimentos partilhados. Um obrigada também pelo interesse que me despertou por esta área de trabalho nas aulas e me fez escolher este tema.

Agradeço ainda à Drª Carla Brazinha pela oportunidade de integrar este projecto.

À Rita Valério, quero agradecer pelo que me ensinou e me ajudou no trabalho laboratorial, pela paciência e disponibilidade, mas também pela motivação, boa disposição e amizade ao longo deste ano.

Agradeço também a todos os membros do grupo BIOENG pela forma como me receberam e me ajudaram sempre que precisei, com especial atenção a Diana Araújo, Sílvia Baptista, João Pereira, Patrícia Reis, Inês Farinha e Filomena Freitas por me terem integrado, ouvido e aconselhado, mas também pelo bom ambiente de trabalho, boa disposição e simpatia.

Aos meus colegas de tese e companheiros no laboratório, Joana Marques, Patrícia Freitas, Sónia Nóbrega, Letícia Fialho, David Cruz, agradeço a companhia, o apoio e também os bons momentos.

Aos meus amigos e companheiros de tese, Eliana Guarda, Juliana Almeida, Liane Meneses, Sara Mateus e Gonçalo Abrantes, um grande obrigada pela paciência, apoio e encorajamento nos momentos de maior desânimo e frustração e nos momentos de mais alegria também. Obrigada pela partilha de conhecimento, pela motivação para a escrita, pela amizade e pelos bons momentos que passámos juntos.

Aos meus amigos Bioqs de Coimbra, Adriana Mamede, Bruna Santos e Ricardo Santo, obrigada pelos momentos de diversão e pelo apoio, mesmo que à distância.

À Professora Margarida Castro, não só uma professora, mas também uma amiga, pelos conselhos, pela preocupação e atenção, muito obrigada.

À minha família, especialmente aos meus pais e à minha irmã, agradeço todo o carinho, paciência, apoio, motivação e compreensão, sem dúvida fundamental ao longo deste ano e de todo o meu percurso académico.

# Abstract

Vanillin is the main component of the vanilla flavor and since natural vanilla coming from the vanilla orchids has a high market price, vanillin can be produced synthetically as alternative. However, nowadays there is a growing interest in natural products and the production of natural vanilla cannot cover the demand. Therefore, over the last years, biotechnological approaches have been studied for vanillin production that, when obtained by this way, can be considered as natural.

In this thesis, the process of bioproduction of vanillin from ferulic acid by the *Amycolatopsis* sp. ATCC 39116 was studied in bioreactor. This microorganism has a remarkable capability to produce vanillin from ferulic acid, since it can tolerate high concentrations of substrate and product.

The objective of this work was to optimize the vanillin production varying some conditions of the standard production that contains 5 $g.L^{-1}$ of yeast extract, 20 $g.L^{-1}$ of glucose and is fed with a single pulse of ferulic acid (10 $g.L^{-1}$). In these conditions, the maximum vanillin concentration achieved was 5.28 $g.L^{-1}$, with a yield of vanillin in ferulic acid of 0.62 $g.g^{-1}$ and a volumetric productivity of vanillin of 0.28 $g.L^{-1}.h^{-1}$.

In the study of the effect of nitrogen concentration in vanillin production, different concentrations were tested (2.00 to 0.25 $g.L^{-1}$) resulting in different cellular concentrations. The best production was obtained with 0.50 $g.L^{-1}$ of nitrogen (coming from 5 $g.L^{-1}$ of yeast extract, standard conditions), giving rise to 5 $g.L^{-1}$ of biomass.

The influence of the nitrogen source was tested by replacing half of the yeast extract that constitutes the cultivation medium by some ammonium salts. Ammonium phosphate was the salt that showed better results, thus had been chosen to perform a bioreactor run. This substitution led to a slower growth and lower vanillin volumetric productivity (0.14 $g.L^{-1}h^{-1}$).

The study of the impact of glucose concentration in vanillin production revealed that the presence of this carbon and energy source in the broth during the transformation phase is important to achieve an accumulation of the product. In its absence, vanillin is consumed at a higher rate and converted into vanillic acid.

Studies with different feeding pulse strategies were performed and the best results were obtained when the broth was removed before the addition of each pulse with biomass recovering, avoiding toxic vanillin concentrations. With this approach it was possible to obtain a higher vanillin volumetric productivity of 0.46 $g.L^{-1}.h^{-1}$ and a yield of vanillin in ferulic acid of 0.69 $g.g^{-1}$ similar to the standard.



## Keywords

Vanillin, Ferulic acid, *Amycolatopsis* sp. ATCC 39116, Nitrogen, Glucose, Pulse feeding.

# Resumo

O principal componente do aroma a baunilha é a vanilina e, uma vez que a baunilha natural, proveniente das orquídeas, tem um valor de mercado muito elevado, a vanilina é produzida, alternativamente, por via sintética. No entanto, hoje em dia, há um crescente interesse por produtos naturais e a produção de baunilha não consegue satisfazer a procura. Ao longo dos últimos anos, têm sido estudadas abordagens biotecnológicas para a produção de vanilina que, ao ser obtida por esta via, pode ser considerada natural.

Nesta tese estudou-se o processo de produção de vanilina em biorreactor a partir de ácido ferúlico por *Amycolatopsis* sp. ATCC 39116. Este microrganismo tem uma capacidade excepcional para produzir vanilina a partir do ácido ferúlico, uma vez que consegue tolerar elevadas concentrações do substrato e do produto.

Este trabalho teve como objectivo optimizar a produção de vanilina, variando algumas condições do processo padrão, que consiste em 5 $g.L^{-1}$ de extracto de levedura, 20 $g.L^{-1}$ de glucose e um único pulso de alimentação (ácido ferúlico 10 $g.L^{-1}$). Nestas condições, a concentração máxima de vanilina obtida foi 5.28 $g.L^{-1}$, com um rendimento de vanilina em ácido ferúlico de 0.62 $g.g^{-1}$ e uma produtividade volumétrica de vanilina de 0.28 $g.L^{-1}.h^{-1}$.

No estudo do efeito da concentração de azoto na produção de vanilina, foram testadas várias concentrações de azoto (de 2.00 a 0.25 $g.L^{-1}$), resultando em diferentes concentrações celulares. A melhor produção foi obtida com 0.5 $g.L^{-1}$ de azoto (proveniente de 5 $g.L^{-1}$ de extracto de levedura, a condição padrão), originando 5 $g.L^{-1}$ de biomassa.

A influência da fonte de azoto foi testada recorrendo à substituição de metade do extracto de levedura que constitui o meio de cultivo por diferentes sais de amónia. O fosfato de amónia foi o que mostrou melhores resultados, tendo sido posteriormente testado em biorreactor. Esta substituição levou a que o crescimento e produção fossem mais lentos, resultando numa menor produtividade volumétrica de vanilina (0.14 $g.L^{-1}h^{-1}$).

O estudo do impacto da concentração de glucose na produção de vanilina revelou que a presença desta fonte de carbono e energia no caldo de fermentação durante a fase de produção é importante para se obter uma acumulação do produto. Na sua ausência, a vanilina é rapidamente consumida e transformada em ácido vanilico.

Foram estudadas diferentes estratégias de alimentação por pulsos, sendo que os melhores resultados foram obtidos quando o caldo de fermentação foi removido, com recuperação da biomassa, antes da adição de cada pulso de substrato, evitando concentrações tóxicas de vanilina. Desta forma, foi possível aumentar a produtividade volumétrica de vanilina para 0.46 $g.L^{-1}.h^{-1}$ mantendo-se um rendimento vanilina em ácido ferúlico de 0.69 $g.g^{-1}$, semelhante ao ensaio padrão.



## Termos chave

Vanilina, Ácido ferúlico, *Amycolatopsis* sp. ATCC 39116, Azoto, Glucose, Alimentação por pulsos.

# List of contents

# List of Figures

# List of tables

## Abbreviations

ATP – Adenosine Triphosphate

CDW – Cell Dry Weight

CoA – Coenzyme A

ECH – Enoyl-CoA Hydratase/Aldolase

FCS – Feruloyl-CoA Synthetase

Glc – Glucose

HPLC – High Performance Liquid Chromatography

N – Nitrogen

$OD_{600}$ – Optical Density at 600 nm

$pO_2$ – Partial Oxygen Pressure (%)

V – Vanillin

VDH – Vanillin Dehydrogenase

YE – Yeast Extract

## Variables

$q_P$ – Specific productivity ($g_{vanillin}.g_X^{-1}.h^{-1}$)

$q_v$ – Specific rate of vanillin production ($g_{vanillin}.g_X^{-1}.h^{-1}$)

$r_c$ – Rate of consumption of a compound ($g_{compound}.L^{-1}.h^{-1}$)

$r_P$ – Volumetric productivity ($g_{vanillin}.L^{-1}.h^{-1}$)

$r_v$ – Rate of vanillin production ($g_{vanillin}.L^{-1}.h^{-1}$)

t – Time (h)

$x$ – Biomass concentration ($g.L^{-1}$)

$x_0$ – Initial biomass concentration ($g.L^{-1}$)

$Y_{P/S}$ – Yield of vanillin in ferulic acid ($g.g^{-1}$)

$Y_{X/S}$ – Yield of biomass in glucose ($g.g^{-1}$)

P – Vanillin produced ($g.L^{-1}$)

$S_1$ – Glucose consumed ($g.L^{-1}$)

$S_2$ – Ferulic acid consumed ($g.L^{-1}$)

μ – Specific growth rate ($h^{-1}$)

# 1. Introduction and Motivation

## 1.1 Vanilla: a natural or a synthetic flavor

Vanilla is one of the most widely used flavors in the world, its major application is in food and beverage industries, flavoring chocolate, ice cream and coffee, for example, but is also extensively applied in perfume and cosmetic industries. The major component of vanilla flavor, vanillin, is also used in pharmaceutical industry for production of some drugs, as L-DOPA, dopamine and papaverine, and due to its antimicrobial and antioxidant activities, it is applied as food preservative (Walton *et al.*, 2003; Ramachandra Rao and Ravishankar, 2000; Fache *et al.,* 2016; Kaur and Chakraborty, 2013). The appliance of vanillin in the industries are illustrated in Figure 1.1.

Figure 1.1 – Industrial applications of vanillin, where ■ represents food and beverage industries (45%), ■ cosmetic and perfume industries (38%), ■ pharmaceutic industry (15%) and ■ other utilizations (2%) (Adapted from Eswaran 2017).

Natural vanilla is extracted from the cured beans of *Vanilla planifolia* and *Vanilla tahitensis*, two species of vanilla orchids, and those cured beans contain only 2% of vanilla flavor that can be extracted (Bomgardner, 2016; Ramachandra Rao and Ravishankar, 2000). The natural vanilla flavor is a complex mixture of flavor components with more than 170 volatile aromatic compounds identified (Ramachandra Rao and Ravishankar, 2000). The main component of this flavor is vanillin, an aromatic aldehyde, which chemical structure is represented in Figure 1.2. The major producer of natural vanilla is Madagascar, with 80% of all production, but it is also produced in lower quantities in Indonesia, India, Uganda and Mexico (Bomgardner, 2016). Natural vanilla is expensive because of the weather conditions and crop dependence, and the production methods that include hand pollination and traditional cure of beans. Furthermore, its production had dropped off for half the normal harvest, making the prices of vanilla rise and eventually return to $1 250 per kilogram, like before 2012 (Bomgardner, 2016).

The production of natural vanilla is about 2 000 tons per year but with the growing of the global market and the interest in natural flavors, this type of production is not enough to cover the demand. Only 1% of vanilla flavor comes from the natural source, the majority is produced synthetically, but due to the complexity of the flavor, only its main component, vanillin, is produced (Bomgardner, 2016; Gallage and Møller, 2015).

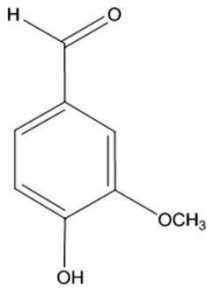



Figure 1.2 – Chemical structure of vanillin.

About 85 % of vanillin with synthetic origin is produced using the petrochemical precursor guaiacol, and almost the rest has lignin as raw material (Fache *et al.*, 2016). Even though synthetic vanillin is cheaper than the natural vanilla, about $10 per kilogram, its production has a negative environmental impact because the organic solvents used in vanillin purification and petrochemical sources are not considered as renewable resources (Bomgardner, 2016; Fache *et al.*, 2016; Gallage and Møller, 2015).

Nowadays there is a rising interest by the consumers for healthy habits where natural food is included, so the demand for natural products has increased with the companies substituting the synthetic vanillin by natural vanilla to label the products as all-natural (Bomgardner, 2016). However, the global production cannot support the demand and the flavor companies are trying to find other sources of natural vanillin, with the quality presented by the vanilla extracted from cured beans (Bomgardner, 2016; Gallage and Møller, 2015). Biotechnological processes are good alternatives for this problem because they are considered natural, according to European and US legislations. In European Union, a substance can be labeled as natural since it is “obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin” and must “correspond to substances that are naturally present and have been identified in nature”(European Commission, 2008). In United States, natural flavor “means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavoring rather than nutritional” (U.S. Food and DrugAdministration).

There are already some biotechnological-derived vanillin in the market which offer an alternative to synthetic vanillin made from petrochemical sources. The company Solvay produces vanillin Rhovanil® Natural, the first to be commercially available, obtained by fermentation of ferulic acid from rice bran by an unmodified strain. This product is considered natural according to US and European regulation (Solvay). De Monchy Aromatics also produces natural vanillin by a bioconversion process from ferulic acid found in rice bran oil and is considered natural by EU regulation (Van Berkel, 2016; De Monchy Aromatics). The company Mane obtains vanillin by bioconversion of eugenol from clove oil and the companies Evolva and International Flavors and Fragrances commercialize vanillin synthetized from glucose by genetic modified microorganisms, eliminating the toxicity of vanillin for the microorganisms with the sugar group, that needs to be removed in order to obtain vanillin (Leffingwell, 2015). These

alternatives are more expensive than the synthetic vanillin obtained from petrochemical source and cheaper than the natural vanilla from cured beans, however the price reaches several hundred dollars per kilogram (Bomgardner, 2016).

## 1.2 Biotechnological production of vanillin

There are other advantages for products obtained by biotechnological processes besides the labelling as natural. When compared with a synthetic approach these advantages include the high specificity for the substrate or the product, in case of the interest in only one of the isomers, and the softer production conditions, being less harmful to the environment. Comparing to the natural aroma, biotechnological production has the advantage of not depending on agriculture conditions, like crop or climate, having an impact in the price. The possibility of a scale-up process and the application on the industry as a controllable process is also a positive point (Krings and Berger, 1998; Kaur and Chakraborty, 2013).

Given the need and the interest in biotechnological production of vanillin there are many studies with different substrates and microorganisms tested to obtain the optimum production conditions and yields.

Biotechnological vanillin can be obtained from biotransformation of natural substrates by fungi, bacteria, genetically modified microorganisms and plant cells. The most studied substrates are ferulic acid, eugenol and isoeugenol, sugars specially glucose, lignin, aromatic amino acids and phenolic stilbenes (Gallage and Møller, 2015). The chemical structures of the substrates for production of vanillin that will be approached in this section are represented in Figure 1.3.

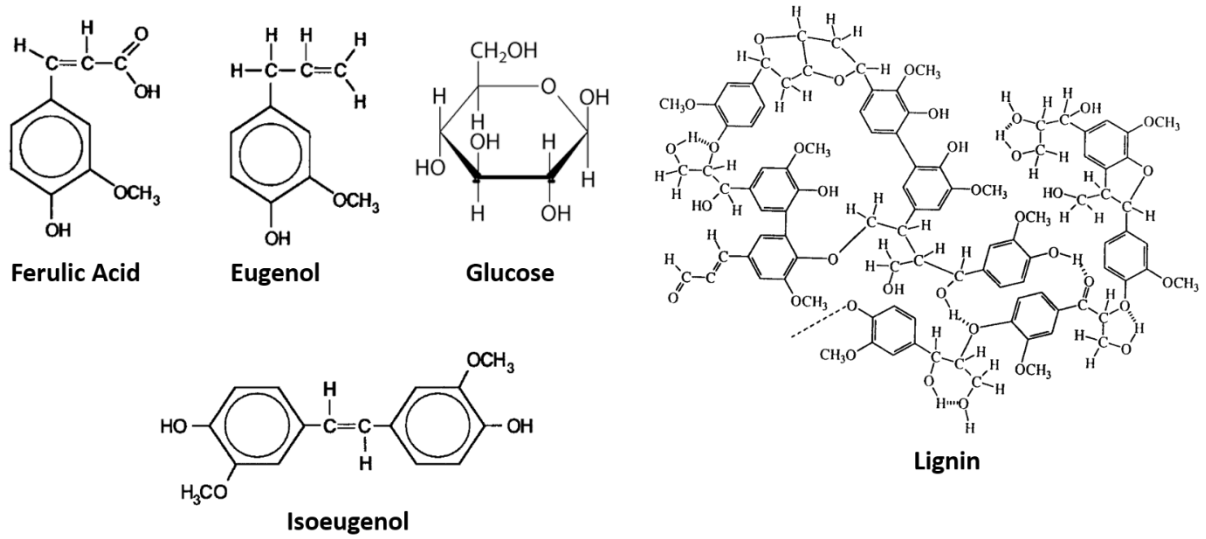



Figure 1.3 – Chemical structures of some of the substrates for vanillin production: ferulic acid, eugenol, isoeugenol, glucose and lignin.

Of all the substrates referred above, ferulic acid is the most studied for vanillin production because it is abundant in nature, present in the plant cell wall, and has a similar chemical structure with the desired molecule (Gallage and Møller, 2015; Priefert *et al.*, 2001). There are several strains of microorganisms that can produce vanillin from this substrate and the most studied that are reported to produce levels above 1 $g.L^{-1}$ are *Amycolatopsis* ATCC 39116 (re-classification of *Streptomyces setonii* ATCC 39116)

(Ma and Daugulis, 2014b; Ma and Daugulis, 2014a; Muheim and Lerch, 1999), *Amycolatopsis sp.* V1 (Ma and Daugulis, 2014a), *Aspergillus niger* and *Pycnoporus cinnabarius* in a two-phase process including these two microorganisms (Zheng *et al.*, 2007).

Eugenol and isoeugenol are present in clove oil as main component and eugenol was the substrate reported for production of vanillin in the first patent for microbiological production of this flavor (Gallage and Møller, 2015; Priefert *et al.*, 2001). Some microorganisms in nature metabolize eugenol and isoeugenol having ferulic acid and vanillin as intermediate products (Gallage and Møller, 2015). The advantages of using eugenol as a substrate are the price, cheaper than ferulic acid, which makes it more attractive, and the identification as GRAS (Generally Recognized as Safe) for usage as food additives. However it has a toxic effect in the microorganisms, inhibiting their metabolism and their growth (Gallage and Møller, 2015; FDA - U.S. Food & Drug Administration; Muheim and Lerch, 1999). The microorganisms that are better studied in eugenol catabolism are *Pseudomonas*, in terms of enzymes and genes involved, however there are not an accumulation of vanillin because it is degraded in other products such as vanillic acid and protocatechuic acid, since vanillin is toxic for microorganisms (Gallage and Møller, 2015; Priefert *et al.*, 2001; Muheim and Lerch, 1999). *Pseudomonas* HR199 with mutations in genes that are involved in degradation of vanillin has been reported in literature as producer of this flavor compound (Overhage *et al.*, 1999; Priefert *et al.*, 2001). A recombinant *Amycolatopsis* sp. HR167, that is a vanillin tolerant microorganism, is also reported as vanillin producer from eugenol, since it became capable of using eugenol as sole carbon source (Overhage *et al.*, 2006; Priefert *et al.*, 2001).

The sugars are very attractive sources for production of vanillin by microorganisms due to the abundance and the low cost as raw material (Hansen *et al.*, 2009; Gallage and Møller, 2015). The most studied sugar for production of this compound is glucose (Gallage and Møller 2015; Kaur and Chakraborty, 2013; Priefert *et al.*, 2001). The advantage of glucose is that, below to certain concentrations, is nontoxic to microorganisms, since it is very commonly used by them as source of carbon and energy, and there are not the problem of the off-taste that can occur when the substrate is an aromatic compound like ferulic acid or eugenol (Gallage and Møller, 2015). However, in nature there is no microorganism reported able to produce vanillin from glucose, so the biosynthesis has been studied in recombinant organisms such as *Escherichia coli*, and yeasts such as *Saccharomyces cerevisiae* (Gallage and Møller, 2015; Kaur and Chakraborty, 2013; Priefert *et al.*, 2001). Some work with recombinant yeasts involve the incorporation of the entire vanillin production pathway, but also the expression of a glycosyltransferase to produce vanillin-β-D-glucoside, that is less toxic to the cells than vanillin, allowing a better accumulation of the compound (Brochado *et al.*, 2010; Hansen *et al.*, 2009). This process for overcoming the toxicity of the compound is based on the natural process for production of vanilla in *Vanilla planifolia*, but a transformation process is needed to transform the product in vanillin (Hansen *et al.*, 2009). In literature, the most reported work with recombinant *E. coli* studied the biotransformation of glucose in vanillic acid by this microorganism, and its subsequent enzymatically reduction in vanillin by enzymes isolated from *Neurospora crassa* (Li and Frost, 1998).

Lignin is an abundant source of aromatic compounds and nowadays a part of synthetic vanillin is obtained chemically by this source, however some studies has been done for its microbiological transformation to obtain vanillin (Fache *et al.*, 2016; Priefert *et al.*, 2001; Sainsbury *et al.*, 2013). White-rot fungi can degrade lignin, due to the presence of the extracellular enzyme lignin peroxidase, and trace amounts of that aromatic compound were identified (Martínez *et al.*, 2005; Priefert *et al.*, 2001). The bacteria *Rhodococcus jostii* RHA1, that can also degrade lignin, was studied for production of vanillin with a deletion of a vanillin dehydrogenase gene that makes the microorganism capable of accumulate the compound (Ahmad *et al.*, 2011; Sainsbury *et al.*, 2013).

The most studied substrates and the corresponding microorganisms used for vanillin production were presented in this section. In the next section, more specific information related to the process that was carried out in this work will be presented.

## 1.3 Transformation of ferulic acid by *Amycolatopsis* sp. ATCC 39116

### 1.3.1 Ferulic acid: the substrate

Ferulic acid is a hydroxycinnamic acid, extremely abundant, found in plant cell walls. It is normally found linked to carbohydrates by covalent bonds, forming esters or ethers bonds with lignin and hemicelluloses, and can also occur as amides. The presence of this molecule confers rigidity and strengthens the cell wall by cross-linking with pentosane chains, arabinoxylans, hemicelluloses and lignin, making these components less vulnerable to hydrolytic enzymes during the development of the plant. The double bond that ferulic acid has in its side chain (the chemical structure can be seen in Figure 1.3) is subjected to *cis*-*trans* isomerization but it is extracted mostly in its *trans*-isomeric form (Tilay *et al.*, 2008; Kumar and Pruthi, 2014; Rosazza *et al.*, 1995).

Ferulic acid is found in fruits, vegetables, grains, leaves and other agricultural products such as beans, corn hulls, wheat, rice and seeds of coffee. Some examples of the content in ferulic acid are 9.1-14.3 mg/0.1 kg in coffee, 9.2-9.9 mg/0.1 Kg in orange, 5.4 mg/0.1 Kg in banana, 1.2-2.8 mg/0.1 kg in carrots, 8.7 mg/0.1 kg in peanuts and 313 mg/0.1 Kg in popcorn (Zhao and Moghadasian, 2008; Kumar and Pruthi, 2014).

To release ferulic acid from the natural sources there are methods for chemical extraction, namely alkaline or acidic hydrolysis (Tilay *et al.*, 2008). Alkaline methods can break ester bonds formed by ferulic acid in plant cell walls. This type of hydrolysis leads to a better yield in releasing some phenolic acids, including ferulic acid, since the acidic hydrolysis can degrade hydroxycinnamic acids as well (Arranz and Saura Calixto, 2010; Pan *et al.*, 2003; Acosta-Estrada *et al.*, 2014; Kim *et al.*, 2006). However, there are other types of chemical linkages, like some types of ether linkages, that are not affected by alkaline treatment, but acidic hydrolysis can cleave them (Xu *et al.*, 2005).

Nowadays, there is a growing interest for healthy and natural products and those chemical methods referred above to extract ferulic acid cannot be considered as natural. So, the alternative can pass through the enzymatic release from natural sources. As ferulic acid is found linked with hemicelluloses,

namely arabinoxylans by ester bonds with arabinose residues, is necessary to break those polysaccharides. For this kind of extraction, a combination of enzymes produced by bacteria and fungi can be used. Such enzymes need to have ferulic acid esterase and carbohydrate hydrolase activities, which includes enzymes such as feruloyl esterase, acetyl xylan esterase, xylanase and arabinoxylanase (Uraji *et al.*, 2013; Dupoiron *et al.*, 2017). However, the results available on literature show that the yields achieved by the enzymatic release are still lower than those obtained by alkaline extraction and the drawback of the process is the difficulty of recovering the enzymes for reuse (Uraji *et al.*, 2013; Dupoiron *et al.*, 2017; He *et al.*, 2015).

Besides its use for vanillin production by biotechnological ways already mention above, ferulic acid and its derivatives have a wide range of functions that allow their application in pharmaceutical and cosmetic industry but also in food industry. It has been reported that, due to its antioxidant activity caused by the formation of a resonance phenoxy radical, ferulic acid shows an anti-inflammatory activity and can act as an anti-diabetic, anti-ageing and anti-cancer agent (Srinivasan *et al.*, 2007; Ou and Kwok, 2004; Acosta-Estrada *et al.*, 2014; Kumar and Pruthi, 2014). This property is also important in food industry, so ferulic acid can be used as additive to preserve food. Due to its capacity as cross-linking agent, ferulic acid can be used to increase the viscosity and form gels from polysaccharides as pectin (Kumar and Pruthi, 2014; Ou and Kwok, 2004).

### 1.3.2 Toxicity of vanillin

Vanillin is an aromatic aldehyde (its chemical structure can be seen in Figure 1.2), and aldehydes are very reactive forming, for example, schiff bases that can lead to the cell death by inhibition of enzymatic activity (Fleige *et al.*, 2013; Gallage and Møller, 2015; Sinha *et al.*, 2008). There are also studies showing that phenolic compounds, as vanillin, can affect or inhibit the DNA, RNA and proteins and its synthesis by formation of covalent bonds with them. It can also affect the glucose uptake, pH homeostasis and ion gradients since the aromatic ring gives hydrophobicity to the molecule, allowing interactions with the cytoplasmic membrane (Fleige and Meyer, 2016). These seems to be the reasons why vanillin cannot be accumulated in high concentrations by the producing microorganisms. Because of its toxicity, vanillin is degraded in some less harmful compounds such as vanillic acid, vanillyl alcohol and guaiacol instead of being accumulated, leading to low production yields, being the main problem with industrial application of the biotechnological process (Fleige *et al.*, 2013; Kaur and Chakraborty, 2013).

Some microorganisms, namely some soil actinomycetes, are being studied for vanillin production from ferulic acid. These microorganisms are *Amycolatopsis* sp. and and *Streptomyces* because of its tolerance to the substrate and the product, allowing a better vanillin accumulation, making these strains interesting to industrial applications (Brunati *et al.*, 2004; Muheim and Lerch, 1999).

In the literature, the most reported strains in production of vanillin are *Amycolatopsis* sp. ATCC 39116, *Amycolatopsis* sp. HR 167 and *Streptomyces* sp. V-1. Although there is a published patent for vanillin production with *Amycolatopsis* sp. HR 167 with a vanillin concentration of 11.5 $gL^{-1}$ and a molar yield of 77.8%, the studies with this bacteria are, in most cases, at molecular level in order to identify genes and

its modification for a better vanillin production (Graham S. Dr. Byng, Rudolf Dr. Hopp, 1996; Jörg Overhage, Alexander Steinbüchel, 2006; Steinbüchel, 2002; S. Achterholt, H. Priefert, 2000). The strain *Streptomyces* sp. V-1 shows vanillin productions of 5.24 $gL^{-1}$ with a molar yield of 74.6% (Hua *et al.* 2007) but better results are also found in literature, with a production of 9.09 $gL^{-1}$ of vanillin and a molar yield of 95.2 $gL^{-1}$ (Ma and Daugulis, 2014a). For *Amycolatopsis* sp. ATCC 39116 some results shown in literature for vanillin productions and molar yields are 9.4 $gL^{-1}$ and 89.2% (Ma and Daugulis, 2014b), 9.18 $gL^{-1}$ and 96.1 % (Ma and Daugulis, 2014a), 12.2 $gL^{-1}$ and 68.6% (Fleige *et al.*, 2016), respectively.

### 1.3.3 *Amycolatopsis* sp. ATCC 39116, an interesting vanillin producer

There are many studies in literature about the actinomycete *Amycolatopsis* sp ATCC 39116, including molecular studies to report the pathways for production and degradation of vanillin and studies related to the optimization and knowledge of the production process. In the most cases, the substrate used for production of vanillin is ferulic acid (Sutherland *et al.*, 1983; Fleige *et al.*, 2016; Fleige *et al.*, 2013; Ma and Daugulis, 2014b; Ma and Daugulis, 2014a; Muheim and Lerch, 1999; Pérez-Rodríguez *et al.*, 2016). There is also a published patent for the biotransformation of ferulic acid to vanillin using this microorganism (Muheim, Andreas; Müller, Bruno; Münch, Thomas; Wetli, 1998).

The pathway for ferulic acid degradation proposed for *Amycolatopsis* sp. ATCC 39116 is coenzyme A (CoA)-dependent as it is shown in Figure 1.4 (Fleige *et al.*, 2013). In the initial step of this pathway, the enzyme Feruloyl-CoA synthetase (FCS) is activated in the presence of the cofactors: adenosine triphosphate (ATP), Coenzime A (CoASH) and magnesium chloride ($MgCl_2$) and catalyze the formation of feruloyl-CoA from ferulic acid. Then, the enzyme Enoyl-CoA hydatase/aldolase (ECH) catalyze the reactions of hydration and removal of acetyl-CoA leading to the formation of vanillin (Gallage and Møller, 2015; Fleige *et al.*, 2013). The gene expression that encodes this enzyme is induced by ferulic acid (Fleige *et al.*, 2016).

As vanillin has a toxic effect to the cells, it is catabolized in vanillic acid by a Vanillin dehydrogenase (VDH). This enzyme as a maximum activity at pH 8.0 and temperature of 44˚C, but it is active at a wide range of temperatures and pHs (BRENDA; Fleige *et al.*, 2013). The discovery of this enzyme and the gene that encodes it, allowed a different approach in studies for improvement of vanillin production, preventing the catabolism of vanillin in vanillic acid. Fleige *et al.* (2013) showed that deletion of the gene which encodes the enzyme VDH do not affect ferulic acid catabolism since it is not part of the cluster with other genes responsible for degradation of that compound. This opened the possibility to study the production and the degradation of vanillin in a *vdh* delection mutant. These studies show that *vdh* mutant can reach a higher vanillin concentration and molar production yield of vanillin in ferulic acid (14.4 ± 1 g/L and 80.9% ± 4.6%) than the wild type (12.2 ± 2.3 g/L and 73.9% ± 9.5%). This happens without a previous production of vanillic acid like other authors described in the wild strain, since vanillin is not catabolized in vanillic acid at the same rate as it is in wild type (Muheim and Lerch, 1999; Fleige *et al.*, 2016; Ma and Daugulis, 2014a). The production, even that low, of vanillic acid may be due to other mechanisms of degradation presents in the cells or an unspecific aldehyde dehydrogenase (Fleige *et al.*, 2016; Fleige *et al.*, 2013). There are also studies for decrease the adaptation phase when ferulic

acid is added to the broth based in genetic modifications. Fleige *et al.* (2016) studied the addition of copies that were constitutively expressed of *fcs* and *ech* that encoded the genes for the two enzymes needed in the beginning of the metabolic pathway. The results showed an enhancement of ferulic acid transformation and no adaptation phase. In the other hand, there were a higher production of vanillic acid than in the *vdh* mutant firstly mentioned.

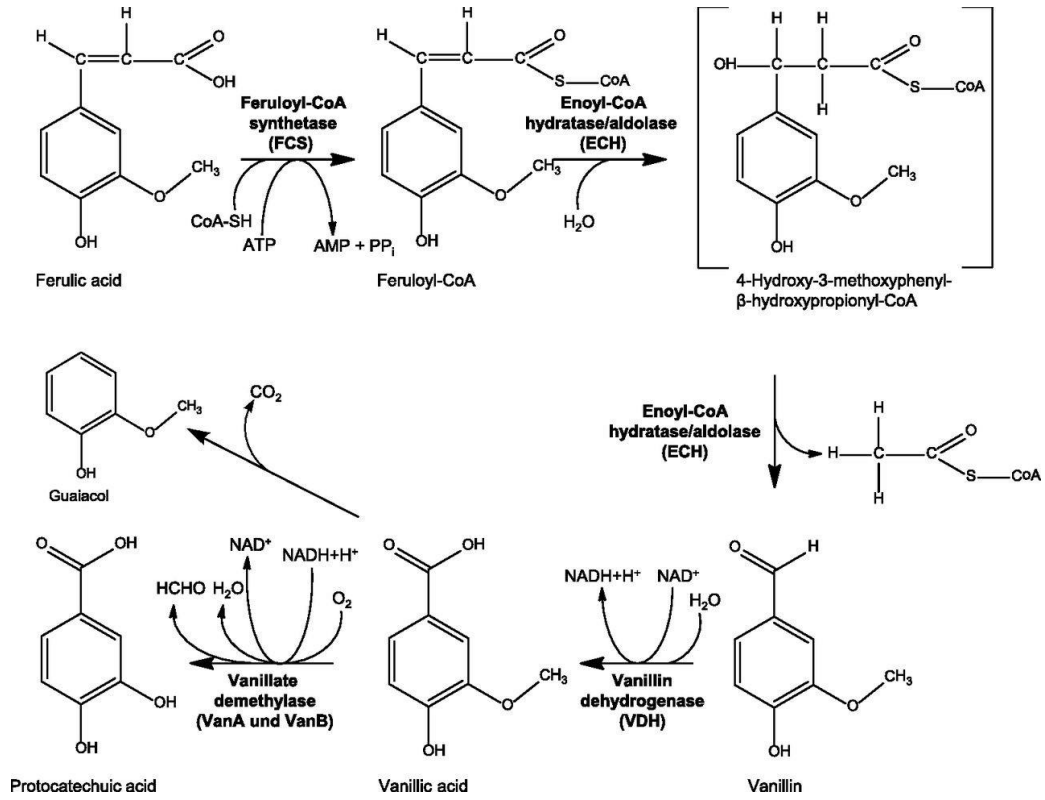



Figure 1.4 – The CoA-dependent catabolic pathway proposed for *Amycolatopsis* sp ATCC 39116 for degradation of ferulic acid via vanillin (Fleige *et al.* 2013).

In the end of the metabolic pathway, the vanillic acid formed during ferulic acid transformation is demethylated to protocatechuic acid by the enzyme Vanillate demethylase (VanA and VanB) or decarboxylated, forming guaiacol (Fleige *et al.*, 2013) (Figure 1.4).

For the great accumulation of vanillin that can be achieved using this strain, two possibilities are presented in literature. Muheim and Lerch (1999), observed that for higher concentrations of ferulic acid, *Amycolatopsis* sp. ATCC 39116 could accumulate vanillin instead of converting it into vanillic acid, suggesting a metabolic bottleneck in that step. These authors also reported an accumulation of vanillic acid until a certain level, after which vanillin starts to be accumulated. Fleige *et al.*(2013) also reached to that last observation and suggested the occurrence of a regulatory mechanism from vanillic acid which influences gene expression for VDH enzyme, or the enzymatic activity, as a possible explanation for the capability of that bacteria to accumulate vanillin instead of degrading it.

Although *Amycolatopsis* sp. ATCC 39116 is a strain with a notable tolerance to vanillin, if this compound is present in the lag or exponential growth phase, it has harmful effects to the cell, inhibiting growth in

lag phase or repressing it in exponential phase. However, the presence of vanillin in stationary phase has no damaging effects in the cell density. So, vanillin production must be performed in stationary phase in order to avoid the negative effect on cells (Ma and Daugulis, 2014b). Besides this tolerance in stationary growth phase, high concentrations of vanillin affect the metabolism of cells (Fleige *et al.*, 2016). Vanillin concentrations higher than 7 $g.L^{-1}$ seems to cause cell death after some hours and exposure to 10 $g.L^{-1}$ have a strong effect in a smaller period of time. In other hand, until 4 $g.L^{-1}$ of vanillin is reached, the microorganism is able to tolerate it, with an initial viable cell decrease. Nevertheless, vanillin concentrations above 10 $g.L^{-1}$ have already been reported in literature, but an adaptation to the presence of vanillin by the microorganisms seems not to happen. The explanation suggested by the authors that carried out these studies was that the induction of the detoxification mechanisms for vanillin in cells were not made by the toxic product but by other compound, for example ferulic acid. This explains the reason for, in the fermentation broth in presence of the substrate, cells can tolerate higher concentrations without such level of cell death even with the production rates strongly decreasing at that time (Fleige *et al.*, 2016).

### 1.3.4 Bioprocess conditions

Nowadays there is a demand for natural products, and biotechnological vanillin can be part of this category, but this product is still much more expensive than the product obtained chemically. In order to try to make vanillin more competitive it is necessary to understand the bioconversion process and try to optimize its yields and productivity. These goals can be reached by the optimization of culture medium, namely: carbon and nitrogen sources and trace elements and growth factors, as well as the operating conditions such as: temperature, pH, aeration and the feeding strategy.

For vanillin production from ferulic acid by *Amycolatopsis* sp. ATCC 39116, there is already a published patent, in which glucose is used as carbon source, yeast extract as nitrogen source, and the need of phosphates, growth factors and trace elements is also expressed (Muheim, Andreas; Müller, Bruno; Münch, Thomas; Wetli, 1998).

In terms of industrial application, besides the low yields and productivities, there are some drawbacks in the medium composition: glucose is a substrate with high purity which increases the production costs as well as yeast extract that is an expensive nitrogen source. But there are not available studies about the medium composition concerning the substitution of these two components or a part of them.

Studies about the optimization of some conditions for vanillin production have been made mainly in shake flasks, trying to understand the process and optimize pH, concentration of glucose and yeast extract in the medium, but also addition of vanillic acid in the beginning of the transformation phase and the feeding strategy (batch or fed-batch) (Pérez-Rodríguez *et al.*, 2016; Ma and Daugulis, 2014a; Muheim and Lerch, 1999). Better productions seem to be achieved neither with a low, nor with a high cell concentration, but in between cell densities (Ma and Daugulis, 2014a; Pérez-Rodríguez *et al.*, 2016) and glucose and yeast extract concentrations can do that control in cell mass (Ma and Daugulis, 2014a). Regarding the concentration of ferulic acid, and because the amount of vanillin produced depends on

the concentration of substrate added to the medium (Muheim and Lerch, 1999), results in literature show that a major concentration of substrate leads to better productions. However, ferulic acid has antimicrobial activity, so there has to be a balance between the maximization of feed and its toxicity, taking into account that with a higher cell density there are more resistance to toxic effects (Pérez-Rodríguez *et al.*, 2016; Ma and Daugulis, 2014a). This conclusion and the experiments made by some authors, lead to the fact that fed-batch has more advantages than batch processes (Pérez-Rodríguez *et al.*, 2016). However, the existing optimization studies, even though being in shake flasks with no control in pH, dissolved oxygen, or stirring, they are all made under different conditions (e.g. temperatures, pHs and medium composition), making it difficult to compare the results. When the process is carried out in bioreactors, a set of other conditions, besides the ones that are referred before, appear with the need for optimization, such as stirring speed and aeration conditions (Ma and Daugulis, 2014b).

## 1.4 Motivation

There is a growing interest by the consumers in natural products and consequently, production of vanillin by a biotechnological approach using microorganisms and natural raw products as substitute of the chemically synthetized one, might be an interesting alternative. Therefore, and having in mind the difficulties to obtain an interesting vanillin production yield and productivity that allow to apply the process at industrial scale (Kaur and Chakraborty, 2013; Priefert *et al.*, 2001), a biotechnological vanillin production process using the vanillin tolerant microorganism *Amycolatopsis* sp. ATCC 39116 and model solutions of ferulic acid as substrate was studied in this thesis.

The objective of this work was to optimize the process in the following parameters:

- From the standard operational conditions, study the influence of nitrogen, with yeast extract, and glucose concentrations in the biotransformation process, trying to reach the best conditions for vanillin production;
- Study the possibility of substituting a part of yeast extract with another nitrogen source, in order to make the process more attractive;
- Study different feeding strategies that allow a better vanillin production and accumulation.

# 2. Materials and methods

## 2.1 Vanillin production in bioreactor

### 2.1.1 Microorganism and growth medium composition

The strain used in all experiments was *Amycolatopsis* sp. ATCC 39116, it was maintained in cryovials containing 20% (v/v) glycerol at -80˚C.

The culture was grown in a growth medium containing: $Na_2HPO_4$, 4 $gL^{-1}$; $KH_2PO_4$, 1 $gL^{-1}$; NaCl, 0.2 $g.L^{-1}$, $MgSO_4.7H_2O$, 0.2 $g.L^{-1}$; $CaCl_2.H_2O$, 0.05 $g.L^{-1}$; glucose anhydrous, 20 $g.L^{-1}$ and yeast extract, 5 $g.L^{-1}$. The medium was sterilized in autoclave at 1 bar, 121˚C for 30 minutes.

### 2.1.2 Inocula preparation

Cells were transferred from the cryovial and grown in plates with the growth medium at 45˚C. For preparation of the pre-inoculum, a colony was transferred to a 500 mL shake flask with 200 mL of growth medium and incubated in an orbital shaker at 45˚C at 200 rpm for 36 h. After that, another 500 mL shake flask with growth medium was inoculated with 10% (v/v) of the pre-inoculum and incubated during 48 h under the same conditions. The manipulation of the microorganisms was performed in a laminar flow chamber to maintain the sterile conditions.

### 2.1.3 Fed-batch bioreactor operation

Experiments for production of vanillin were carried out in 2 L bioreactors (BioStat B-plus, Sartorius, Germany) with an initial volume of 1.8 L. The bioreactors containing the growth medium were inoculated with about 10% (v/v) of inoculum prepared as it was described above.

The temperature was automatically controlled at 45˚C and as the experiments were performed at a high temperature (45 ˚C), in order to prevent the evaporation losses, the bioreactor condenser was maintained at 3˚C. The aeration rate was kept constant during all experiments at 2 vvm and the dissolved oxygen concentration (or partial oxygen pressure, $pO_2$) was automatically controlled at 30% by the automatic variation of the stirrer rate, between 500 and 1000 rpm, provided by two six-blade impellers. To prevent the foam formation an antifoam Y-30 emulsion was automatically added to the broth.

The process is divided in two phases: the growth phase, when cells are cultivated in the growth medium in sterile conditions and the bioconversion phase, when ferulic acid is added and sterile conditions are not maintained. During the growth phase pH was controlled at 7.2±0.02, and in the bioconversion phase, pH was adjusted and controlled at 8.2±0.02, by the addition of 5 M NaOH and/or 5 M HCl.

After the end of the exponential growth phase the biotransformation phase starts by feeding the bioreactor with a single pulse (of about 220 mL) of a ferulic acid solution (90 $g.L^{-1}$) to give a concentration of 10 $g.L^{-1}$. The ferulic acid stock solution was prepared by dissolving the phenolic compound in 0.5 M NaOH.

Culture broth samples were periodically taken to monitor the cell growth by measuring the optical density at 600 nm ($DO_{600}$). The samples were then centrifuged, cell pellets were used for determination of cell dry weight (CDW) and supernatants were used for quantification of ferulic acid, vanillin, vanillic acid, glucose and total nitrogen concentrations.

## 2.2 Effect of nitrogen concentration

In order to study the effect of nitrogen concentration in the process, experiments were performed in bioreactors with different concentrations of this element.

The composition of the growth medium was the same as described in section 2.1.1 with the exception of nitrogen concentration. Different total nitrogen concentrations in study were achieved using yeast extract and a combination of yeast extract and $(NH_4)_2SO_4$ as represented in Table 2.1.

Table 2.1 – Total nitrogen concentration ($N_{total}$) studied in the experiments performed and the corresponding concentrations of yeast extract and ammonium sulphate ($(NH_4)_2SO_4$) used to achieve the desired $N_{total}$.

| Run | $N_{Total}$ ($g.L^{-1}$) | Yeast extract ($g.L^{-1}$) | $(NH_4)_2SO_4$ ($g.L^{-1}$) |
|---|---|---|---|
| **1** | 2 | 10 | 4.7 |
| **2** | 1 | 10 | - |
| **3** | 0.75 | 7.5 | - |
| **4** | 0.25 | 2.5 | - |

The pre-inoculum and the inoculum of *Amycolatopsis* sp. ATCC 39116 for this set of experiments were performed as described in section 2.1.2.

The bioreactors operation was carried out in the same conditions of the standard bioreactor, described in section 2.1.3 as well as the feeding mode.

## 2.3 Influence of nitrogen source

### 2.3.1 Shake flask experiments

#### 2.3.1.1 Growth medium

Shake flasks experiments were performed in order to study the substitution of a part of yeast extract with another nitrogen source (ammonium salts). The nitrogen sources used in these experiments, besides yeast extract, were $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $NH_4Cl$. The compounds were added in appropriate concentrations to have a total nitrogen content of 0.5 $gL^{-1}$ with 0.25 $gL^{-1}$ coming from yeast extract and 0.25 $gL^{-1}$ coming from the others nitrogen sources, as presented in Table 2.2. The other medium components were used in the same concentrations as described in section 2.1.1. The medium was previously sterilized in autoclave at 1 bar, 121˚C for 20 minutes.

Table 2.2 – Concentration of ammonium salts and yeast extract used in shake flasks experiments.

| Experiment | Ammonium salts | Yeast extract ($gL^{-1}$) | Ammonium salts ($gL^{-1}$) |
|---|---|---|---|
| **1** | - | 5 | - |
| **2** | $(NH_4)_2SO_4$ | 2.5 | 1.20 |
| **3** | $(NH_4)_2HPO_4$ | 2.5 | 1.18 |
| **4** | $NH_4Cl$ | 2.5 | 0.95 |

#### 2.3.1.2 Inocula preparation

For preparation of pre-inoculum for the shake flasks experiments, the plates were prepared in the same way described in section 2.1.2. Then, a colony was transferred to a 500 mL baffled shake flask with 200 mL of growth medium described in section 2.1.1 and incubated in an orbital shaker at 45˚C at 200 rpm for 24 h. The inoculum was prepared by transferring 20 mL of the pre-inoculum to another 500 mL baffled shake flask with 200 mL of the growth medium and incubated as pre-inoculum. After this, the mediums containing the different ammonium salts, with pH around 7.2, were inoculated with 20 mL of the inoculum. The sterile conditions were maintained during this process.

#### 2.3.1.3 Shake flasks assays

The cells were grown at 45˚C in an orbital shaker during approximately 24 h and, afterwards they were fed with 22 mL of a 90 $gL^{-1}$ ferulic acid stock solution, prepared as described in section 2.1.3. After the feed with ferulic acid, pH was adjusted to 8.2 and whenever the samples were taken, pH was readjusted to 8.2 with 5 M NaOH or 5 M HCl. Samples of 2 or 3 mL were periodically taken during the whole process and the pH measure, the cell growth was accompanied by measurement of optical density at 600 nm ($DO_{600}$). The samples were centrifuged, supernatant was recovered for quantification of ferulic acid, vanillin, vanillic acid, glucose and total nitrogen and pellet for biomass quantification.

### 2.3.2 Bioreactor experiment

To complete the studies for the substitution of a part of the yeast extract with other nitrogen source, a bioreactor run was performed. In this experiment, the nitrogen source used was the ammonium salt that achieved better results in shake flask experiments, giving a nitrogen concentration of 0.25 $gL^{-1}$ and the yeast extract giving the same concentration as the ammonium salt, to have a total nitrogen content of 0.5 $g.L^{-1}$.

The inocula preparation and the bioreactor operation were carried out as explained in section 2.1.2 and 2.1.3, respectively.

## 2.4 Impact of glucose concentration

In order to evaluate the influence of glucose concentration in the process of vanillin production two different glucose concentrations (10 and 15 $g.L^{-1}$) were tested. The growth medium used for these experiments was the same described in section 2.1.1, apart from the glucose concentration.

The pre-inoculum and inoculum were performed as described in section 2.1.2, as well as the operation of the bioreactor, in section 2.1.3.

## 2.5 Pulse feeding strategy

To improve vanillin production a pulse feeding strategy was used. The conditions for the bioreactor cultivation run are described in section 2.1. Ferulic acid pulses were fed to the bioreactor taking into consideration the $pO_2$ rise or the acid addition to the broth, which indicate the end of growth phase or ferulic acid biotransformation. In these studies, the pulse feeding was made to give a concentration around 10 $gL^{-1}$ of ferulic acid in the bioreactor.

### 2.5.1 Fed-batch with multiple pulse feedings

After growth phase the first ferulic acid pulse was fed, as described above (section 2.1.3). Then, when the stirring started to decrease and/or $pO_2$ increasing, another pulse was given. This process was repeated until the cells were unable to perform the biotransformation, seen by the increase in $pO_2$ values (rising above 30 %, until 100 %)

### 2.5.2 Fed-batch with multiple pulse feedings and biomass recovery

Other approach tested was the feeding with two pulses of ferulic acid as already explained in section 2.5.1, but before the third pulse, the broth was centrifuged during 20 minutes at 4 424 x g to separate the cells from the medium. Then, the biomass was resuspended in a solution of 8 $gL^{-1}$ of glucose and immediately reintroduced in the bioreactor. Right after, cells were fed with an appropriate volume of ferulic acid stock solution to give the desired concentration. This process of biomass centrifugation and reintroduction in the bioreactor was repeated until the cells were unable to continue the vanillin production.

### 2.5.3 Multiple pulse feedings with biomass recovery

This feeding strategy consisted in performing a centrifugation step before every given pulse, including the first one, and resuspending cells in a new medium containing only glucose. The centrifugation conditions were the same described in section 2.5.2, as well as the medium used to resuspend the biomass. As in the previous sub-section this process was executed until the biomass was unable to continue the biotransformation.

## 2.6 Analytical techniques

### 2.6.1 Cell growth

During the experiments, the cell growth was monitored by measurement of optical density at 600 nm in a UV-Visible spectrophotometer (VWR V-1200 spectrophotometer) and the samples were diluted in deionized water whenever it was necessary. The measurements were done in duplicate.

### 2.6.2 Biomass quantification

For biomass quantification, the resulting cell pellets from each sample taken, were washed twice with deionized water (two cycles of resuspension with deionized water and centrifugation in a VWR Mini Star Silverline centrifuge), frozen in liquid nitrogen and lyophilized (ScanVac CoolSafe™, LaboGene) at -110˚C during 48 h. The lyophilized cell pellets were weighted to obtain the cell dry weight (CDW). This analysis was done in duplicate.

### 2.6.3 Phenolic compounds quantification

To determine the concentration of ferulic acid, vanillin and vanillic acid, the supernatant of each sample taken during the bioconversion phase of the bioprocess were analyzed by high performance liquid chromatography (HPLC; Alliance) equipped with a Nova-Pak® C18 3.9 mm×150 mm column (particle diameter of 4 mm), the UV detector was set at 280 nm. The injection volume of each sample was 20 µL. The compounds were separated at 30˚C with a gradient elution program at the flow rate of 0.5 $mL.min^{-1}$. The mobile phase was a mixture of two eluents, one of them (A) constituted by 10 % (v/v) methanol, 2%(v/v) acetic acid in Milli-Q water and the other (B) constituted by 90 % methanol, 2% acetic acid in Milli-Q water. The gradient elution program was 0% B (0–10 min), from 0 to 15 % B (10–25min), from 15 to 50 % B (25–35 min) and 50 to 0% B (35-38 min). The samples were diluted in a matrix solution constituted by 3 $g.L^{-1}$ tartaric acid and 0.075 $mg.L^{-1}$ catechin. The standards used to make the calibration curves (that are represented in appendix, as example in A.1, A.2 and A.3) were prepared in matrix solution with ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid, Sigma-Aldrich, ≥99%), vanillin (4-hydroxy-3-methoxybenzaldehyde, Sigma Aldrich, 99%) and vanillic acid (4-hydroxy-3-methoxybenzoic acid, Sigma-Aldrich, ≥97%), within concentrations of 0.006 and 0.2 $g.L^{-1}$.

### 2.6.4 Glucose quantification

For glucose quantification, the supernatant of the samples taken during the bioprocess were filtered in centrifuge filters (with a pore size of 0.2 µm) and analyzed by HPLC (VWR Hitachi Organizer (Pump L-2130, Auto sampler L-2200, Column Oven L-2350)) with a detector Merck Differential Refractometer RI-71. The pre-column and the column used was VARIAN Metacarb 87H and Biorad aminex HPX -42A 125-0129, respectively. The injection volume of each sample was 99 µL. The samples were separated during 15 min at 30 ˚C with 0.01 N $H_2SO_4$ as eluent and a gradient elution program at the flow rate of 0.5 $mL.min^{-1}$. The standard solution with glucose (D(+)-glucose anhydrous, Scharlau) was prepared in eluent, and the standards prepared to make a calibration curve (that are represented, as example in in appendix A.4) with concentrations range between 1 and 0.06 $gL^{-1}$.The samples were diluted in eluent.

### 2.6.5 Nitrogen quantification

Total nitrogen was quantified using the Hach Lange Laton® total nitrogen ($TN_b$) kit protocol. The samples were diluted in deionized water to have a nitrogen concentration within the range measured by the kit (between 20 and 100 $mgL^{-1}$). After the procedure indicated in the protocol, the total nitrogen was measured in the spectrophotometer DR 2800™ by recognition of the bar code present in the sample cell.

### 2.6.6 Calculation method

The specific growth rate (μ, $h^{-1}$) was calculated using the following equation:

$$ln\left(\frac{x}{x_0}\right) = \mu t \qquad \text{Equation 2.1}$$

where $x$ and $x_0$ ($g.L^{-1}$) represent the cell concentration in the moment $t$ and in the beginning of the assay, respectively.

The yield of biomass on substrate ($Y_{X/S}$, $g.g^{-1}$) was calculated using the equation presented below:

$$Y_{X/S} = \frac{\Delta X}{\Delta S_1} \qquad \text{Equation 2.2}$$

where $\Delta X$ ($g.L^{-1}$) represents the biomass produced during the process and $\Delta S_1$($g.L^{-1}$) represents the substrate consumed during the cell growth, in this case the substrate is glucose.

The yield of product on substrate ($Y_{P/S}$, $g.g^{-1}$) was determined by the following equation:

$$Y_{P/S} = \frac{\Delta P}{\Delta S_2} \qquad \text{Equation 2.3}$$

where $\Delta P$ ($gL^{-1}$) is the maximum concentration of the product formed during the bioprocess, in this case the product is vanillin, and $\Delta S_2$ ($g.L^{-1}$) is the concentration of substrate consumed to give rise to that product concentration, in this case the substrate is ferulic acid.

The volumetric productivity ($r_p$, $g.L^{-1}.h^{-1}$) of vanillin was determined as following:

$$r_p = \frac{\Delta P}{\Delta t} \qquad \text{Equation 2.4}$$

where $\Delta P$ ($g.L^{-1}$) corresponds to the variation of product concentration, vanillin, in an interval of time $\Delta t$ (h), since the beginning of the run and the maximum production of vanillin.

For calculation of specific productivity ($g.g_X^{-1}.h^{-1}$), the equation used was the following:

$$q_p = \frac{r_p}{X} \qquad \text{Equation 2.5}$$

where $X$ ($g.L^{-1}$) is the cell concentration, in dry weight.

The rate of production of vanillin ($r_v$, $g.L^{-1}.h^{-1}$) was calculated as by the following equation:

$$r_v = \frac{\Delta P}{\Delta t}$$ Equation 2.6

where $\Delta P$ (g.L$^{-1}$) is the variation of the product vanillin concentration, in an interval of time $\Delta t$ (h), that begin by the time that ferulic acid is fed.

The consumption rate of vanillin or ferulic acid ($r_c$, g.L$^{-1}$.h$^{-1}$), was determined by the equation below:

$$r_c = \frac{\Delta C}{\Delta t}$$ Equation 2.7

where $\Delta C$ (g.L$^{-1}$) represents the variation of the desired compound concentration (ferulic acid or vanillin), in an interval of time $\Delta t$ (h). This interval of time for ferulic acid begins by the time that is added to the broth and for vanillin begins when the product reaches its maximum concentration.

The specific rate of vanillin production ($q_v$, g.g$_X$$^{-1}$.h$^{-1}$) was calculated with the following equation:

$$q_v = \frac{r_v}{X}$$ Equation 2.8

where $X$ (g.L$^{-1}$) represents the cell concentration on dry weight.

# 3. Results and discussion

## 3.1 Vanillin production in bioreactor

In order to study the vanillin production from ferulic acid by *Amycolatopsis* sp. ATCC 39116 a bioreactor run was performed with 5 $g.L^{-1}$ of yeast extract as nitrogen source and 20 $g.L^{-1}$ of glucose as carbon source, as it is described in section 2.1.1. At the end of exponential growth phase, around 10 $g.L^{-1}$ of ferulic acid was added to the bioreactor in a single pulse. The conditions used in this run were those used in previous work (data not shown), and from now on, they will be denominated as standard conditions. Figure 3.1 depicts the standard run for vanillin production, where the profiles of cell growth with the consumption of glucose and nitrogen and the production of vanillin and vanillic acid from ferulic acid are represented over time, as well as the partial oxygen pressure ($pO_2$).

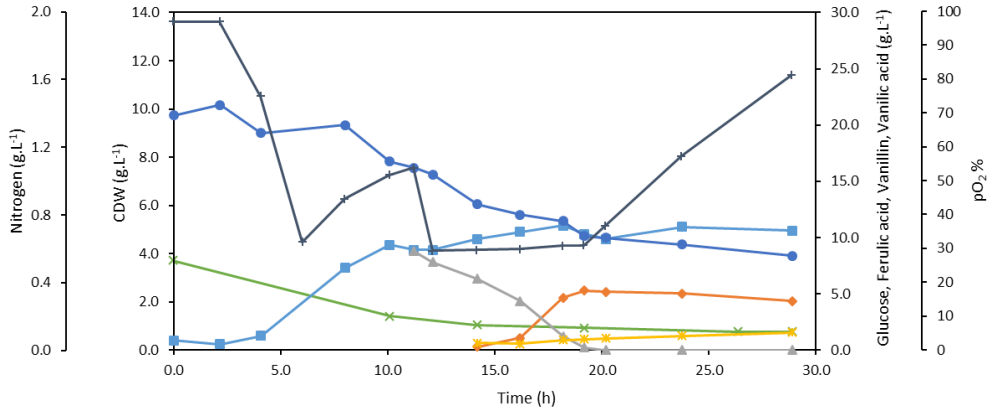



Figure 3.1 – Cultivation profile of *Amycolatopsis* sp. ATCC 39116 in standard conditions, showing the concentrations of CDW (■), glucose (●), nitrogen (×), ferulic acid (▲), vanillin (♦), vanillic acid (✳) and $pO_2$ (+) during the run time.

*Amycolatopsis* sp. ATCC 39116 started to grow exponentially after 4 h of lag phase, reaching a maximum cell mass of 4.36 $g.L^{-1}$ in 10 hours. At that time, $pO_2$ started to increase, indicating the beginning of the stationary growth phase. At this moment, according to Ma and Daugulis (2014b), ferulic acid should be added to the broth.

During the exponential growth phase 4.11 $g.L^{-1}$ of glucose and 0.33 $g.L^{-1}$ of nitrogen were consumed. The yield of biomass in substrate, glucose, ($Y_{X/S}$) obtained was 0.96 $g.g^{-1}$, as presented in Table 3.1.

At the time that ferulic acid was added, in a concentration of 8.79 $g.L^{-1}$, $pO_2$ decreased and returned to the minimum controlled, 30%, showing that the cells turned more active again. The production of vanillin started slowly after an adaptation phase (around 3 h), in which the cells are probably activating the mechanisms required to catabolize ferulic acid. A maximum vanillin concentration of 5.28 $g.L^{-1}$, was reached after 19 h since the beginning of the cultivation run and 8 h since the feeding. The yield of vanillin in ferulic acid ($Y_{P/S}$) for the maximum vanillin concentration was 0.62 $g.g^{-1}$ (that corresponds to a molar yield of 78.8%). This molar yield is within the values reported by Ma and Daugulis (2014b) and

Fleige and Meyer (2016) (89.2% and 73.9%, respectively) for vanillin production in bioreactors. However, the production conditions and the feeding strategy were different in all of these processes, the medium composition used by Ma and Daugulis (2014b) was similar to the used in this work, but the biotransformation of ferulic acid into vanillin was performed at 28˚C and with a stirring speed of 280 and 370 rpm. The medium used by Fleige and Meyer (2016) was constituted by 5 $g.L^{-1}$ of glucose and the same concentration of yeast extract, with a continuous feeding of ferulic acid. In the standard run, considering the time frame since the beginning of cultivation until maximum production of vanillin (19.2 h) the vanillin volumetric productivity ($r_p$) obtained was 0.28 $g.L^{-1}.h^{-1}$, similar to the obtained by Ma and Daugulis (2014b) (0.27 $g.L^{-1}.h^{-1}$). The vanillin specific productivity ($q_p$) obtained for the standard conditions was 0.06 $g_{vanillin}.g_x^{-1}.h^{-1}$ and after the feeding (11.2 h after the beginning of the run, and in the next 8 h of transformation), vanillin was produced at a rate ($r_v$) of 0.66 $g.L^{-1}h^{-1}$ until its maximum concentration (Table 3.1).

The assay was ended when $pO_2$ started increasing, reaching high values (around 80%), indicating a decrease in cellular activity. This increase in $pO_2$ occurred after the maximum production of vanillin. Right after the total consumption of ferulic acid, vanillin started to decrease slowly which was due to its conversion into vanillic acid, as can be seen in Figure 3.1.

Furthermore, when vanillin started to be produced, vanillic acid was already present in a concentration of 0.63 $g.L^{-1}$, and was rising until the end of the process, reaching a concentration of 1.57 $g.L^{-1}$. The vanillic acid production before vanillin was also previously reported by Muheim and Lerch (1999) and Fleige *et al.* (2013) and it occurs probably for detoxification from the aldehyde formed, since it is very reactive. Based on the presence of vanillic acid before vanillin, some authors proposed that the vanillin accumulation is possible because of a bottleneck in the oxidation step to form vanillic acid (Muheim and Lerch, 1999) and others suggested that an accumulation of the vanillic acid until a certain value activates a regulatory mechanism interfering with the gene that encodes VDH enzyme or with the activity of the enzyme, avoiding the degradation of vanillin (Fleige *et al.*, 2013).

In order to avoid the transformation of vanillin into vanillic acid, the process should be stopped before the depletion in vanillin concentration, making the process more attractive in terms of yield and productivity. This can be done by online monitoring the process. In fact, during the production phase it was noticed an addition of acid to the broth (pH was automatically controlled to 8.2 at this phase), coincident with the maximum concentration of vanillin. This alkalization was also reported by Ma and Daugulis (2014a) in the end of the biotransformation phase and by Pérez-Rodríguez *et al.* (2016) during the same phase and can be caused by the total consumption of ferulic acid from the medium. Therefore, pH seems to be a good indication to stop the process.

As it can be seen in Figure 3.1, glucose was not entirely consumed in the growth phase and a substantial concentration remained for the transformation phase (16.75 $g.L^{-1}$). During this phase, an increase in cell mass (from 4.16 to around 5 $g.L^{-1}$) was noticed, due to the glucose consumption (7.82 $gL^{-1}$) and the slightly consumption of nitrogen (0.09 $gL^{-1}$). This nutrient remained at a residual level in the last hours

of the run (0.11 $g.L^{-1}$), in opposition of glucose concentration that remained high (8.38 $g.L^{-1}$). Even with the consumption of glucose, there is a consumption of ferulic acid, indicating that this substrate is degraded by the secondary metabolism since it is not used, in this case, as source of carbon and energy for growth. However, ferulic acid and vanillin can be used by the *Amycolatopsis* sp. ATCC 39116 for growth. Fleige *et al.* (2013) used ferulic acid and vanillin as sole source of carbon and energy for growth in both substrates, but growth rate was very low with a long lag phase (30 to 40 h).

Giving the excess of glucose in the end of the process, it can be seen that the concentration of nitrogen was the limiting factor for growth. Therefore, in order to avoid this limitation and to evaluate its impact in biomass concentration and yield in vanillin production, different nitrogen concentrations were tested.

## 3.2 Effect of nitrogen concentration

The cellular growth is supported by a source of carbon, nitrogen and oxygen. In the standard growth conditions discussed above (section 3.1), nitrogen seems to limit the growth since it was consumed reaching a residual level while a significant concentration of glucose remained in the broth. So, in order to maximize vanillin production, different initial nitrogen concentrations (2, 1, 0.75 and 0.25 $g.L^{-1}$) were tested in bioreactors, while all the other conditions were maintained similar to the standard. Figure 3.2 shows the cultivation profile of *Amycolatopsis* sp. ATCC 39116 with different nitrogen concentrations, from run 1 to 4. The desired concentrations of nitrogen were achieved by adding different concentrations of yeast extract, knowing that 10% of the extract is nitrogen. However, to obtain 2 $g.L^{-1}$ of nitrogen, the concentration of yeast extract needed was 20 $g.L^{-1}$ and this concentration had a negative impact on the cell growth (data not shown). Therefore, that concentration was obtained with half of the nitrogen coming from the yeast extract and the other half from ammonium sulphate, this ammonium salt was used in literature in the cultivation medium of the *Amycolatopsis* sp. ATCC 39116 (Sutherland, 1986).

The run 1 (Figure 3.2 a.) was performed with the highest initial nitrogen concentration of all the experiments, 2 $g.L^{-1}$, achieved with 10 $g.L^{-1}$ of yeast extract and 1 $g.L^{-1}$ of nitrogen (from ammonium sulphate). In these conditions, cells grew during 11.9 h, after a lag phase of around 2 h, reaching 13.39 $g.L^{-1}$ of cell mass, higher than in the standard, which confirms that previous conditions were nitrogen growth limiting. During growth exponential phase 1.02 $g.L^{-1}$ of nitrogen and 17.49 $g.L^{-1}$ of glucose (the totality of glucose present in the medium) were consumed with a yield of biomass in glucose of 0.62 $g.g^{-1}$ (Table 3.1). When biomass reached the stationary phase, the $pO_2$ started to increase, indicating the moment for the addition of ferulic acid. The higher cell concentration obtained in this assay was clearly due to the higher nitrogen present which allowed that glucose was completely consumed. A clear indication of the glucose exhaustion was revealed by the rise in $pO_2$ values.

After the ferulic acid feeding (that was present in a concentration of 8.97 $g.L^{-1}$ in the broth), the biomass concentration decreased, due to the dilution effect, and kept constant in around 10 $g.L^{-1}$, until the maximum vanillin concentration was achieved. Then biomass concentration started to decrease, indicating cell death, accompanied by an increase in total nitrogen. Regarding the maximum production of vanillin, 2.90 $g.L^{-1}$ (Table 3.1) was reached 5.2 h after the ferulic acid feeding and 21.6 h after the

beginning of the run. After this, vanillin concentration decreased rapidly, until all the vanillin has been consumed with the production of vanillic acid, that achieved a concentration of 2.60 $g.L^{-1}$ in the end of the run (Figure 3.2 a.). The yield of vanillin in ferulic acid obtained was 0.38 $g.g^{-1}$ and the vanillin volumetric productivity was 0.13 $g.L^{-1}.h^{-1}$, these values were calculated considering the time until maximum production of vanillin (Table 3.1). The nitrogen was not totally consumed and in the end of the run, 1.04 $g.L^{-1}$ still remained in the broth. Though nitrogen had been consumed during the growth phase and during the production phase, is was then released in the end of the run. This release was likely caused by cellular lysis.

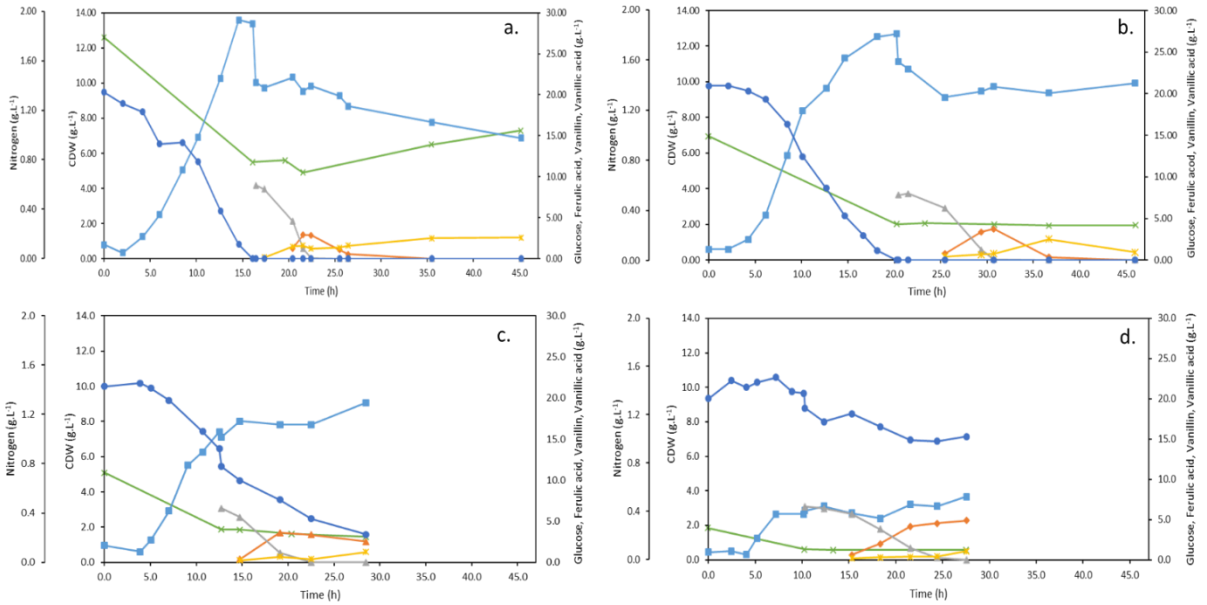



Figure 3.2 – Cultivation profiles of *Amycolatopsis* sp. ATCC 39116 at different initial nitrogen concentrations: a) Run 1: 2 $g.L^{-1}$, b) Run 2: 1 $g.L^{-1}$, c) Run 3: 0.75 $g.L^{-1}$ and d) Run 4: 0.25 $g.L^{-1}$. The concentrations of CDW (■), glucose (●), nitrogen (×), ferulic acid (▲), vanillin (♦) and vanillic acid (✳) are represented throughout the run time.

In run 2 (Figure 3.2 b.), the total nitrogen concentration was 1 $g.L^{-1}$, achieved with 10 $g.L^{-1}$ of yeast extract, in these conditions cells grew, after a lag phase of around 4 h, during 16.2 h with 12.68 $g.L^{-1}$ of cell mass being achieved, similar to the run 1, with a yield of vanillin in ferulic acid of 0.58 $g.g^{-1}$ as can be seen in Table 3.1. Glucose (20.97 $g.L^{-1}$) and nitrogen (0.99 $g.L^{-1}$) present in the medium were consumed for growth. In this run, similar to the first one, the rising in $pO_2$ values coincided with the ending of the substrate.

When ferulic acid was added to the broth, in the beginning of the stationary growth phase (reaching a concentration of 7.81 $g.L^{-1}$), the biomass concentration decreased and kept constant at around 9 $g.L^{-1}$. Maximum vanillin production was 3.74 $g.L^{-1}$ (Table 3.1) and occurred after 30.7 h from the beginning of the experiment and 10.3 h after the feeding. The yield of vanillin in ferulic acid obtained was 0.48 $g.g^{-1}$ and a vanillin volumetric productivity was 0.12 $g.L^{-1}.h^{-1}$ (these parameters were calculated considering the time frame since the beginning of the run until the maximum vanillin concentration has been achieved). Then, as in previous run, vanillin was transformed in vanillic acid, reaching a maximum

concentration of 2.51 $g.L^{-1}$. During the production phase, nitrogen was not consumed, since glucose was not present in the broth, remained 0.28 $g.L^{-1}$ of nitrogen in the end of the run.

Table 3.1 – Parameters of growth and production and maximum concentration of vanillin obtained in standard conditions and in the cultivation runs for study the effect of the nitrogen concentration in vanillin production.

| Run | $N_{total}$ ($g.L^{-1}$) | $Y_{(X/S)}$ ($g.g^{-1}$) | $\mu$ ($h^{-1}$) | $Y_{(P/S)}$ ($g.g^{-1}$) | $r_v$ ($g.L^{-1}.h^{-1}$) | $r_P$ ($g.L^{-1}.h^{-1}$) | $q_P$ ($g.g_X^{-1}.h^{-1}$) | $V_{max}$ ($g.L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| **Standard** | 0.5 | 0.96 | 0.31 | 0.62 | 0.66 | 0.28 | 0.057 | 5.28 |
| **(1) 10 $g.L^{-1}$ YE + 1 $g.L^{-1}$ N** | 2 | 0.62 | 0.32 | 0.38 | 0.57 | 0.13 | 0.014 | 2.90 |
| **(2) 10 $g.L^{-1}$ YE** | 1 | 0.58 | 0.34 | 0.48 | 0.36 | 0.12 | 0.013 | 3.74 |
| **(3) 7.5 $g.L^{-1}$ YE** | 0.75 | 0.85 | 0.37 | 0.66 | 0.56 | 0.19 | 0.024 | 3.59 |
| **(4) 2.5 $g.L^{-1}$ YE** | 0.25 | - | 0.37 | 0.74 | 0.28 | 0.18 | 0.048 | 4.87 |

In these two runs, a higher biomass concentration was achieved, as anticipated, since there was a higher nitrogen concentration that allowed a higher consumption of glucose. However, the yields of vanillin in ferulic acid (run 1: 0.38 $g.g^{-1}$, run 2: 0.48 $g.g^{-1}$) and the vanillin volumetric productivities (run 1: 0.13 $g.L^{-1}.h^{-1}$, run 2: 0.12 $g.L^{-1}.h^{-1}$) were considerably lower than those achieved in the standard conditions (0.62 $g.g^{-1}$ and 0.28 $g.L^{-1}.h^{-1}$, respectively), as it can be seen in Table 3.1.This results were obtained with almost the double of the biomass concentration comparing with the standard conditions, opening the possibility that such higher biomass concentrations are unfavorable to the process. This behavior had already been reported by Ma and Daugulis (2014a) and Pérez-Rodríguez *et al.* (2016). Pérez-Rodríguez *et al.* (2016) suggested that high cell densities, and the inherent enzymatic machinery, support a quicker ferulic acid conversion, but also a vanillin degradation with higher rate, not allowing the accumulation of this product and the achievement of high concentrations. However, the results obtained in the present work, in terms of ferulic acid consumption, are not in accordance with the reported by Pérez-Rodríguez *et al.* (2016). These two runs presented here, performed with different concentrations of nitrogen, even with similar values of biomass concentration and a similar profile of glucose consumption, showed different rates of ferulic acid consumption ($r_c$), 1.49 $g.L^{-1}.h^{-1}$ for run 1 and 0.76 $g.L^{-1}.h^{-1}$ for run 2. For the standard conditions, the rate obtained was between the values for the previous runs (0.98 $g.L^{-1}.h^{-1}$) indicating that a higher value of biomass does not necessary lead to a higher rate of substrate consumption, but suggesting that is related to the capability of the cells to activate the machinery necessary for the transformation. Regarding the vanillin consumption, in the runs 1 and 2, the rate was significantly higher (0.36 and 0.57 $g.L^{-1}.h^{-1}$, respectively) than in the standard conditions (0.097 $g.L^{-1}.h^{-1}$). In these three runs, the difference, besides the biomass concentration, was the presence of glucose in the transformation phase in the standard run, so it is possible that not only the biomass concentration, but also the presence of glucose can influence the consumption of vanillin.

In fact, vanillin and ferulic acid can be used as carbon and energy sources by this microorganism (Fleige *et al.*, 2013), and without glucose, it is possible that vanillin is being consumed at the same time that is produced, resulting in no net vanillin accumulation. Further studies in this work evaluated the influence of the glucose concentration in the process (section 3.4).

Taking into account the possibility that such high biomass concentration had a negative impact in the production, two other concentrations of yeast extract, a little above and below from the standard conditions, were tested, 7.5 $g.L^{-1}$ and 2.5 $g.L^{-1}$.

In run 3 (Figure 3.2 c.), the total nitrogen concentration of 0.75 $g.L^{-1}$ was achieved with 7.5 $g.L^{-1}$ of yeast extract. After a lag phase of around 4 h, the biomass concentration started to increase, reaching 7.42 $g.L^{-1}$ in 12.5 h since the beginning of the run, with a yield of biomass in glucose of 0.85 $g.g^{-1}$ (Table 3.1). In the exponential growth phase 7.59 $g.L^{-1}$ of glucose and 0.46 $g.L^{-1}$ of nitrogen were consumed, reaching a higher biomass concentration than in standard conditions but lower than in the experiments presented above, as expected in accordance with the yeast extract added.

Ferulic acid was fed in the beginning of the stationary growth phase, achieving a concentration of 6.55 $g.L^{-1}$. At that time, glucose had not been totally consumed, being present in the broth in a significant quantity (11.65 $g.L^{-1}$). The presence of the carbon source in the medium allowed, along with the nitrogen, a slight increase in biomass concentration that reach 8 $g.L^{-1}$ and then kept constant. The glucose continued to be consumed (with a total consumption of 8.26 $g.L^{-1}$ in the vanillin production phase), probably for maintenance similar to what happened in the standard run. In the end of the run 3.39 $g.L^{-1}$ of glucose and 0.21 $g.L^{-1}$ of nitrogen remained in the broth. The maximum vanillin concentration achieved was 3.59 $g.L^{-1}$, 6.3 h after the feeding with ferulic acid, and 19.1 h after the beginning of the run. Comparing with the standard run (Table 3.1), the present assay had a similar yield of vanillin in ferulic acid (0.66 $g.g^{-1}$) but a lower rate of vanillin production (0.56 $g.L.^{-1}.h^{-1}$) and vanillin productivity (0.19 $g.L.^{-1}.h^{-1}$), allowing to conclude that the biomass concentration affects the production (these parameters were calculated considering the time frame since the beginning of the run until the maximum vanillin concentration has been achieved). Still, the conditions performed in this run had less deleterious effect than the previous ones. After the maximum production of vanillin had been reached, its transformation into vanillic acid was lower than in the run 1 and 2, and had a more similar behavior with the run in standard conditions. The difference between those runs, besides the cellular density, was the presence of glucose in the standard and in the run 3, so it is possible that the presence or absence of glucose influence the consumption of vanillin, as referred above.

The run 4 (Figure 3.2 d.) was performed with the lowest nitrogen concentration tested, 0.25 $g.L^{-1}$, obtained with 2.5 $g.L^{-1}$ of yeast extract. After a lag phase of about 4 h, the cells grew exponentially reaching 2.85 $g.L^{-1}$, 10.3 h after the beginning of the run, obtaining the lowest value of biomass, as anticipated, accordingly with the amount of nitrogen added to the medium. In the end of the exponential growth phase, 0.08 $g.L^{-1}$ of nitrogen were present in the broth, a residual level that prevented further growth.

When ferulic acid was added to the bioreactor (6.62 $g.L^{-1}$), in the beginning of the stationary growth phase, a significant quantity of glucose still remained in the broth. During the production phase there was no consumption of nitrogen, but there was a consumption of glucose for maintenance. In the end of the process, when the $pO_2$ started to increase (reaching values of 69%) the concentration of vanillin was 4.87 $g.L^{-1}$. At that time, the ferulic acid had just been consumed in its totality so, in line with the other experiments, this would be the maximum vanillin concentration and after that time it would started to be consumed. The yield of vanillin in ferulic acid and vanillin volumetric productivity obtained for this run was 0.74 $g.g^{-1}$ and 0.18 $g.L^{-1}.h^{-1}$, respectively (Table 3.1). With a low biomass concentration, the vanillin production rate was slow (0.28 $g.L^{-1}.h^{-1}$), decreasing the productivity of the process. Even though the yield of vanillin in ferulic acid was higher than the obtained in standard conditions, these conditions do not improve greatly the vanillin production, since the productivity is low, compared with the obtained in standard conditions.

The results obtained with these different nitrogen concentrations, which resulted in different biomass concentrations, allowed to understand that the production of vanillin is affected by high and also low cellular densities and this could influence the production rate and consumption of vanillin. From the conditions tested, the standard conditions that allow the generation of 5 $g.L^{-1}$ of biomass, are those which supported a better production, with the highest productivity values along with a good yield of vanillin in ferulic acid, that are in accordance with those obtained by Pérez-Rodríguez *et al.* (2016). Since the specific rate of growth was similar in all the runs performed, is possible to conclude that there was not inhibition of growth by any conditions tested.

## 3.3 Influence of nitrogen source

Yeast extract can be used as nitrogen source, but is expensive for use in industrial scale, so it is important try to replace it for other compounds. However, yeast extract is not only a source of nitrogen, but also a source of peptides and amino acids, vitamins (vitamin B complex), sugars and minerals that stimulate the bacterial growth, so for its replacement several compounds are needed.

In section 3.2, the quantity of nitrogen that allows a production of vanillin with a better productivity and a good yield of vanillin in ferulic acid was determined, 5 $g.L^{-1}$. Based in these results, and in order to replace a part of yeast extract with other source of nitrogen, ammonium salts, some experiments in shake flasks and in bioreactor were performed. In these experiments, only half of the yeast extract needed was replaced to ensure that the growth factors, even in a lower concentration, were present in the medium.

To perform the replacement, it was necessary choose the ammonium salt that would be used as nitrogen source. Different ammonium salts, ammonium sulphate, ammonium phosphate and ammonium chloride, were tested in shake flasks experiments and compared with the control experiment that had the same medium composition as the standard, having yeast extract as the only source of nitrogen. Table 3.2 presents the yield of vanillin in ferulic acid and the rate of vanillin production for the control experiment and the experiments with different ammonium salts tested.

Results show that vanillin is produced faster when only yeast extract is used (Table 3.2). This is likely due to the presence of higher concentration of the trace elements present in the extract, which are missing when the ammonium salts replace a part of the yeast extract. But considering the assays with ammonium salts, when part of the nitrogen comes from ammonium phosphate, a better yield and a higher rate of vanillin production were obtained (Table 3.2). It seems that is better for the cells to increase the quantity of phosphate present in the medium than the sulphate or chloride.

Table 3.2 – Yield of vanillin in ferulic acid and rate of vanillin production obtained for each nitrogen source tested: the control experiment and the ammonium salts that represent a half of the nitrogen source, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $NH_4CL$.

| **Nitrogen source** | **$Y_{P/S}$ ($g.g^{-1}$)** | **$r_v$ ($g.L^{-1}.h^{-1}$)** |
|---|---|---|
| **YE** | 0.46 | 0.034 |
| **YE + $(NH_4)_2SO_4$** | 0.22 | 0.017 |
| **YE + $(NH_4)_2HPO_4$** | 0.46 | 0.021 |
| **YE + $NH_4Cl$** | 0.13 | 0.018 |

Considering the best results obtained in shake flask experiments, a bioreactor run was performed with half of the initial nitrogen coming from ammonium phosphate and the other part from yeast extract, with an initial nitrogen concentration of 0.5 $g.L^{-1}$, maintaining the other conditions similar to the standard. The cultivation profile of this run (run 5) is represented in Figure 3.3 and the comparison of the parameters obtained in this run and in standard run are shown in Table 3.3.

In these conditions, *Amycolatopsis* sp. ATCC 39116 started to growth exponentially after a lag phase of around 3h, reaching 6.07 $g.L^{-1}$ of biomass in the end of the exponential growth phase, 15 h after the beginning of the run. Despite the lag phase was shorter in this run, the specific cell growth rate was lower (0.22 $h^{-1}$) than in standard run (0.31 $h^{-1}$), showing that the reduction in yeast extract had influence in cell growth, since the components that stimulated growth present in the extract were present in a lower concentration. For the cellular growth, 9.19 $g.L^{-1}$ of glucose and 0.32 $g.L^{-1}$ of nitrogen were consumed, the yield of biomass in substrate obtained was 0.58 $g.g^{-1}$, significantly lower than in standard run.

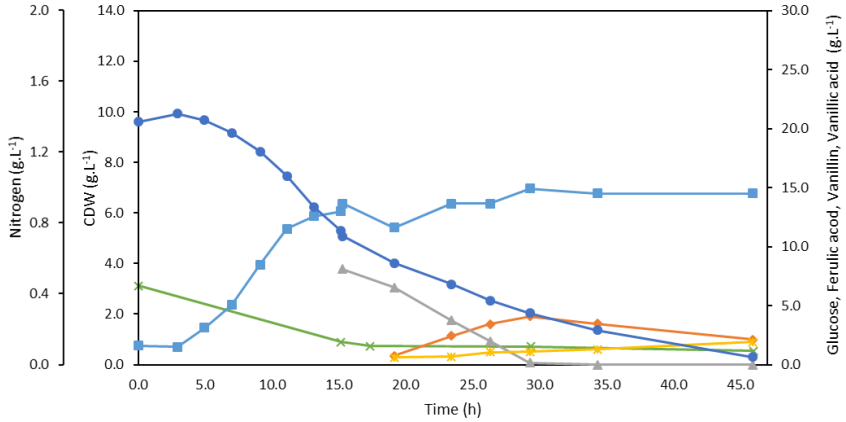



Figure 3.3 – Cultivation profile of *Amycolatopsis* sp. ATCC 39116 in a medium containing 0.25 g.L$^{-1}$ of nitrogen coming from yeast extract and 0.25 g.L$^{-1}$ coming from ammonium phosphate (Run 5). The concentrations of CDW (■), glucose (●), nitrogen (×), ferulic acid (▲), vanillin (♦) and vanillic acid (✳) are represented throughout the run time.

In the beginning of stationary phase, ferulic acid was added to the medium (8.10 g.L$^{-1}$) resulting in a maximum vanillin concentration of 4.11 g.L$^{-1}$, achieved 14 h after that and 29.3 h after the run has started. Considering the time frame of vanillin production (and not the part of the run when vanillin was consumed), the yield of vanillin in ferulic acid obtained was lower (0.52 g.g$^{-1}$) than in standard conditions (0.62 g.g$^{-1}$) as well as the rate of vanillin production (0.29 g.L$^{-1}$.h$^{-1}$), that was less than a half lower, and the vanillin volumetric productivity of the process (0.14 g.L$^{-1}$.h$^{-1}$), that was a half of the obtained in the standard, as it is represented in Table 3.3. Since all the process was slower in the condition tested than in the standard one, it is possible to conclude that yeast extract has an important role in the growth and production of vanillin not because of the nitrogen supply, but because the other components that stimulate the cells metabolism. Therefore, for substitution of yeast extract is important to find which components are essential for the cells and provide a solution with this components to the growth medium.

Table 3.3 – Comparison of growth and vanillin production obtained in standard conditions and in the run performed with the nitrogen coming from yeast extract and ammonium phosphate.

| **Run** | **$Y_{(X/S)}$ (g.g$^{-1}$)** | **μ (h$^{-1}$)** | **$Y_{(P/S)}$ (g.g$^{-1}$)** | **$r_v$ (g.L$^{-1}$.h$^{-1}$)** | **$r_P$ (g.L$^{-1}$.h$^{-1}$)** | **$q_P$ (g.g$_X^{-1}$.h$^{-1}$)** | **$V_{max}$ (g.L$^{-1}$)** |
|---|---|---|---|---|---|---|---|
| **Standard** | 0.96 | 0.31 | 0.62 | 0.66 | 0.28 | 0.057 | 5.28 |
| **(5) 0.25 g.L$^{-1}$ N (YE) + 0.25 g.L$^{-1}$ N ($(NH_4)_2HPO_4$)** | 0.58 | 0.22 | 0.52 | 0.29 | 0.14 | 0.020 | 4.11 |

## 3.4 Impact of glucose concentration

The source of carbon and energy is used for cellular growth but also for cellular maintenance. In the vanillin production process, *Amycolatopsis* sp. ATCC 39116 can use glucose and ferulic acid at the same time as it was discussed in section 3.1. In the standard run, a significant concentration of glucose remained in the broth in the end of the experiment, so it is important to decrease the quantity of this source of carbon and energy wasted in the process, which can affect production costs. However, in the studies performed with the aim to evaluate the effect of nitrogen concentration in vanillin production (section 3.2), there was noticed that the behavior of cells in vanillin consumption varied in some circumstances, which could be linked to the presence or absence of glucose in the broth. Thus, in order to understand if glucose can affect the rate of vanillin degradation and if the concentration used can be decreased, two experiments in bioreactors were performed with different concentrations of that source of carbon and energy, 15 and 10 $g.L^{-1}$, maintaining the other conditions similar to the standard. The cultivation profiles of the runs are presented in Figure 3.4 and the parameters of growth and production obtained can be compared with those obtained in standard run in Table 3.4. The parameters for the production phase presented in the Table 3.4 were calculated taking into account the period of time until the maximum concentration of vanillin was reached.

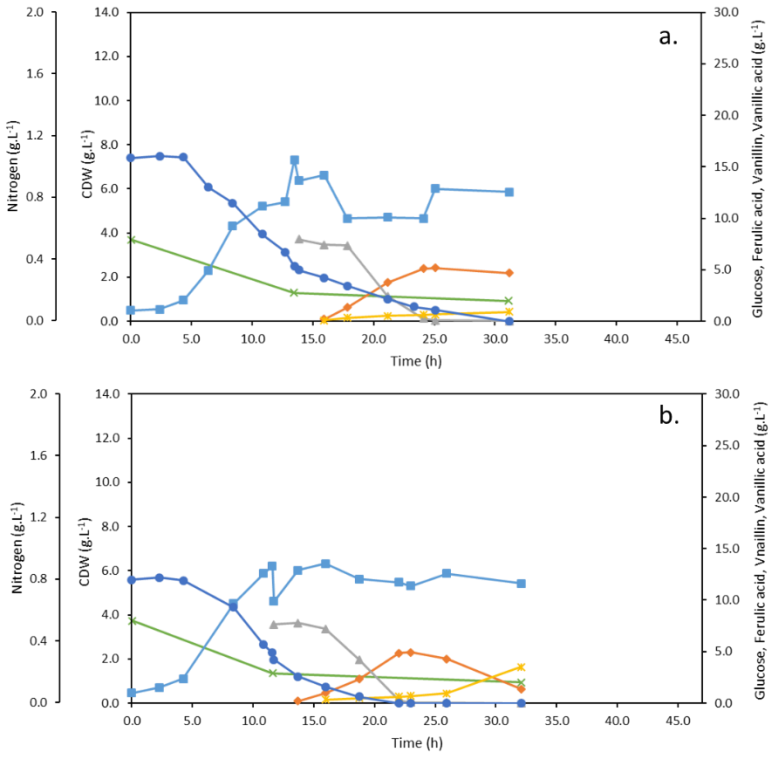



Figure 3.4 – Cultivation profiles of *Amycolatopsis* sp. ATCC 39116 at different initial glucose concentrations: a) Run 6: 15 $g.L^{-1}$, b) Run 7: 10 $g.L^{-1}$. The concentrations of CDW (■), glucose (●), nitrogen (×), ferulic acid (▲), vanillin (♦) and vanillic acid (✳) are represented throughout the run time.

The run with the intermediate concentration of glucose, 15 $g.L^{-1}$, (run 6, Figure 3.4 a.) had a growth profile with a lag phase similar to other experiments performed, around 4 h, and cells grew exponentially reaching a concentration of around 6 $g.L^{-1}$, 13.9 h after the beginning of the run. At that time, the bioreactor was fed with ferulic acid (7.97 $g.L^{-1}$). For the growth, 10.54 $g.L^{-1}$ of glucose was consumed, with a yield of biomass in glucose of 0.65 $g.g^{-1}$ (Table 3.4), having consumed 0.34 $g.L^{-1}$ of nitrogen, showing a higher consumption of glucose than in the standard conditions. In the vanillin production phase, around 6 $g.L^{-1}$ of biomass were present in the broth, similar to the standard, and in the beginning of that phase 4.96 $g.L^{-1}$ of glucose were available to be used for cellular maintenance. Concerning vanillin, it reached a maximum of 5.20 $g.L^{-1}$, 11.2 h after the addition of the substrate to the broth and 25.1 h after the beginning of the run. The yield of vanillin in ferulic acid (0.66 $g.g^{-1}$) obtained was similar to that obtained in standard conditions, but the vanillin volumetric productivity was lower (0.21 $g.L^{-1}.h^{-1}$) as it can be observed in Table 3.4, as well as the vanillin production rate (0.46 $g.L^{-1}h^{-1}$).

Table 3.4 – Comparison of growth and vanillin production parameters obtained in standard conditions and in the cultivation runs performed with different glucose concentrations.

| Run | $Y_{(X/S)}$ ($g.g^{-1}$) | $\mu$ ($h^{-1}$) | $Y_{(P/S)}$ ($g.g^{-1}$) | $r_v$ ($g.L^{-1}.h^{-1}$) | $r_P$ ($g.L^{-1}.h^{-1}$) | $q_P$ ($g.g_X^{-1}.h^{-1}$) | $V_{max}$ ($g.L^{-1}$) |
|---|---|---|---|---|---|---|---|
| **Standard** | 0.96 | 0.31 | 0.62 | 0.66 | 0.28 | 0.057 | 5.28 |
| **(6) 15 $g.L^{-1}$ glc** | 0.65 | 0.37 | 0.66 | 0.46 | 0.21 | 0.034 | 5.20 |
| **(7) 10 $g.L^{-1}$ glc** | 0.81 | 0.34 | 0.65 | 0.44 | 0.21 | 0.040 | 4.92 |

In the run with the lower initial glucose concentration, 10 $g.L^{-1}$ (run 7, Figure 3.4 b.) the growth profile was similar to that obtained in standard conditions, with a lag phase that lasted around 4 h and an exponential phase during 7.5 h, that lead to a biomass concentration of 6.22 $g.L^{-1}$. During the exponential phase, 7.10 $g.L^{-1}$ of glucose were consumed, giving rise to a yield of biomass in glucose of 0.81 $g.g^{-1}$ (Table 3.4) and having been consumed 0.34 $g.L^{-1}$ of nitrogen. The ferulic acid added was 7.64 $g.L^{-1}$. For the vanillin production phase, around 6 $g.L^{-1}$ of biomass were present and 4.20 $g.L^{-1}$ of glucose was still available in the broth, but was totally consumed until the time that ferulic acid ended. Vanillin reached its maximum concentration of 4.92 $g.L^{-1}$, 11.3 h after the start of the production phase and 23 h since the beginning of the run. The yield of vanillin in ferulic acid was similar (0.65 $g.g^{-1}$) to the obtained in the run 6 and in the standard run, as can be seen in Table 3.4, but the vanillin production rate and volumetric productivity (0.44 and 0.21 $g.L^{-1}.h^{-1}$) were lower than in the standard and similar to run 6.

Results suggest that rate of vanillin production is favored by higher concentrations of glucose in the broth, since the production was faster in the standard run, where 16.20 $g.L^{-1}$ of glucose are available by the time that the reactor was fed, different from these runs where only around 4 $g.L^{-1}$ of the substrate were available. Based on these results it is possible to conclude that the initial concentration of glucose in the medium should not be reduced.

Considering the profile of the runs 6, 7 and standard, until the point of maximum production of vanillin, the three runs had a very similar behavior. However, the profile started to be different in run 7 after the peak of vanillin production, when no glucose was present in the broth and the concentration of vanillin started do decrease rapidly. In the standard run and in the run performed with 15 $g.L^{-1}$ of initial glucose (Figure 3.1 and Figure 3.4 a, respectively), it can be seen that glucose is present until the end of the run. In run 7 (Figure 3.4 b), the run with 10 $g.L^{-1}$ of initial glucose, that substrate ended when vanillin reached its maximum concentration. Along with these observations, it is possible to notice a difference in the profile of vanillin in the standard run and in run 6, where vanillin suffered a slight decrease unlike the run 7 where the consumption of vanillin was higher and faster. In fact, in the standard run the rate of vanillin consumption ($r_c$) was 0.097 $g.L^{-1}.h^{-1}$, similar to the obtained in the run 6, 0.084 $g.L^{-1}.h^{-1}$, but in the run 7 vanillin was consumed at 0.39 $g.L^{-1}.h^{-1}$, a rate four times higher than the obtained when glucose is present. Vanillin is likely degraded by the microorganism for its own detoxification, since is a reactive aldehyde, but could also been used as source of carbon and energy (Fleige *et al.*, 2013). Therefore, is possible that when no glucose is present in the medium, the cells start to use vanillin as substrate for cellular maintenance, and in this case the rate of consumption increases, as it was anticipated in section 3.2.

## 3.5 Pulse feeding strategy

Until this point, in the current work, the effect of nitrogen and glucose concentrations were studied aiming at understanding and improving the process of vanillin production. These studies were performed in processes with addition of ferulic acid in a single pulse. Giving the previous results, in the following experiments the pulse feeding strategy was studied to understand if a higher concentration of vanillin could be produced with a better productivity, by feeding a higher amount of ferulic acid.

As a phenolic compound, ferulic acid has toxic properties to the cells, so, in this pulse feeding approach, it is important that this substrate do not accumulate in the broth, affecting cellular viability. For that reason, it is necessary to know when ferulic acid was totally consumed, which corresponds to the maximum production of vanillin, not only to evaluate the time to supply the next pulse of the substrate, but also to evaluate and compare the production in each pulse. However, it is also important not to lose vanillin during the process by conversion into vanillic acid. In the experiments performed until this point of work, it was noticed that in some of the runs, namely standard and runs 1, 3 and 5, acid was added to the broth near to the point of maximum vanillin concentration and the increase in $pO_2$ values was also observed in standard run and in runs 1, 2, 5 and 6 after that point. So, in this set of experiments, these two signs were considered to perform the feeding, along with the duration of the production phase in the standard run.

The run 8 (Figure 3.5 a.) was performed in standard conditions, accordingly with the previous studies in this work, having a growth phase that lasted 9.2 h, with 4 h of lag phase, reaching a biomass concentration of 4.20 $g.L^{-1}$. In this phase 4.64 $g.L^{-1}$ of glucose and 0.09 $g.L^{-1}$ of nitrogen were consumed, allowing a yield of biomass in glucose of 0.71 $g.g^{-1}$. Regarding the consumption profile of glucose in this

run, the first point is lower than the following points and this may be due to the non-homogeneity of the medium by the time that the sample was taken.

When the stationary growth phase started, ferulic acid was added, achieving a concentration of 6.63 $g.L^{-1}$. This substrate was consumed during 8.1 h leading to the production of 4.47 $g.L^{-1}$ of vanillin, with a yield of vanillin in ferulic acid of 0.67 $g.g^{-1}$ (as can be seen, along with the rate and the specific rate of vanillin production, in the Table 3.5). After that time, $pO_2$ started to rise, followed by the addition of acid and another pulse of ferulic acid was given. However, at that time, the ferulic acid from the first pulse had not been totally consumed, 0.10 $g.L^{-1}$ had remained in the broth, so the concentration of the substrate in the medium was 8.07 $g.L^{-1}$. After 7.7h since the beginning of the second feeding pulse, $pO_2$ started to increase, indicating the moment to perform the third pulse. During the second pulse, the addition of acid was frequent, not showing the relation with the production that was noticed before in this run and in other runs. At the end of second pulse, 9.34 $g.L^{-1}$ of vanillin were present in the broth, 5.24 $g.L^{-1}$ of them being produced during this pulse, with a yield of vanillin in ferulic acid of 0.71 $g.g^{-1}$ (Table 3.5). When the third pulse was given the substrate reached a concentration of 9.06 $g.L^{-1}$ in the broth, resulted from the accumulation with 0.68 $g.L^{-1}$ that remained from the previous pulse. However, after this pulse, $pO_2$ values did not return to the minimum controlled (30%), increasing with time (until 100%, the end of the run), indicating a loss of activity. In fact, in the last 21.4 h of the run 3.85 $g.L^{-1}$ of ferulic acid were consumed with a vanillin production of 2.19 $g.L^{-1}$ and a production yield of vanillin in ferulic acid of 0.57 $g.g^{-1}$, but at a low rate 0.10 $g.L^{-1}.h^{-1}$.

In the first pulse of ferulic acid, there was an adaptation phase of 3 h when the concentration of ferulic acid does not vary significantly, as it happened in the other runs performed in this work, during which the cells are probably activating the mechanisms necessary to metabolize ferulic acid. However, when the second pulse are fed, this phase does not exist leading to an increase in the rate of production as can be seen in Table 3.5.

During third pulse, it was observed a decrease in cellular activity, in first place, by high $pO_2$ values (increasing from 36 % to 100% in the end of the process), but also because of the low glucose consumption during the whole pulse (1.02 $g.L^{-1}$) and the slow vanillin production, with few ferulic acid being consumed. This loss of activity may has been due to the high vanillin concentration present in the broth, which is reported to have antimicrobial activity. In the end of the second pulse, the concentration of vanillin present in the broth was 9.34 $g.L^{-1}$, and for the beginning of the third pulse it had a small decrease to 7.71 $g.L^{-1}$ due to the removal of some volume to allow the addition of the substrate. Actually, Fleige and Meyer (2016) studied the toxicity of the vanillin in *Amycolatopsis* sp. ATCC 39116, concluding that this compound is tolerated by cells in concentrations up to 4 $g.L^{-1}$ with an initial reduction in the viable cells, but has an antimicrobial effect for concentrations above 7 $g.L^{-1}$. For these concentrations they reported a complete cell death within 6 h, and for 10 $g.L^{-1}$, 90% of cell death in 1 h, and when 3 h had passed no viable cells were present.

In Table 3.6 the parameters obtained for the total production process were calculate taking into account the two first pulses, since there was inhibition by product in the third pulse. In this run an average of 4.85 $g.L^{-1}$ of vanillin were produced in each ferulic acid pulse and vanillic acid reached a concentration of 1.34 $g.L^{-1}$ in the end of the run. Comparing this results with those obtained in standard conditions, in which just one pulse was fed, the run 8 had a better vanillin volumetric productivity, 0.38 $g.L^{-1}.h^{-1}$ (in the standard the vanillin volumetric productivity was 0.28 $g.L^{-1}.h^{-1}$), that represents an increase of 35 %, with a similar yield of vanillin production in ferulic acid (0.62 $g.g^{-1}$ in the standard run and 0.69 $g.g^{-1}$ in the run 8). So, it is possible to conclude that an approach in which ferulic acid is fed in various pulses is advantageous. However, the pulse feeding strategy needs to be different from the performed in run 8 in order to avoid the inhibition by vanillin.

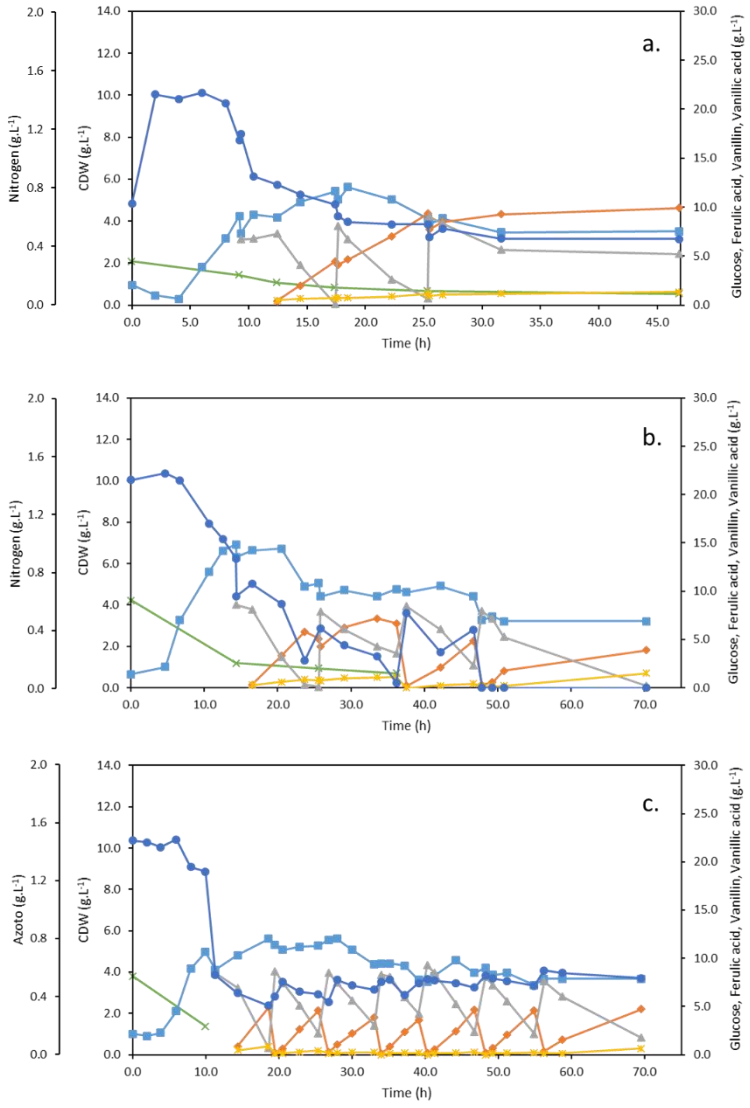



Figure 3.5 – Cultivation profiles of *Amycolatopsis* sp. ATCC 39116 with different feeding pulse strategies: a) Run 8: multiple pulse feeding, b) Run 9: multiple pulse feeding and biomass recovery and c) Run 10: multiple pulse feeding with biomass recovery. The concentrations of CDW (■), glucose (●), nitrogen (×), ferulic acid (▲), vanillin (♦) and vanillic acid (✳) are represented throughout the run time.

To overcome this limitation, run 9 (Figure 3.5 b.) was performed in the same conditions than run 8, but when the production of the second pulse was ended, the broth was removed from the bioreactor and centrifuged. The biomass was recovered and resuspended in a solution with 8 $g.L^{-1}$ of glucose (the glucose concentration consumed during the production phase in standard conditions), that was found to be necessary for the production process and avoid the consumption of vanillin (section 3.4), and reintroduced in the bioreactor along with the ferulic acid to start the conversion. The reintroduction of biomass was repeated one more time, to fed the fourth pulse of ferulic acid.

Run 9 had a growth phase of 14.3 h, 4.7 h of them being the lag phase. At the end of exponential growth phase 6.92 $g.L^{-1}$ of biomass were present in broth. For this biomass production, 8.14 $g.L^{-1}$ of glucose were consumed, with a yield of vanillin in glucose of 0.77 $g.g^{-1}$ (Table 3.6) along with the consumption of 0.43 $g.L^{-1}$ of nitrogen.

The first pulse of ferulic acid reached a concentration of 8.58 $g.L^{-1}$ in the broth and the maximum production of vanillin was 5.80 $g.L^{-1}$, with a yield of vanillin in ferulic acid of 0.70 $g.g^{-1}$, 9.3 h after the feeding and 23.7 h after the beginning of the process (Table 3.5). During this pulse, the biomass concentration decreased for 5 $g.L^{-1}$ in the end of the transformation. The second pulse was performed, similar to what has been done for run 8, when $pO_2$ started to increase, however, at that time vanillin had reached its maximum and its concentration had decreased (to 5.09 $g.L^{-1}$). After the second pulse, the concentration of ferulic acid in the broth was 7.89 $g.L^{-1}$, leading to a production of 2.92 $g.L^{-1}$ vanillin, accumulating 7.17 $g.L^{-1}$ of vanillin in this pulse, 7.7 h after the feeding. However, this second pulse had a lower production than its homologous in run 8. This might have happened due to a little higher vanillin production achieved in the first pulse performed in this run, that could had achieved the toxic level, even with vanillin being consumed, that could have motivated a decrease in the cellular viability, due to a higher exposure time, making the vanillin production rate lower (0.37 $g.L^{-1}.h^{-1}$, as presented in Table 3.5) and turning impossible the total consumption of ferulic acid, remaining in the broth 3.58 $g.L^{-1}$ when the $pO_2$ started to increase. Similar to what happened in the first pulse, but without the total consumption of ferulic acid, the vanillin reached its maximum concentration and started to decrease with the production of vanillic acid, for its detoxification.

The third pulse, after the centrifugation step, started with a ferulic acid concentration of 8.46 $g.L^{-1}$ and with a similar biomass concentration to the present in the previous pulse (4.5 $g.L^{-1}$). The production took 9.1 h with 4.67 $g.L^{-1}$ of vanillin being produced, with a yield of vanillin in ferulic acid and a specific rate of vanillin production comparable with the other pulses (0.76 $g.g^{-1}$ and 0.12 $g.g_X^{-1}.h^{-1}$, respectively), showing that the removal of vanillin from the broth allow to continue vanillin production with similar rates and yields, even if the cells were exposed to high vanillin concentrations for a short period of time. However, this exposure affected the cells since $pO_2$ during this pulse did not decrease to the minimum controlled (30 %) maintaining constant at around 40%, indicating that cellular metabolism was affected. Nevertheless, a fourth pulse was given, after a centrifugation step, with ferulic acid reaching a concentration of 7.92 $g.L^{-1}$. In this last pulse, $pO_2$ values never stopped to increase, reaching 100% in the end of the run, indicating that cells lost its viability, probably due to assay long duration, since the

fourth pulse was given 47.9 h after the beginning of the run. In fact, production of vanillin was lower in this pulse (3.58 $g.L^{-1}$) and with a low yield of vanillin in ferulic acid and vanillin specific production rate (0.46 $g.g^{-1}$ and 0.05 $g.g_X^{-1}.h^{-1}$, respectively, Table 3.5).

The growth and production parameters for the global process are represented in Table 3.6, and were calculated taking into account just the three first pulses, since in the fourth, the production was affected by the loss of cellular viability. The vanillin production yield from ferulic acid obtained (0.71 $g.g^{-1}$) were comparable with those obtained in the standard run and in run 8 (0.62 $g.g^{-1}$ and 0.71 $g.g^{-1}$, respectively), but the global vanillin productivity (0.29 $g.L^{-1}.h^{-1}$) was lower than the obtained for run 8 (0.38 $g.L^{-1}.h^{-1}$), and similar to the standard (0.28 $g.L^{-1}.h^{-1}$). This low productivity, compared with run 8, may be due to the concentration of vanillin that turned toxic to the cells, affecting the metabolism of ferulic acid in the second pulse.

In run 9 it was possible to understand that centrifugation is advantageous for the vanillin production since product withdraw from the medium avoids its accumulation and consequently cellular inhibition. Therefore, cells can continue producing vanillin. In this run it was also possible to realize that in two ferulic acid pulses a toxic concentration of vanillin in broth can be achieved, affecting the cellular metabolism. So, this approach was not the right one to increase the vanillin production and productivity. The removal of the broth before the addition of each pulse can be advantageous, since the concentration of vanillin would not achieve toxic concentrations, this was the approach performed in run 10.

In run 10 (Figure 3.5 c.) the growth phase took 10 h, being produced 4.95 $g.L^{-1}$ of biomass and having consumed 0.35 $g.L^{-1}$ of nitrogen. When $pO_2$ values started to increase indicating the beginning of the stationary growth phase, broth was removed from the bioreactor in order to centrifuge cells and reintroduce them in the bioreactor resuspended in a solution with glucose (8 $g.L^{-1}$).

In run 10, the second pulse and the following pulses were fed when acid started to be added in the broth. It was noticed, in a similar run (data not shown), that if the pulses were given when $pO_2$ started to increase, there was enough time for vanillin production and its consumption in vanillic acid and even consumption of the acid produced. It was observed that the maximum vanillin concentration was achieved by the time that acid started to be added to the broth, so in run 10 this was the signal taken into account to perform the next pulse. Therefore, was also possible to understand that if the production phase was stopped by the time that maximum vanillin production was achieved, it would be possible to increase the number of pulses given, since cells started to lose its capability of degrade ferulic acid after 50 h from the beginning of the run, similar to what happened in run 9.

The first pulse of ferulic acid given in run 10, started with a substrate concentration of 8.45 $g.L^{-1}$ that led to 4.92 $g.L^{-1}$ of vanillin produced, reached 7.3 h after the feeding, with a yield of vanillin in ferulic acid of 0.63 $g.g^{-1}$ and a vanillin specific production rate of 0.12 $g.g_X^{-1}.h^{-1}$ (Table 3.5), that includes 3h of adaptation phase for ferulic acid degradation. When acid start being added to the broth, ferulic acid had been almost totally consumed, remaining 0.67 $g.L^{-1}$. However, in the following pulses, a higher concentration of ferulic acid remained in the broth. Regarding glucose, during the transformation time

of this pulse, 3.16 $g.L^{-1}$ was consumed, that was less than the expected considering the other experiments. In the next pulses, the amount of glucose consumed in each pulse was even lower, about 1 $g.L^{-1}$.

In the second pulse, ferulic acid was present in a concentration of 8.64 $g.L^{-1}$, being produced 4.37 $g.L^{-1}$ of vanillin in 5.9 h with a yield of vanillin in ferulic acid and a vanillin specific production rate of 0.68 $g.g^{-1}$ and 0.14 $g.g_X^{-1}.h^{-1}$, respectively (Table 3.5). In this pulse there was not an adaptation phase for degradation of ferulic acid, it started to be degraded by the time that was added to the broth, but when the broth was removed from the bioreactor, the ferulic acid concentration present was 2.23 $g.L^{-1}$.

Table 3.5 – Parameters of vanillin production calculated for each pulse of ferulic acid fed in the runs performed for the study of the pulse feeding strategy: yield of vanillin in ferulic acid, rate and specific rate of vanillin production and maximum vanillin concentration reached in the pulse.

| | **(8) No centrifugation** | | | | **(9) centrifugation after the 2nd pulse** | | | | **(10) centrifugation before each pulse** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Pulse** | **$Y_{(P/S)}$ ($g.g^{-1}$)** | **$r_v$ ($g.L^{-1}.h^{-1}$)** | **$q_v$ ($g.g_X^{-1}.h^{-1}$)** | **$V_{max}$ ($g.L^{-1}$)** | **$Y_{(P/S)}$ ($g.g^{-1}$)** | **$r_v$ ($g.L^{-1}.h^{-1}$)** | **$q_v$ ($g.g_X^{-1}.h^{-1}$)** | **$V_{max}$ ($g.L^{-1}$)** | **$Y_{(P/S)}$ ($g.g^{-1}$)** | **$r_v$ ($g.L^{1}.h^{-1}$)** | **$q_v$ ($g.g_X^{-1}.h^{-1}$)** | **$V_{max}$ ($g.L^{-1}$)** |
| **1** | 0.67 | 0.55 | 0.10 | 4.47 | 0.70 | 0.63 | 0.13 | 5.80 | 0.63 | 0.68 | 0.12 | 4.92 |
| **2** | 0.71 | 0.68 | 0.17 | 5.24 | 0.81 | 0.37 | 0.09 | 2.92 | 0.68 | 0.74 | 0.14 | 4.37 |
| **3** | 0.57 | 0.10 | 0.03 | 2.19 | 0.76 | 0.51 | 0.12 | 4.67 | 0.65 | 0.58 | 0.13 | 3.58 |
| **4** | – | – | – | – | 0.46 | 0.16 | 0.05 | 3.58 | 0.85 | 0.67 | 0.18 | 3.45 |
| **5** | – | – | – | – | – | – | – | – | 0.65 | 0.69 | 0.18 | 4.47 |
| **6** | – | – | – | – | – | – | – | – | 0.74 | 0.66 | 0.20 | 4.39 |
| **7** | – | – | – | – | – | – | – | – | 0.75 | 0.33 | 0.09 | 4.40 |

In the third pulse, ferulic acid reached an initial concentration of 8.50 $g.L^{-1}$ and 3.58 $g.L^{-1}$ of vanillin was produced in 6.2 h, with a yield of vanillin in ferulic acid of 0.65 $g.g^{-1}$ and a vanillin specific production rate of 0.13 $g.g_X^{-1}.h^{-1}$ (Table 3.5), remaining 3.02 $g.L^{-1}$ of ferulic acid in the broth.

The ferulic acid concentration in the fourth pulse was 8.31 $g.L^{-1}$, having been produced 3.45 $g.L^{-1}$ of vanillin in 5.2 h, remaining 4.25 $g.L^{-1}$ of the substrate, with a yield of vanillin in ferulic acid and a vanillin specific production rate of 0.85 $g.g^{-1}$ and 0.18 $g.g_X^{-1}.h^{-1}$, respectively (Table 3.5). During this pulse, the biomass concentration decreased to around 4 $g.L^{-1}$, maintaining constant until the end of the run.

The fifth pulse, had an initial ferulic acid concentration of 9.27 $g.L^{-1}$, that was consumed during 6.5 h, remaining 2.38 $g.L^{-1}$ of the substrate in the broth, leading to a production of 4.47 $g.L^{-1}$ of vanillin, with a

yield of vanillin in ferulic acid of 0.65 $g.g^{-1}$ and a vanillin specific rate of production of 0.18 $g.g_X^{-1}.h^{-1}$ (Table 3.5)

The sixth pulse started with ferulic acid concentration of 8.05 $g.L^{-1}$. After 6.6 h, 4.39 $g.L^{-1}$ of vanillin were present in the broth along with 2.14 $g.L^{-1}$ of ferulic acid obtaining a yield of vanillin in ferulic acid and a vanillin specific production rate of 0.74 $g.g^{-1}$ and 0.20 $g.g_X^{-1}.h^{-1}$, respectively (Table 3.5). However, during this pulse, the $pO_2$ values increased, oscillating between 40 % and 50%, indicating that the cells were started to lose its activity, as was expected, in line with what was notice in other runs, since this pulse was performed 48.3 h after the beginning of the process.

A seventh pulse, and the last one, was performed 56.3 h after the beginning of the run, started with a ferulic acid concentration of 7.61 $g.L^{-1}$. After 13.3 h since the feeding, 4.40 $g.L^{-1}$ of vanillin had been produced, remaining in broth 1.77 $g.L^{-1}$ of the substrate. During this pulse, the $pO_2$ values were high, increasing from 70 % to 100% in the end of the process, demonstrating that the cells lost viability. In fact, the vanillin specific production rate obtained was lower than those obtained for the other pulses, 0.09 $g.g_X^{-1}.h^{-1}$, as can be seen in Table 3.5.

In this run, taking into account the first six pulses, since in the last one the cells were not able to metabolize ferulic acid at the same rate as the other pulses, an average of 4.20 $g.L^{-1}$ of vanillin were produced in each pulse, with a total production yield of vanillin in ferulic acid of 0.69 $g.g^{-1}$, and a total vanillin volumetric productivity of 0.46 $g.L^{-1}.h^{-1}$, as can be seen in Table 3.6. The productivity achieved in this run, was the best of all the runs performed in this work and even the bioreactor having been fed with a higher total concentration of ferulic acid, the total production yield was similar to obtained in the standard run. In fact, with this pulse feeding strategy, there is only a growth phase and one adaptation phase for the ferulic acid degradation and cells are viable during a little more than 50 h, allowing the feeding with various pulses of ferulic acid. The centrifugation was advantageous for the process since a high vanillin concentration, that can affect the cellular viability, was never achieved or maintained in the broth for a long time, making the production yield, the production rate and the specific rate of production similar in all the pulses added (Table 3.5). The removal of the broth before each pulse, avoiding the accumulation of vanillin was also important because the transformation of vanillin in vanillic acid was also reduced, in the total of the six pulses considered, only 1.72 $g.L^{-1}$ of vanillic acid were produced, that is a low value compared with the production in run 8 in just two pulses (1.34 $g.L^{-1}$). The vanillin volumetric productivity achieved in this run, represents an increase of 64% comparing with that obtained in the standard process and an increase of 21 %, comparing with the pulse feeding process without centrifugation (run 8). However, the ferulic acid fed was not totally consumed for almost the pulses in the run and it is important to avoid the waste of the substrate, so it could be important analyze a sample of the broth taken by the time that acid starts to be added to understand if the substrate is still present, before the addition of the next pulse.

Table 3.6 – Comparison of the overall parameters of the growth and production obtained in the vanillin production process, in the standard run and in the runs performed for the study of the pulse feeding strategy and the vanillin produced in each run.

| Run | $Y_{(X/S)}$ ($g.g^{-1}$) | $\mu$ ($h^{-1}$) | $Y_{(P/S)}$ ($g.g^{-1}$) | $r_P$ ($g.L^{-1}.h^{-1}$) | Vanillin ($g.L^{-1}$) |
|---|---|---|---|---|---|
| **Standard** | 0.96 | 0.31 | 0.62 | 0.28 | 5.28 |
| **8** | 0.71 | 0.27 | 0.69 | 0.38 | 4.85 [a] |
| **9** | 0.77 | 0.27 | 0.71 | 0.29 | 4.47 [a] |
| **10** | - | 0.33 | 0.69 | 0.46 | 4.20 [a] |

[a] An average value of vanillin produced in each ferulic acid pulse.

Studies for vanillin removal from the broth with the aim of avoid accumulation of product in toxic concentrations are already reported in literature (Ma and Daugulis, 2014b; Hua *et al.*, 2007). The volumetric productivity achieved in this work was very similar to the obtained by Ma and Daugulis (2014b). In their work, polymer beads (Hytrel® G4078W) were added to the bioreactor to remove vanillin from the broth, preventing the contact with cells and its consequent consumption. Ma and Daugulis (2014b) approach led to a vanillin concentration of 19.5 $g.L^{-1}$ and a volumetric productivity of 0.45 $g.L^{-1}.h^{-1}$. The centrifugation method discussed in this thesis presents similar results, showing that is also feasible. These two approaches in terms of production are the ones that show better results using non-modified stains.

# 4. Conclusions and future work

This thesis aiming at studying and optimize the production of vanillin in bioreactor by *Amycolatopsis* sp. ATCC 39116. Studies were performed to evaluate the effect of nitrogen concentration and source, the impact of the glucose concentration and the use a pulse feeding strategy on the yield and process productivity.

To investigate the effect of nitrogen concentration, different quantities of nitrogen (obtained from yeast extract and, for the maximum nitrogen concentration tested, from yeast extract and ammonium sulphate) were added to the culture medium (0.25 to 2.00 $g.L^{-1}$). High concentrations of this nutrient supported a high cellular growth but a lower vanillin volumetric productivity, comparing with the standard run (0.5 $g.L^{-1}$ of nitrogen from 5 $g.L^{-1}$ of yeast extract) and low concentrations of nitrogen lead to low biomass concentrations and also a lower vanillin volumetric productivity. The biomass concentration that lead to a better vanillin volumetric productivity (0.28 $g.L^{-1}.h^{-1}$) along with a good yield of vanillin in ferulic acid (0.62 $g.g^{-1}$) was 5 $g.L^{-1}$, achieved by the presence of 5 $g.L^{-1}$ of yeast extract in the medium. The results obtained in this study raised the possibility that glucose plays an important role in the transformation of ferulic acid into vanillin, since different quantities of nitrogen led to different quantities of glucose present in the production phase and different cellular behaviors, in terms of production and accumulation of vanillin.

Knowing the right nitrogen concentration needed for the vanillin production process and in order to decrease the quantity of yeast extract in the process for costs reduction, the influence of the nitrogen source was investigated by replacing half of the yeast extract by ammonium salts (ammonium sulphate, ammonium phosphate and ammonium chloride). The experiments, performed in shake flaks, allowed to choose ammonium phosphate to perform a bioreactor run. This study shown that a reduction in the yeast extract had a negative impact in growth and in the vanillin production. The growth was slower than in the other experiments, presenting a lower specific growth rate (0.22 $h^{-1}$). The production was also slower, presenting a lower vanillin volumetric productivity (0.14 $g.L^{-1}.h^{-1}$) than the obtained in the standard conditions (0.28 $g.L^{-1}h^{-1}$). The reduction in yeast extract concentration, concomitantly with the decrease in the growth factors and nutrients from the extract present in the medium turned the process slow and with a low production.

In order to reduce the production costs, further studies should be performed to decrease the yeast extract concentration, understanding which components present in the extract are essential for bacterial growth and vanillin production.

The use of different glucose concentrations in the process showed that the presence of this carbon and energy source is important to increase vanillin production. These studies showed that glucose is needed for cellular maintenance and in its absence, vanillin starts to be consumed and converted into vanillic acid, preventing the accumulation of vanillin.

The pulse feeding strategy was studied in order to increase the quantity of ferulic acid fed and the vanillin produced. The pulses of ferulic acid, were of around 10 $g.L^{-1}$ and, in this study, glucose (8 $g.L^{-1}$) was added to the feeding solution whenever a centrifugation step was performed. With these studies, it was possible to understand that due to the toxicity of vanillin it is important to remove vanillin from the broth before each pulse in order to avoid that vanillin reaches a toxic concentration (around 7 $g.L^{-1}$, accordingly with Fleige and Meyer (2016) or even less) that affect the capability of the cells to degrade ferulic acid and produce vanillin. It was also possible to conclude that, in the conditions tested, *Amycolatopsis* sp. ATCC 39116 is viable and capable to produce vanillin during around 50 h. The removal of the broth before each pulse of ferulic acid given and the reuse of the biomass allowed the increasing of the vanillin volumetric productivity of the entire process to 0.46 $g.L^{-1}$, with the feeding of six pulses, that represents an increase of more than 60 % comparing to the feeding with a single pulse. However, in this pulse feeding strategy with broth removal, in the end of the production of each pulse, some ferulic acid remained in the broth. Thus, in future studies it is important to know the moment when ferulic acid is totally consumed to perform the next pulse, in order to avoid the waste of substrate. This could be done by online HPLC analysis of samples taken by the time that acid is added to the broth, which was the signal taken into account for the performance of a new pulse feeding.

For future work, it should be tested a feeding strategy with a higher concentration of ferulic acid pulses, in order to confirm if the concentration of ferulic acid fed and the vanillin produced are toxic to the cells and reduce the steps of centrifugation during the process.

The recovery of biomass and removal of the broth using other processes, besides the centrifugation, such as processes using membranes, for example, should be tested in order to simplify the process.

Runs were performed at 45 ˚C, this high temperature lead to higher energetic costs, that can be a disadvantage for an industrial application. However, it can also be an advantage, since high temperatures can avoid contamination problems, that is an important issue in processes performed with pure cultures. In literature, there are reports of processes performed at lower temperatures (28 ˚C by Ma and Daugulis (2014a,b) and 30 ˚C by Pérez-Rodríguez *et al.* (2016)), and for future work, studies with lower temperatures should be performed to understand if the production process is affected or if it brings advantages in terms of costs.

# 5. References


Acosta-Estrada, B.A., Gutiérrez-Uribe, J.A. & Serna-Saldívar, S.O., 2014. Bound phenolics in foods, a review. *Food Chemistry*, 152, pp.46–55.

Ahmad, M. *et al.*, 2011. Identification of DypB from *Rhodococcus jostii* RHA1 as a Lignin Peroxidase. *Biochemistry*, 50, pp.5096–5107.

De Monchy Aromatics, Vanillin Natural. Available at: http://demonchyaromatics.com/en/products/vanillin-natural-(ex-ferulic-acid)/ [Accessed January 29, 2017].

Arranz, S. & Saura Calixto, F., 2010. Analysis of polyphenols in cereals may be improved performing acidic hydrolysis: A study in wheat flour and wheat bran and cereals of the diet. *Journal of Cereal Science*, 51, pp.313–318.

Van Berkel, M., 2016. Biobased platform chemicals in flavours & fragrances. *Specialty Chemicals Magazine*, pp.30–31.

Bomgardner, M.M., 2016. The problem with vanilla - After vowing to go natural, food brands face a shortage of the favored flavor. *Chemical & Engeneering News, Volume 94 Issue 36*, pp.38–42.

BRENDA, Information on EC 1.2.1.67 - vanillin dehydrogenase. Available at: http://www.brenda-enzymes.org/enzyme.php?ecno=1.2.1.67&Suchword=&reference=&UniProtAcc=&organism%5B%5D=&show_tm=0 [Accessed May 28, 2017].

Brochado, A.R. *et al.*, 2010. Improved vanillin production in baker's yeast through in silico design. *Microbial cell factories*, 9(1), p.84.

Brunati, M. *et al.*, 2004. Biotransformations of cinnamic and ferulic acid with actinomycetes. *Enzyme and Microbial Technology*, 34, pp.3–9.

Dupoiron, S. *et al.*, 2017. A novel and integrative process: From enzymatic fractionation of wheat bran with a hemicellulasic cocktail to the recovery of ferulic acid by weak anion exchange resin. *Industrial Crops and Products*, 105, pp.148–155.

Eswaran, A.K., Fermentation-derived vanillin could be a sustainable natural alternative to synthetic vanillin. *Beroe*. Available at: https://www.beroeinc.com/article/fermentation-derived-vanillin/ [Accessed July 7, 2017].

European Commission, 2008. *L 354/34*,

Fache, M., Boutevin, B. & Caillol, S., 2016. Vanillin Production from Lignin and Its Use as a Renewable Chemical. *ACS Sustainable Chemistry and Engineering*, 4, pp.35–46.

FDA - U.S. Food & Drug Administration, Food Additive Status List. Available at: http://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm091048.htm#ftnE [Accessed February 5, 2017].

Fleige, C. *et al.*, 2013. Investigation of the *Amycolatopsis* sp. strain ATCC 39116 vanillin dehydrogenase and its impact on the biotechnical production of vanillin. *Applied and Environmental Microbiology*, 79(1), pp.81–90.

Fleige, C., Meyer, F. & Steinbüchel, A., 2016. Metabolic Engineering of the Actinomycete *Amycolatopsis* sp. Strain ATCC 39116 towards Enhanced Production of Natural Vanillin. *Applied and Environmental Microbiology*, 82(11), pp.3410–3419.

Gallage, N.J. & Møller, B.L., 2015. Vanillin-bioconversion and bioengineering of the most popular plant flavor and its de novo biosynthesis in the vanilla orchid. *Molecular Plant*, 8, pp.40–57.

Graham S. Dr. Byng, Rudolf Dr. Hopp, J.D.R., 1996. Process for the preparation of vanillin and suitable microorganisms - EP 0761817 B1.

Hansen, E.H. *et al.*, 2009. De novo biosynthesis of Vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*). *Applied and Environmental Microbiology*, 75(9), pp.2765–2774.

He, F., Zhang, S. & Liu, X., 2015. Immobilization of feruloyl esterases on magnetic nanoparticles and its potential in production of ferulic acid. *Journal of Bioscience and Bioengineering*, 120(3), pp.330–334.

Hua, D. *et al.*, 2007. Enhanced vanillin production from ferulic acid using adsorbent resin. *Appl. Microbiol. Biotechnol.*, 74, pp.783–790.

Jörg Overhage , Alexander Steinbüchel, H.P., 2006. Harnessing eugenol as a substrate for production of aromatic compounds with recombinant strains of *Amycolatopsis* sp . HR167. *Journal of Biotechnology*, 125, pp.369–376.

Kaur, B. & Chakraborty, D., 2013. Biotechnological and molecular approaches for vanillin production: a review. *Applied Biochemistry and Biotechnology*, 169, pp.1353–1372.

Kim, K.H. *et al.*, 2006. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry*, 95, pp.466–473.

Krings, U. & Berger, R.G., 1998. Biotechnological production of flavours and fragrances. *Applied Microbiology and Biotechnology*, 49, pp.1–8.

Kumar, N. & Pruthi, V., 2014. Potential applications of ferulic acid from natural sources. *Biotechnology Reports*, 4, pp.86–93.

Leffingwell, D.L.J.C., 2015. Flavours & fragrances : Recent advances in biotechnology. *Specialty Chemicals Magazine*, pp.32–34.

Li, K. & Frost, J.W., 1998. Synthesis of vanillin from glucose. *Journal of the American Chemical Society*, 120, pp.10545–10546.

Ma, X.K. & Daugulis, A.J., 2014a. Effect of bioconversion conditions on vanillin production by *Amycolatopsis* sp. ATCC 39116 through an analysis of competing by-product formation. *Bioprocess and Biosystems Engineering*, 37, pp.891–899.

Ma, X.K. & Daugulis, A.J., 2014b. Transformation of ferulic acid to vanillin using a fed-batch solid-liquid two-phase partitioning bioreactor. *Biotechnology Progress*, 30(1), pp.207–214.

Martínez, Á.T. *et al.*, 2005. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*, 8, pp.195–204.

Muheim, Andreas; Müller, Bruno; Münch, Thomas; Wetli, M., 1998. Process for the production of vanillin - EP 0 885 968 B1.

Muheim, A. & Lerch, K., 1999. Towards a high-yield bioconversion of ferulic acid to vanillin. *Applied Microbiology and Biotechnology*, 51, pp.456–461.

Ou, S. & Kwok, K., 2004. Ferulic acid : pharmaceutical functions, preparation and applications in foods. *Journal of the Science of Food and Agriculture*, 84, pp.1261–1269.

Overhage, J. *et al.*, 1999. Biotransformation of eugenol to vanillin by a mutant of *Pseudomonas* sp. strain HR199 constructed by disruption of the vanillin dehydrogenase (*vdh*) gene. *Applied microbiology and biotechnology*, 52, pp.820–828.

Overhage, J., Steinbüchel, A. & Priefert, H., 2006. Harnessing eugenol as a substrate for production of aromatic compounds with recombinant strains of *Amycolatopsis* sp. HR167. *Journal of Biotechnology*, 125, pp.369–376.

Pan, G.X., Thomson, C.I. & Leary, G.J., 2003. The role of ferulic acid removal during bleaching of wheat straw mechanical pulp with peroxygen reagents. *Holzforschung*, 57, pp.282–288.

Pérez-Rodríguez, N. *et al.*, 2016. Ferulic acid transformation into the main vanilla aroma compounds by *Amycolatopsis* sp. ATCC 39116. *Applied Microbiology and Biotechnology*, 100, pp.1677–1689.

Priefert, H., Rabenhorst, J. & Steinbuchel, A., 2001. Biotechnological production of vanillin. *Applied Microbiology and Biotechnology*, 56, pp.296–314.

Ramachandra Rao, S. & Ravishankar, G. a., 2000. Vanilla flavour: production by conventional and biotechnological routes. *Journal of the Science of Food and Agriculture*, 80, pp.289–304.

Rosazza, J.P.N. *et al.*, 1995. Review: Biocatalytic transformations of ferulic acid: An abundant aromatic natural product. *Journal of Industrial Microbiology*, 15, pp.457–471.

S. Achterholt, H. Priefert, A.S., 2000. Identification of *Amycolatopsis* sp. strain HR 167 genes, involved in bioconversion of ferulic acid to vanillin. *Appl. Microbiol. Biotechnol.*, 54, pp.799–807.

Sainsbury, P.D. *et al.*, 2013. Breaking down lignin to high-value chemicals: The conversion of lignocellulose to vanillin in a gene deletion mutant of *Rhodococcus jostii* RHA1. *ACS Chemical Biology*, 8, pp.2151–2156.

Sinha, A.K., Sharma, U.K. & Sharma, N., 2008. A comprehensive review on vanilla flavor: extraction, isolation and quantification of vanillin and others constituents. *International journal of food sciences and nutrition*, 59(4), pp.299–326.

Solvay, Rhovanil® Natural. Available at: http://www.solvay.com/en/markets-and-products/featured-products/rhovanil-natural.html [Accessed January 29, 2017].

Srinivasan, M., Sudheer, A.R. & Menon, V.P., 2007. Ferulic Acid : Therapeutic Potential Through Its Antioxidant Property. *Journal of Clinical Biochemestry and Nutrition*, 40, pp.92–100.

Steinbüchel, H Priefert, S.A.A., 2002. Transformation of the Pseudonocardiaceae *Amycolatopsis* sp . strain HR167 is highly dependent on the physiological state of the cells. *Appl. Microbiol. Biotechnol.*, 58, pp.454–460.

Sutherland, J.B., 1986. Demethylation of Veratrole by Cytochrome P-450 in *Streptomyces setonii*. *Applied and Environmental Microbiology*, 52(1), pp.98–100.

Sutherland, J.B., Crawford, D.L. & Pometto, a L., 1983. Metabolism of cinnamic, p-coumaric, and ferulic acids by *Streptomyces setonii*. *Canadian journal of microbiology*, 29, pp.1253–1257.

Tilay, A. *et al.*, 2008. Preparation of ferulic acid from agricultural wastes: Its improved extraction and purification. *Journal of Agricultural and Food Chemistry*, 56, pp.7644–7648.

U.S. Food and DrugAdministration, U.S. Government Publishing Office. *Title 21: Food and Drugs PART 501—ANIMAL FOOD LABELING. Subpart B—Specific Animal Food Labeling Requirements. Revised as of April 1, 2016.* Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=101.22 [Accessed February 5, 2017].

Uraji, M. *et al.*, 2013. Enzymatic production of ferulic acid from defatted rice bran by using a combination of bacterial enzymes. *Applied Biochemistry and Biotechnology*, 171(5), pp.1085–1093.

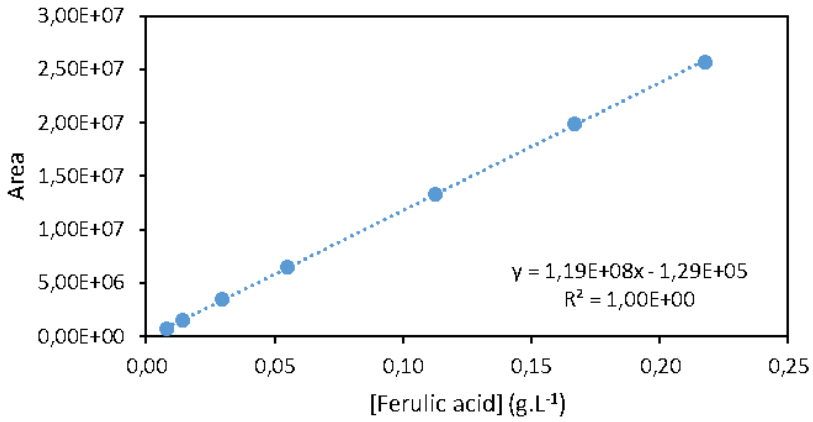
Walton, N.J., Mayer, M.J. & Narbad, A., 2003. Vanillin. *Phytochemistry*, 63, pp.505–515.

Xu, F. *et al.*, 2005. Determination of cell wall ferulic and p-coumaric acids in sugarcane bagasse. *Analytica Chimica Acta*, 552, pp.207–217.

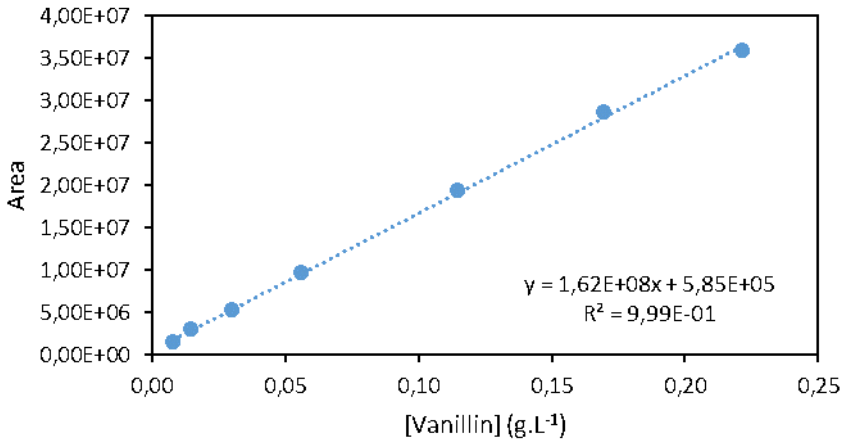
Zhao, Z. & Moghadasian, M.H., 2008. Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review. *Food Chemistry*, 109, pp.691–702.

Zheng, L. *et al.*, 2007. Production of vanillin from waste residue of rice bran oil by *Aspergillus niger* and *Pycnoporus cinnabarinus*. *Bioresource Technology*, 98, pp.1115–1119.

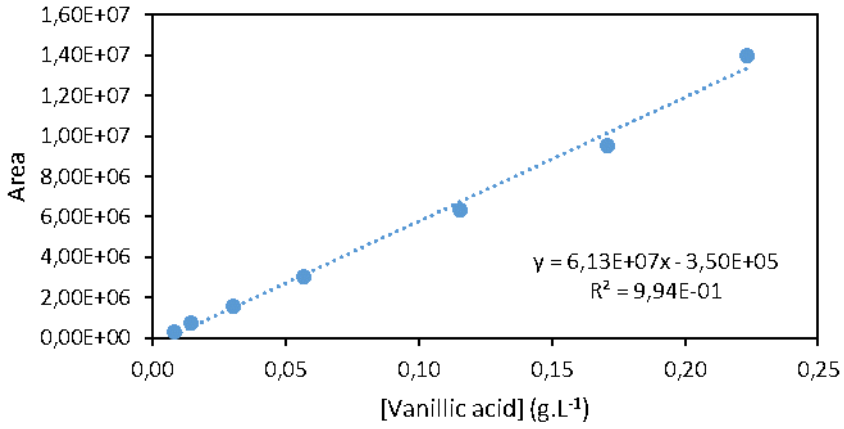
# 6. Appendix



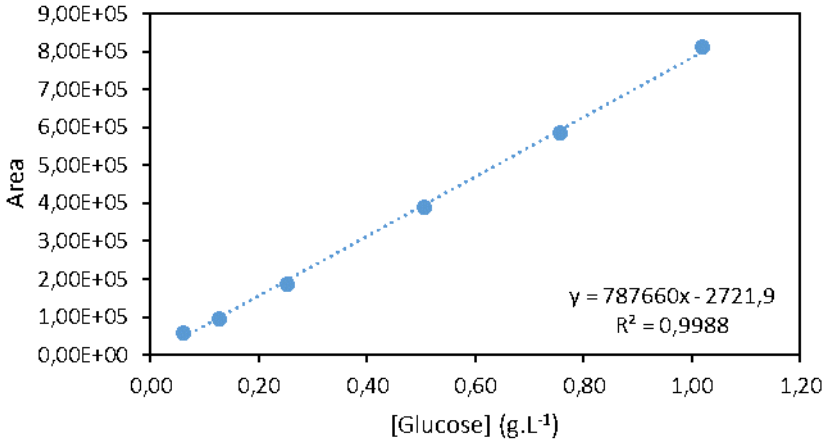

A.1 – Ferulic acid calibration curve.




A.2 – Vanillin calibration curve.




A.3 – Vanillic acid calibration curve.




A.4 – Glucose calibration curve.