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BIOTECHNOLOGICAL PRODUCTION OF VANILLIN USING MICROBIAL CELLS

(CHIM/11)

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Index

Abstract

This PhD research project focused on the optimization of the biotechnological process for vanillin production from ferulic acid using recombinant *Escherichia coli* cells.

Nowadays flavours market covers about one fourth of the global food additives market with a 25 million dollar turnover and a 5.5% growth rate. Flavouring compounds are generally produced by chemical synthesis or extractive methods from natural sources. Flavours produced by chemical synthesis, are classified as “artificial flavours”; also these processes are damaging for environment and cause some problems with unwanted compounds, with reduction of the process efficiency and rise of the product recovery costs. On the other hand, extraction processes from plants are often more expensive because of the low concentrations of the molecules target in the raw material. Moreover cost of aromatic compounds extracted from plants depends on uncontrollable factors such as plant diseases and weather conditions. The drawbacks of both methods and the increasing interest of consumers in natural product (Sinha et al., 2008), reported in recent market surveys, have led a great interest in the exploration of more “eco-friendly” procedures for production of natural flavours.

Vanillin is the major compound responsible for vanilla aroma. It is one of the most commonly used aromatic compounds in drugs and food industry. Since vanillin extracted from cured vanilla beans is very expensive and its availability depends on plantations production, curing process duration and labour costs. Synthetically produced vanillin is the most commonly used in vanilla flavoured products, and covering 99% of the global market. According to the regulation No 1334/2008 of the European Parliament and of the Council, vanillin produced by biotechnology from natural feedstocks can be classified as ‘natural flavoring’, provided that the source is always specified. All these factors make vanillin an important commercial target for biotechnological industry, and bases and applied research.

Since vanillin is an intermediate product of the ferulic acid catabolism (a compound present in significant quantities in the lignocellulosic material) it is possible to confer the ability to convert ferulic acid to vanillin, through metabolic engineering, to strains unable to degrade ferulic acid.

Unfortunately these bioconversion processes are not economically competitive yet; the high chemical activity and toxicity of both the product (vanillin) and the substrate (ferulic acid) cause low yield in the bioconversion process.

With this research project, aiming at develop a competitive bioconversion process for vanillin production, many factors influencing the system productivity and selectivity, have been

optimized. Bioconversion experiments carried out using resting cells of *E. coli* demonstrated that composition and pH of bioconversion buffer affected the formation of vanillin and unwanted products such as vanillyl alcohol. Using moderately alkaline bioconversion buffer (pH 9.0) it has been possible to double the amount of final product, with respect to the systems until now described.

Using the statistical approach of the Response Surface Methodology (RSM) it has been possible to evaluate the synergic effect of ferulic acid concentration and stirring speed on the productivity and selectivity of the bioconversion process. Our results demonstrated that the highest vanillin title obtained incubating cells at 135-165 rpm range and initial ferulic acid concentration of 12-18 mM. Under optimized working conditions, vanillin yield increased from 8.51 ± 0.02 to 11.63 ± 0.1 mM, while, ferulic acid concentration higher than 20 mM cause a drastic decrease in vanillin production.

Finally, to maintain low initial concentration of ferulic acid in the bioconversion buffer a two-phase (solid-liquid) system for the controlled release of the substrate has been developed. Using agarose gel cylinders containing ferulic acid, in conjunction with optimized buffer and nutrient amendments (LB medium), we demonstrated that, compared to previous results (Barghini et al., 2007), it is possible to increase vanillin final title (up 20%); to reduce the bioconversion time from 4 to 1 day; to increase the final vanillin concentration in the liquid phase of 5-fold. The maximum amount of accumulated vanillin in the liquid phase under optimized conditions was 20.57 ± 0.05 mM, the highest found in the literature for recombinant *E. coli* strains.

In conclusion, results obtained demonstrated that vanillin production by *resting* cells of *E. coli* can be increased significantly by acting on several parameters, including the bioconversion buffer formulation and the way to modulate the substrate concentration.

Riassunto

Questo progetto di tesi ha riguardato lo studio e l'ottimizzazione della produzione biotecnologica di vanillina a partire da acido ferulico utilizzando ceppi ricombinanti di *Escherichia coli*.

Attualmente, il mercato degli aromi copre circa un quarto del mercato mondiale degli additivi alimentari, e il suo giro d'affari è stimato in 25 milioni di dollari, con un tasso di crescita annuo del 5.5%. I composti aromatici vengono generalmente prodotti per sintesi chimica o per via estrattiva da matrici naturali. Le sostanze aromatizzanti ottenute per sintesi chimica, anche se a partire da materie prime naturali, sono classificate come "aromi artificiali"; inoltre questo tipo di processi possono essere dannosi per l'ambiente e comportare alcuni problemi come la formazione di composti non desiderati, con conseguente riduzione nell'efficienza del processo e aumento dei costi di recupero del prodotto. D'altra parte i processi di estrazione da matrici naturali sono spesso costosi a causa della bassa concentrazione delle molecole di interesse. In questo caso i maggiori svantaggi sono legati al fattori ambientali e climatici che comportano un prezzo di mercato molto alto. Gli svantaggi dei metodi estrattivi e di sintesi, e l'interesse crescente dei consumatori per prodotti naturali (Sinha et al. 2008), ha portato alla ricerca di strategie ecosostenibili per la produzione di aromi naturali, come la vanillina.

La vanillina è il composto caratterizzante dell'aroma di vaniglia, e rappresenta una delle principali sostanze aromatizzanti impiegate nell'industria alimentare e farmaceutica. La vanillina estratta dai baccelli curati dell'orchidea *Vanilla planifolia* è molto costosa, poiché la sua produzione è limitata dall'areale di diffusione della pianta, da fattori climatici e ambientali che condizionano lo sviluppo della pianta, dall'impollinazione che deve essere effettuata manualmente e dalla durata del processo di cura dei baccelli di vaniglia. Nei prodotti aromatizzati alla vaniglia si utilizza quasi esclusivamente vanillina ottenuta per sintetica chimica, che di fatto ricopre circa il 99% del mercato mondiale. In accordo con il Regolamento Europeo 1334/2008 del Parlamento e del Consiglio, la vanillina prodotta per via biotecnologica a partire da materie prime naturali è classificabile come aroma naturale a condizione che la matrice di partenza venga sempre specificata. Questi fattori rendono la vanillina un target commerciale importante per le industrie biotecnologiche, e per la ricerca di base e applicata.

Poiché la vanillina è un intermedio del catabolismo dell'acido ferulico (composto presente in abbondanza negli scarti lignocellulosici), ed i geni che codificano per gli enzimi responsabili della degradazione dell'acido ferulico sono stati caratterizzati, è possibile conferire la capacità

di convertire l'acido ferulico in vanillina, tramite tecniche di ingegneria metabolica, anche a ceppi che non sono in grado di degradare questo composto.

Purtroppo i processi per la produzione biotecnologica di vanillina non sono ancora competitivi dal punto di vista economico; l'elevata reattività della vanillina e la tossicità sia del prodotto che del substrato (acido ferulico) comportano basse rese e alti costi nel recupero del prodotto, nonché un alto impatto ambientale a una selettività poco elevata, il che può portare alla formazione di prodotti indesiderati la cui presenza può essere incompatibile con l'uso alimentare.

In questo lavoro di tesi è stata valutata la possibilità di utilizzare cellule *resting* di *E. coli* per produrre vanillina a partire da acido ferulico, andando a valutare parametri importanti che influenzano la produttività e la selettività del sistema catalitico. I risultati ottenuti hanno evidenziato che l'efficacia del processo di bioconversione è strettamente collegata alla formulazione e al pH del mezzo di bioconversione. Utilizzando tamponi di bioconversione moderatamente alcalini (pH 9.0) è possibile ottenere un incremento di due volte nella quantità di prodotto finale rispetto ai sistemi fino ad oggi descritti.

Inoltre, utilizzando l'approccio statistico della Superficie di Risposta (Response Surfece Methodology, RSM) è stato possibile valutare l'effetto sinergico della concentrazione iniziale di acido ferulico e della velocità di agitazione sulla resa molare e la selettività del sistema catalitico. Tali prove hanno dimostrato che entrambe le variabili influenzano il processo e che i risultati migliori sono ottenuti fissando la velocità di agitazione tra 135-165 rpm e la concentrazione di acido ferulico tra 12-18 mM. Nelle condizioni ottimali individuate nel presente lavoro la vanillina prodotta con cellule *resting* di *E. coli* FR13 è aumentata da 8.51 ± 0.02 a 11.63 ± 0.1 mM. Concentrazioni iniziali di acido ferulico superiori a 20 mM causano un drastico decremento nella quantità massima di vanillina.

Infine, per controllare la concentrazione di acido ferulico nel mezzo di bioconversione è stato sviluppato un sistema a due fasi (solido-liquido) per il rilascio controllato del substrato nel mezzo di bioconversione. Utilizzando acido ferulico intrappolato all'interno di cilindri di agarosio è stato possibile, rispetto a quanto ottenuto in studi precedenti (Barghini et al., 2007), incrementare la quantità massima di vanillina prodotta del 20%; ridurre la durata del processo da 4 ad un giorno; aumentare la concentrazione di vanillina nel mezzo liquido di cinque volte, fino a 20.57 mM , la quantità più alta attualmente ottenuta in letteratura sfruttando cellule *resting* di *E. coli*.

In conclusione i dati mostrati indicano che la produzione di vanillina mediante cellule *resting* di *E. coli* può essere incrementata in modo significativo agendo su più parametri di processo,

inclusi la formulazione del mezzo di bioconversione e la modalità di somministrazione del substrato.

1. Introduction

1.1 The history of food flavourings

Flavourings are a major category of ingredients intentionally added to food. Their history begins when people discovered that components characteristics of the aroma of natural products could be enriched by simple methods. Today's food flavouring industry has continually evolved due to the ever expanding knowledge of natural raw materials, gastronomy, cooking techniques and procedures adopted from the perfumery industry. Progress made by the chemical industry during the industrial revolution has also played a crucial role.

Throughout history, men have sought to make their food more appetizing since well before recorded history, first by using spices and herbs and then by the spirits of fruits and aromatic plants or essential oils, but the flavour industry has developed only after the past 170 years from small beginnings in companies specializing in the processing and marketing of natural botanicals such as the herbs and spices, vanilla beans, vegetables drugs and the distillation of essential oils and aromatic essences, the isolation of aromatic chemicals from these products and drug extraction.

The known utilization of spices and herbs extends over some 5000 years, starting with the ancient Egyptian when people used perfumed balms in religious ceremonies. The Egyptians were the first to embrace odoriferous raw materials, creating elaborate perfumes with mastic, juniper, myrrh and cypress as ingredients to scent atmosphere during rituals, while rose and peppermint were stepped in oils to create an unguent. They also seasoned and flavoured their dishes.

Theophrastus, a Greek philosopher, reported the use of artificial flavourings in foods in the 3rd century BC. In the 2nd century AD the respected physician, Galen, warned against the adulteration of herbs and spices. Sodium chloride or common salt has been added to foods as a preservative for thousands of years. Spices like nutmeg and cinnamon, used to flavour foods, have been sort after and traded throughout history. The trade routes between Asia, the Middle East and Europe flourished as the demand for these additives grew.

Jews, according to the Old Testament, ate grilled paschal lamb flavoured with bitter herbs before crossing the Red Sea.

The Romans also made great strides, adding strong and elaborate sauces to their meat dishes containing such delicacies as, *garum* and various sweet and sour sauces. Roman wealthy citizen were kidnapped for ransom, not of gold or jewels, but exotic spices.

New found skills contributed to the evolution of flavouring in daily life; for example, the crusaders from the Near East introduced extraction techniques such as distillation for aromatic and odoriferous substances. Many essential oils currently used by perfumers and flavourists were originally prepared by distillation in pharmacies in the 13th and 14th centuries. Systematic development began in the 13th century, when pharmacies started to prepare so-called remedy oils and later recorded the properties and physiological effects of these oils in pharmacopeia.

In the medieval age, mostly monks were pioneers in the art of capturing natural essences and transforming them in food flavouring substances.

Soon after, exotic ingredients such as spices and aromatic herbs came to the fore, enriching the variety of raw materials at our disposal. The aromatic ingredients coming from the Asian continent were shipped by boats circumnavigating the African continent, following the sea route discovered in 1497 by Vasco de Gama. America came to the world's attention when Christopher Columbus sailed off in 1492 to seek a faster route to Far Eastern spice sources.

During the 16th century, alcohol (ethyl alcohol) was increasingly used to manufacture aromatic extracts of spices and herbs. This period also saw the appearance of essential oils such as those derived from citrus fruits.

In the 1800s, German and Swiss businesses were the first to expand the flavour market significantly, through the development of synthetic aromatic chemicals that served as the basis for many new artificial flavours. Most of these early commercially produced flavouring substances were derived from or based on substances recurring naturally.

Then, in the 19th century, industrialisation and urbanisation changed the living conditions, nutritional habits and food requirements of the world's population. The advances in organic chemistry knowledge permitted to produce synthetic flavouring products that were used in place of certain hard-to-find or expensive ingredients.

It was not until the latter half of the 19th century that chemicals began to realize the flavouring possibilities of synthetic aromatic chemicals. The foundation of the modern flavour industry was established in 1843 with the synthesis of methyl salicylate (methyl 2-hydroxybenzoate), followed by cinnamic aldehyde ((2*E*)-3-phenylprop-2-enal) in 1856, and benzaldehyde in 1863. In 1869, the first book of artificial flavouring formulations was published anonymously in Philadelphia. The synthesis of vanillin, the key ingredient in flavour creativity, in 1872 was the explosion for the flavour industry.

Vanillin was cultivated for the first time by the ancient Totonac Indians of south-eastern Mexico. They used it in rituals, as a medicine and as a perfume. Interestingly enough, they

didn’t use it for flavouring. It was adopted by the Aztecs after the Totonacs were conquered in the 15th century. The Aztecs mixed it with chocolate to make their tasty drink chocolaty. When the Spaniards conquered Mexico, Cortez brought vanilla and cocoa back to Europe where only rich people could afford them. It wasn’t until 1602, that vanilla was actually used as a flavouring.

At the beginning of the 20th century, a growing number of food and beverage companies created more and more demand for commercial flavours.

These revolutionary advancements led to far-reaching changes in the field of food production. Food craft grew and manufacturing at an industrial level gained momentum. Tins for preserving fruit and vegetables were developed and the term “convenience food” made its humble beginnings.

Our diets also became more diverse. Tasty and sophisticated foods were no longer the privilege of the rich. Everyone, rich and poor alike, had access to tasty, industrially produced foods. In those years scientists or business people founded the first flavour and fragrance companies. Many of them still exist, either as such or as the nucleus of larger firms that changed during the decades. This industry has developed into a very profitable market. It includes food and beverage, cosmetics, household products and fragrance industries companies. Today the industry is dominated by several very large multinational flavour and fragrance companies mainly prime in natural products and/or synthetic chemicals which are used in the compounding of an almost limitless range of flavourings and fragrance products.

Figure 1.1 Egyptian ancient evidences of the use of fragrances and flavours

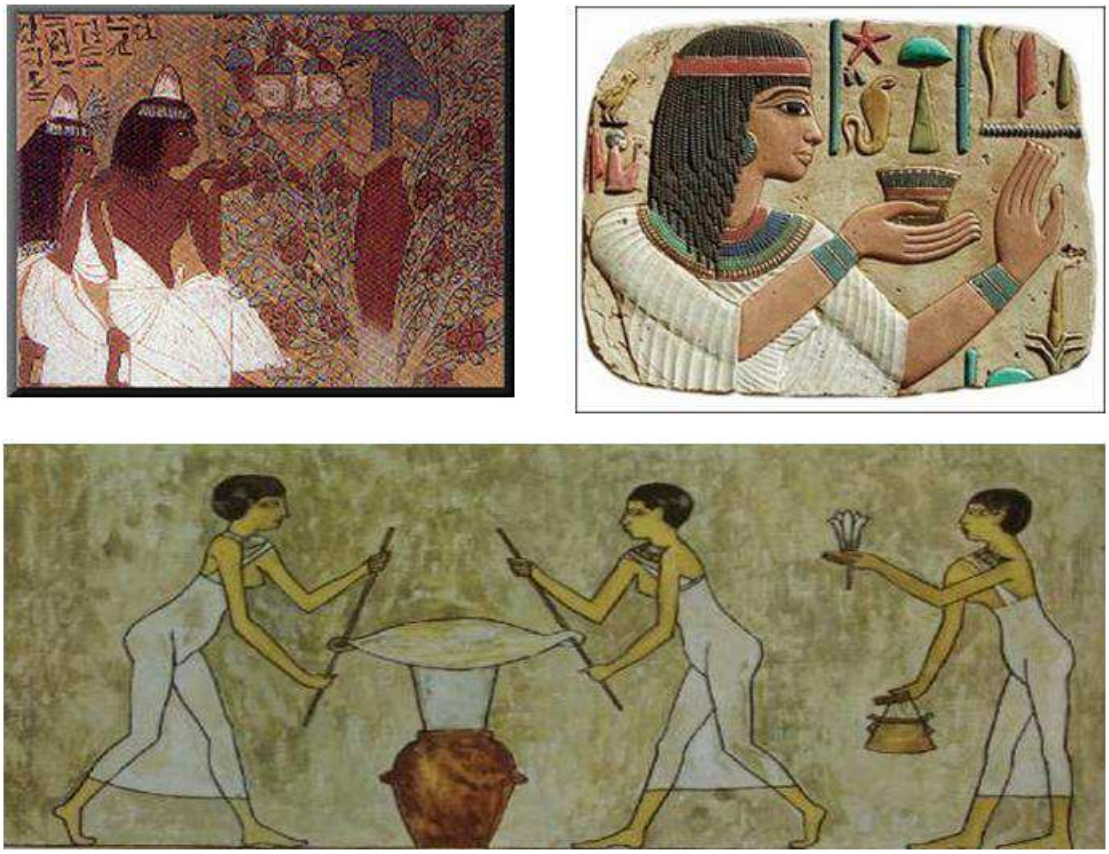


Figure 1.2 Drawing of Vanilla from the Florentine Codex (ca. 1580) and description of its use and properties written in the Nahuatl language

1.2 Food flavours market

Food flavouring, liquid extracts, essences, and flavours added to foods to enhance their taste and aroma, are used to import a pleasant test and/or appealing to foods and beverages mainly, but not only; they are also used in pets food, human care products, drugs, tobacco, etc. Flavours may be composed by individual molecules; more often they consist of a mixture of hundreds of compounds.

The global flavour market is divided into two broad categories: natural and synthetic aromas. The first is composed of essential oils (these are vegetable products obtained especially by distillation), natural extracts (these are substantially obtained by extraction from plants and flowers) and natural aromatic substance (these aromas are isolated from natural sources different from the original). Synthetic flavourings are those molecules produced by chemical synthesis, already existing in nature or created ex-novo in the lab.

The worldwide flavor and fragrance market was worth an estimated $21.8 billion in 2011. This market is projected to exceed $30 billion in 2017, a compound annual growth rate (CAGR) of 5.6% between 2012 and 2017 (BCC Research). This means 25 % of the total food additives market. The market shares between the flavour and the fragrance parts are almost equal. About 10,000 volatiles have been mentioned in food products that are representative of the complexity of the investigations in this field. 2000 synthetic flavors are available on the market and about 400 natural flavors. Beverages and cooked products represent around 70% of the total use of flavor additives. Up to 90% of natural flavour are used in beverages and only 20% in sweets and candies.

The flavor and fragrance industry's total demand for ingredients was an estimated $7.6 billion in 2011 and growing at a projected CAGR of 5.7%. At this rate, the market should reach $10.7 billion by 2017. The major markets are Europe, Africa, and Middle East region (36%) and North America (32%), followed by Asia-Pacific (26%) and South America (6%). In the Asia–Pacific regions, it will reach 6.480 billion of US dollars in 2014 with a rate of 5.3% per year during 2009-2014. The fastest growth will be registered in China and India where the account cover one-third of total value gains. In this way Asia-Pacific regions will become the second largest consumer of flavours and fragrances after North America where only the USA cover a quarter of the global demand. In Central and South America, in Africa/Mideast regions and in Eastern Europe the demand growth will be higher than the global average.

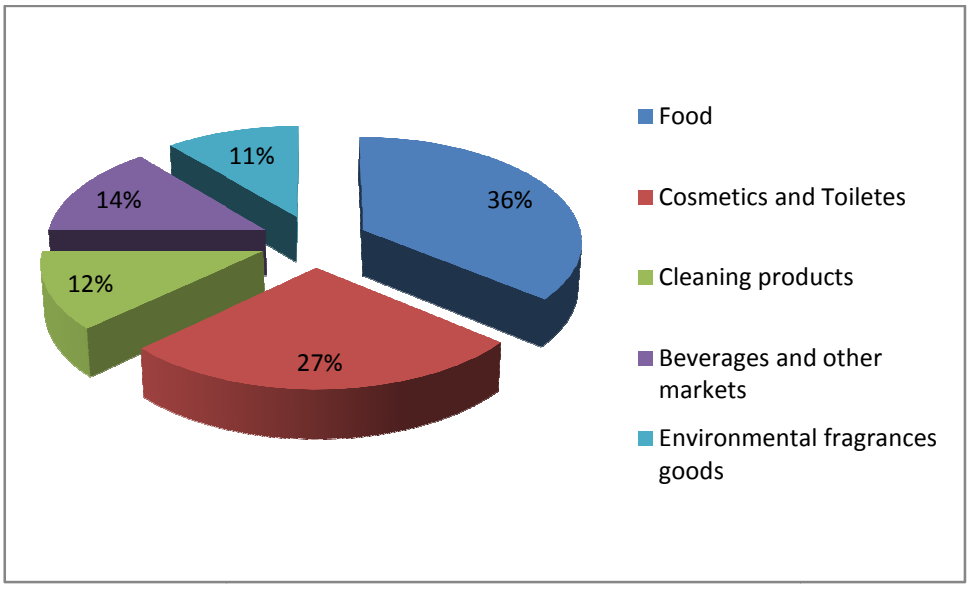
Flavour and fragrances industrial structure is characterized by a number of giants. It is estimated that a dozen companies cover two-thirds of world flavourings demand. The two

largest flavour and fragrance companies are Givaudan (Swiss company established in 1991) and International Flavors & Fragrances (IFF, American company established in 1958), followed by Firmenich (Swiss company founded in 1885) and Symrise (German company, founded in 2003), Quest International (founded in 1987 by the merger of two companies), Takasago (most Japanese manufacturer of flavors and fragrances), T. Hasegawa (Japanese company founded in 1903), Sensient Technologies (American company established in 1882), Danisco (Danish company), and Mane Fils (French company founded in 1926) . The top two companies have a turnover in excess of $2 billion, the next three companies have a turnover in excess of $1 billion each (Givaudan source). The rest of the market is covered by smaller producers, specialized in the production of individual commodities.

In the food industry, flavours are often integral part of the product's market success or failure, in fact they have traditionally been used to drive sales, and indeed today's more sophisticated consumers actively look for new flavour experiences from the products they buy. The final taste of a product is determined after several formulation revisions. Only then the product will be launched for consumer testing, test market or to some other form of organized consumer use study. Flavour formulas are simple or complex mixtures of many compounds. Scientists consider many factors for the creation of a flavour. They must know the physical form of the flavour that is specific to the complete formulation of the end-product, the availability of raw materials, the processing technologies, the classification requirements and legislation restrictions.

Food and beverage manufacturers use flavours to change or specify a product taste but also because they can provide several benefits to the end-product. A flavour can often reduce the cost of a product by giving developers the option of replacing more expensive ingredients. In this case a small amount of ingredient can be used for label purposes, whereas natural flavour can be added back to create the fuller taste profile of the product.

Figure 1.3 World Flavours and Fragrances Market (2009-2014)



Source Freedonia group http:www.freedoniagroup.com

Table 1.1 Food Flavour demand (1997-2010)

FOOD: FLAVOUR DEMAND (million dollars) 1997 – 2017

Item	1997	2002	2007	2012	2017
Food Shipments (bil $)	398.1	433.1	547.1	628.0	719.0
$ flavors/000$ food	2.9	3.0	2.9	3.1	3.2
Food Flavor Demand	1160	1307	1612	1925	2280
Processed Food	402	475	560	655	770
Bakery Products	216	233	278	315	345
Dairy Products	196	212	277	340	420
Candy & Confectioneries	181	197	245	305	375
Other	165	190	252	310	370
% food	37.4	36.5	36.6	36.5	36.2
Total Flavor & Fragrance Demand	3100	3580	4400	5270	6300

Freedonia group http:www.freedoniagroup.com

1.2.1 Vanilla market

Vanilla remains the world's most popular flavour, but supply issues are constant challenge for an ingredient mainly sourced from Madagascar. Vanillin was one of the very first artificial flavors created. Vanillin was first isolated as a relatively pure substance in 1858 by Nicolas-Theodore Gobley, who obtained it by evaporating a vanilla extract to dryness, and recrystallizing the resulting solids from hot water. In 1874, the German scientists Ferdinand Tiemann and Wilhelm Haarmann deduced its chemical structure, at the same time finding a synthesis for vanillin from coniferin, a glycoside of isoeugenol found in pine bark.

There are three products on the market from different background: the "pod" of vanillin, the real one: the best known, but not the most used; the products of natural vanillin, but not coming from the original pod (rice bran, lignin in the wood) and the Vanilla Products, made from the chemical synthesis of vanillin.

About 60% of the vanillin goes into food and beverage, 33% into perfumes and cosmetics and 7% into pharmaceuticals. The price of natural vanillin extracted from vanilla is estimated to be between $1200 and $4000 per kilogram. Natural vanillin derived from microbial production has a price of about $1000 per kilogram. Synthetic vanillin cost about $11-15 per kilogram (Berger, 2007).

While the natural trend is on the rise, the food ingredients industry will never be able to meet demand for a flavor that is used in anything from bakery and dairy to confectionery and beverages. The trendy concept of sustainability is also somewhat of a paradox for an ingredient that requires significant transportation costs and an intense extraction process from vanilla pods prior to being used; 1 kg of vanillin requires approximately 500 kg of vanilla pods and hand pollination of approximately 40,000 flowers. The annual world market for naturally-sourced vanillin is approximately $240 million; an estimated 200,000 people are involved in the production of about 2,000-3,000 metric tons of cured vanilla beans. This of course leads to a high carbon footprint. To cope with the demand for vanilla flavor, the aroma chemical vanillin absolutely defeats natural vanilla in terms of usage.

The global market for vanillin is estimated to be between 15-16,000 tons per year. The synthetic vanillin market saw total industry-wide price increases of between 23% and 25% from early 2005 up to present. Vanillin contained in vanilla beans represents just 50 tons a year around 0.5% of global vanillin demand. Global demand for synthetic vanillin is reportedly increasing 3% to 4% per year. Vanilla beans are clearly not able to cover the

world's demand for vanillin. For example, with beans only, the amount of accessible and affordable ice-cream and chocolate would be vastly reduced.

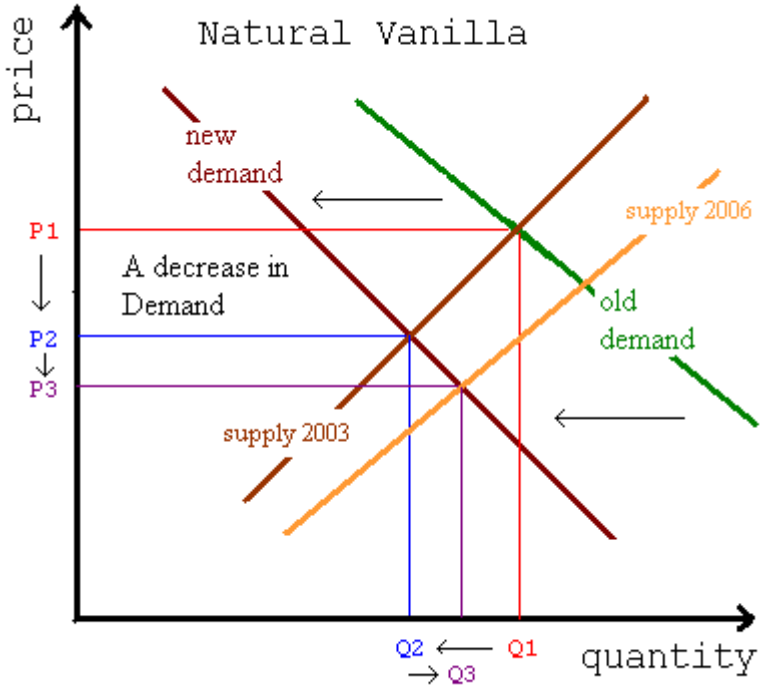
Growth demand in China, however, is in excess of 10% per year estimated at 2,200 tons. There are between 50 and 100 million new Chinese consumers for vanillin each year, especially in applications such as in food and personal care formulated with vanilla flavors. China accounts for 40 percent of the global vanillin supply although several Chinese producers have already exited the vanillin market, driven by skyrocketing benzene cost and growing environmental and health concern associated with the manufacture of vanillin that uses the o-nitrochlorobenzene (ONCB, the main industrial method for synthesizing vanillin) route (Rhodia source).

From an ecological and environmental point of view the flavour and fragrance industry's weak points are emissions, the use of chemicals and chemical reactions and the production of wastewater. Moreover the use of flavours for foods and beverages is not so easily understood by a certain part of the population. Companies have started sustainable development activities as measurable reduction of energy and emissions. Another important point is the search for sustainable raw materials.

The price difference between vanillin from beans and vanillin from other sources is probably in the range of the factor 50, and in recent years, a strong upsurge occurred in the demand for natural products, including natural flavors. This has created a number of opportunities for the employment of new biotechnological processes for the production of flavours. Biocatalysis are useful to catalyse a wide range of stereo and region-selective chemical reactions that are not easily carried out with the less selective classical synthetic procedures. Many microbial processes to produce flavours have been described, but their application in the industrial field is limited because of the low yield obtained. However the high costs for down-stream processing for the recovery of the microbial flavours in low concentration from fermentations broths could be compensated by the fact that the market price of natural aromas is 1:100 times higher than that of synthetic aromas. The price of microbial flavours has to range between 200 and 2000 US $/kg to be competitive (Berger, 2009).

Problems related to the application of the biotechnology on a large scale make the industrial applications difficult. An important challenge for researchers in the biotechnological production of flavours is the development of specific fermentation techniques and recovery methods.

Figure 1.4 Supply and demand in natural vanilla market



Vanilla pods prices dropped from over US $300 per kilogram in 2003 to less than $50 in 2005 as a result of adverse climatic conditions and the use of alternative sources for the production of vanilla aroma.

1.3 Vanilla flavour: production and applications

Vanilla is an odoriferous extract prepared from the processed pod of the *Vanilla planifolia* orchid. Originally harvested in Mexico, vanilla is now grown in various tropical countries such as Madagascar, Indonesia, Uganda, Comoro, Tahiti and India. Each of these growth sites yields vanilla with different flavour characteristics. Madagascar produces more than half of the world production of vanilla beans (1000 – 1200 tons). Indonesia is the second largest producer with some 350 tons. Due to some plant diseases and strong international competition, including that from new production regions, Indonesian production has considerably decreased in the past few years, but the quality has increased (Berger, 2007).

Since it originates from a plant, vanilla consists of a complex assortment of organic, or carbon-based, compounds. Around 85% of the volatiles are vanillin and around 130 different chemical compounds that contribute to the nuances of the vanilla flavour have been identified in the fermented fruit (phenols, phenol ether, alcohols, carbonyl compounds, acids, ester, lactones, aliphatic and aromatic carbon hydrates and heterocyclic compounds). Vanilla additionally contains 25% sugar, 15% fat, 15 to 30 % cellulose and 6% minerals. Water content is unusually high (35%).

Vanilla extract is a complex prepared by an alcohol – water or any other permitted solvent system from cured vanilla beans and other naturals; its cost depends on its concentration and vanillin amount and varies from US $30 to 100 per kilogram and the supercritical CO_2 extract are even higher priced. Various methods of extraction have been used to elicit the flavour from the cured bean. The concentration of an extract is noted by its ‘fold’; a single fold of vanilla extract contains 100g of extractable material per litre. Vanilla flavour is generally obtained through two main extraction methods: percolation and oleoresin method.

The percolation method consists of circulating a solvent, which is an ethanol/water solution in the range 35-50:65-50 (v/v), over and through the beans under vacuum. This process may take between 48 and 72 hours. By using this process, an approximately four fold strength vanillin can be obtained.

The oleoresin method consists of pulverising whole beans and then circulating ethanol over beans under vacuum, at about 45°C. The excess alcohol is removed by evaporation. This process takes about 8-9 days. However, by using the oleoresin process, an approximately 10-fold strength vanillin may be obtained. Post extraction processing involves clarification by centrifugation or filtration followed by aging of the extract for 1 year.

Concentrated vanilla extract (or flavour) is made by removing some of the solvent usually by vacuum distillation until the desired concentration or 'fold' is reached. Each fold must correspond to an original 380g of beans in the starting extract before concentration, so a two-fold would have the extractable of 760g of beans. Higher folds such as 10x or 20x are made by diluting oleoresins, which do not contain solvents. Distillation destroys some of the aromatic substances of vanilla flavour.

Many variables influence the flavour characteristics of vanilla extract, including country of origin, crop year, curing techniques, storage conditions, lots, extraction method and manufacturer. For many years, flavour profiles of vanillin were described by their origin. Today, profiles within the origins are changing. Vanilla preference is subjective. Vanilla users therefore, prefer to specify flavour profile they want rather than origin. Basic flavour characters used to describe vanilla are vanillin, resinous/leathery, woody pruney, fruity, chocolate, smoky and bourbon/rummy. A bourbon vanilla is marked by moderate bourbon/rummy notes, slight to moderate resin and slight vanillin, woody, pruney. A low quality vanilla extract is moderately smoky, woody and leathery, with very slight vanillin and bourbon/rummy notes.

The vanilla flavour used by the food industry especially by the ice cream industry largely takes into account the entire vanilla profile, as ice cream retains all flavour characteristics. Extraction technique greatly affects finished vanilla profile. Other factors governing the vanilla extract profile are the vanilla species, origin, bean quality and curing, extraction and concentration of additional flavours.

According to industry reports, vanilla produced from Madagascar (Bourbon) beans has been considered the industry's gold standard, but the quality of beans from the area has declined over the last two decades due to political and economic instability. Vanillin levels in Madagascar beans have decreased up to 40% in some cases. On the other hand, according to the sources, Indonesian vanilla quality has improved greatly over the last 20 years and the vanillin levels have equalled or surpassed that of Madagascar beans.

Vanilla beans can be used in their whole or ground form; however, they are most commonly used for producing extracts, flavours, oleoresins and powders.

Most vanilla is used in the food industry and dairy products, followed by beverage. The type of vanilla used depends on the product, the ingredients in the base formulation and the desired flavour profile. The main application of natural vanilla is for flavouring ice cream and soft drinks. It is estimated that nearly 300 m^2 of vanilla beans are used in USA every year in the preparation of cola type drinks, in addition to the complex of spice and citrus notes. Cream

sodas, root beer and some fruit beverages also contain vanilla. In whiskey products vanillin is one of the chemicals extracted from the oak barrels in which the product age.

Vanilla is often used to enhance fruit flavours in many dairy and beverage applications. it is generally used as a background note in a variety of sweet and fruit flavours to round out the flavour profile. It also enhances the sweetness perception of foods, especially in bakery products, at the same time it reduces burning and biting sensation developing due to bitterness in some food products.

Pure vanilla extract is generally not used for baking because the aromatic components of extracts begin to volatilize at about 140 °C, a temperature that is readily attained in cookie baking. Cakes rarely exceed 100°C internally, so an extract or blends of extracts are used successfully. Vanilla-vanillin extracts (is a vanilla extract to which one of vanillin has been added for every fold of vanilla extract) and artificial flavourings are generally recommended for baking applications. Natural and/or artificial flavours give food product designers the added benefit of blending vanilla with various flavour notes such as buttery, nutty and brown sugar.

The potential use of vanilla extract in savoury applications is limited only by the developer's creativity. Vanilla is an exotic, complex flavour that is liked throughout the world. Food product designer have come up with several tastes from appetizers to desserts that incorporate vanilla extract and are continually discovering new uses for all ingredients.

Figure 1.5 Longitudinal section of a vanilla flower

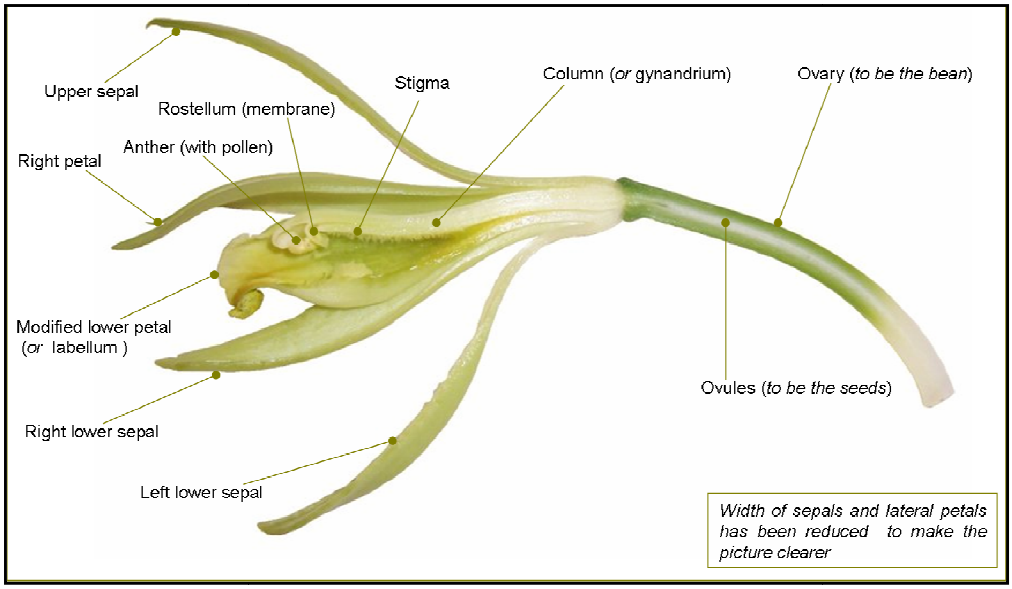


Figure 1.6 A: fresh vanilla pods; B: cured vanilla pods

Table 1.2 Parameters of laboratory curing processes under traditional Indonesian conditions

Stage	Temperature (°C)	Relative humidity (%)	Time
Scalding (killing)	70		1,5 min
Autoclaving	60	95	3 h
	55	95	3 h
	50	95	3 h
	45	95	3 h
Sunning/sweating	40	70	1 h
	47.5	62.5	3 h
	55	55	2 h
	50	95	6 h
	42.5	95	12 h
Slow drying	30	80	3 weeks

Figure 1.7 Vanilla producers countries

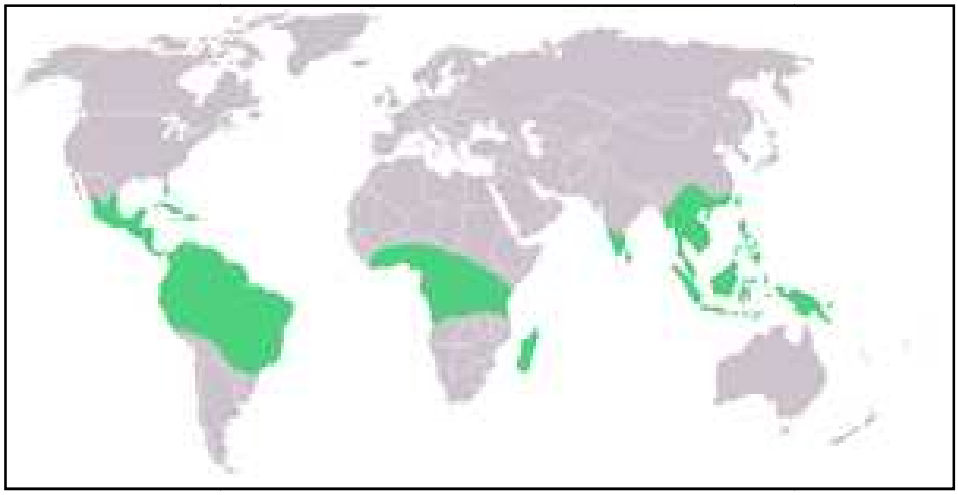


Table 1.3 Major components of cured vanilla pods

Component	g/Kg (dry weight basis)
Vanillin	20
Vanillic acid	1
p-Hydroxybenzaldehyde	2
p-Hydroxybenzyl methyl ether	0.2
Sugars	250
Fats	150
Cellulose	150-300
Minerals	60
Water	350

Table 1.4 Natural vanilla extract; the table reports the ratio values quantity of the major component in vanilla flavour

Vanilla flavour components	
Vanillin/*p*-hydroxybenzaldehyde	10-20
Vanillin/*p*-hydroxy benzoic acid	40-110
Vanillin/Vanillic acid	12-29
p-hydroxy benzoic acid/ *p*-hydroxy benzaldehyde	0.15-0.35
Vanillic acid/*p*-hydroxy benzaldehyde	0.53-1.50

Source: "Note d'information" N° 2003-61 (June 16th, 2003) Vanillin content should be 1.6-2.4% (ISO 5565-2, 1999)

1.4 Vanillin production

Vanillin is the most important flavour compound in vanilla aroma, and is often used to replace the extract. Vanilla extract and vanillin are very versatile flavours, at any concentration they are acceptable, and most people enjoy the flavour, making it the world's most popular flavour. Its odour threshold for humans is 11.8 x 10^{-14} M (Buccellato, 2005). It has the unique characteristic that even at very high dose the flavour is still pleasant. In addition to being an important flavor molecule, vanillin is valued also for other properties, including anti-oxidant, antimicrobial, and anti-inflammatory properties. The antimicrobial effects on the fungi *Aspargillus flavus*, *A. niger*, *A. ochraeus* and *A. parasiticus* and the bacteria *Escherichia coli*, *Bacillus subtilus* and *Staphylococcus aureus* were reviewed by Tipparaju et al. in 2004. Thanks to this property it can be used as food preservative for a wide variety of products like diary products, soft drinks and fruit juices (Walton et al., 2003; Fitzgerald et al., 2004).
Vanillin can be obtained from vanilla beans, after an elaborate processing which takes about six months (the exact reactions during curing are not know, but apparently enzymes play an important role in flavour formation), but because of the high costs of the beans, various other production methods have been developed. These include microbial production and genetic engineering of microorganisms and plants.

Figure 1.8 Vanillin molecule

1.4.1Synthesis of vanillin

Natural vanilla is a complex mixture of flavour components, and the major constituent of this aroma is vanillin. The limited supply and high price of the natural flavour compound increased the production of synthetic vanillin, which currently satisfies 99% of the world

demand for vanilla flavouring; it is a major intermediate in the production of various chemicals, including medicines and herbicides.

Basically, economic reasons lead up to the use of artificial aroma, as chemical synthesis is the cheapest production method. The artificial vanillin is in all its properties perfectly equal to the natural vanillin contained in the vanilla bean (isotope ratios of hydrogen (D/H) and carbon $^{13}C/^{12}C$ isotopes are used to determine the source of vanillin), but aldehyde made in this way cannot be labelled as natural.

The first chemical process to obtain vanillin was developed in 1872 starting from coniferin; this process was developed by Haarmann and Tiemann, and is not cheap because the yield is about 350 g/Kg of coniferin. Coniferin, the glucoside of coniferyl alcohol was obtained from fir or pine trees; after it was oxidized in the presence of a mixture of potassium dichromate and sulphuric acid to obtain vanillin (Rao & Ravishankar, 2000a).

The Remier-Tiemann reaction is also used to produce another form of vanillin called ethyl vanillin. Ethyl vanillin is the ethyl ether of 4-hydroxybenzoic acid, 4-hydroxy-3-ethoxybenzaldehyde ($(CH_3CH_2O)(OH)C_6H_3CHO$). It is a close chemical relative of natural vanillin in which the methyl ($-CH_3$) group of natural vanillin is replaced by an ethyl ($-CH_2CH_3$) group. Ethyl vanillin is also known as artificial vanilla or synthetic vanilla. Its flavor is about three times as strong as that of methyl vanillin and is used to fortify or replace natural and lignin vanillin.

Actually, vanillin can be obtained by other chemical cheaper methods using the starting materials eugenol, lignin and guaiacol.

Preparation of vanillin from eugenol is a process developed in 1876 by Tiemann. Eugenol is a member of the phenylpropanoids class of chemical compounds. Currently there are three methods to prepare vanillin from eugenol two of which pass through the isomerization to isoeugenol.

Vanillin can also be obtained by decomposition of sulphite liquor from the cellulose industry. These mother liquors are rich in lignin which is degraded with either sodium or calcium hydroxide solution and simultaneously oxidized in air in the presence of a catalyst. This process require very alkaline pH, high temperature and pressures, making the system not ecofriendly. When the reaction is complete, the solid wastes are removed and vanillin is extracted from the acidified solution with a solvent (Rao & Ravishankar, 2000a).

Condensation of guaiacol with glyoxylic acid followed by oxidation of the resulting mandelic acid to the corresponding phenylglyoxylic acid and, finally, decarboxylation continues to be a competitive industrial process for vanillin synthesis (Rao & Ravishankar, 2000a). At the end

of the process, compared with lignin-derived production, the vanillin obtained is nearly absent of by-products, simplifying purification and reducing the environmental impact.

Today, a small amount of synthetic vanillin is made from lignin wastes, most of the synthetic vanillin is made from guaiacol, which is a petrochemical precursor. From a scientific point of view, the origin of a chemical cannot affect its bioactivity; however, food laws world-wide reflect the 'natural/artificial discrimination made by consumers (Berger, 2009).

Figure 1.9 Production of vanillin via eugenol

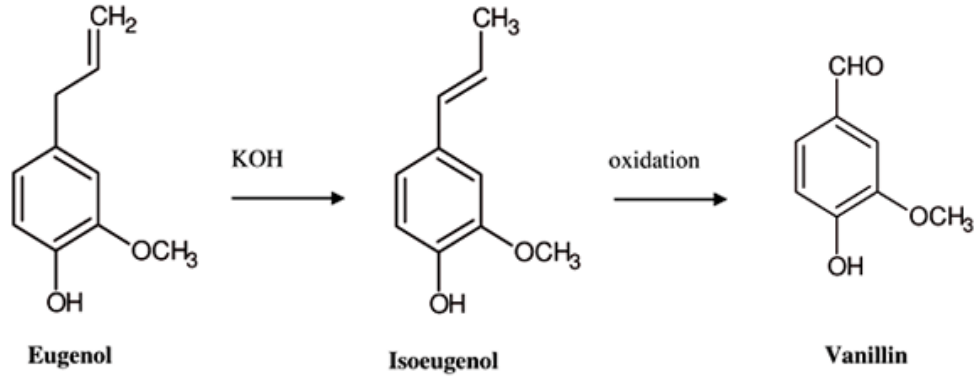


Figure 1.10 Vanillin production by waste sulphite liquor

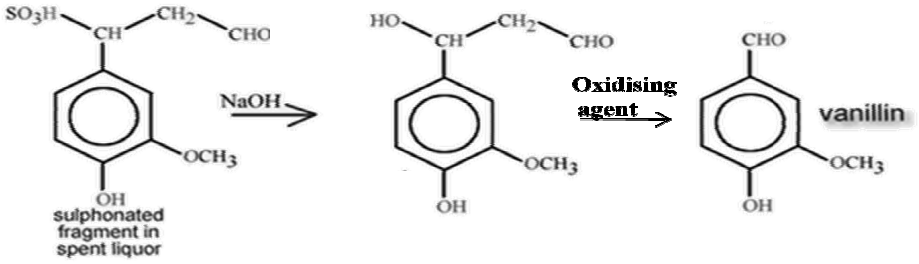
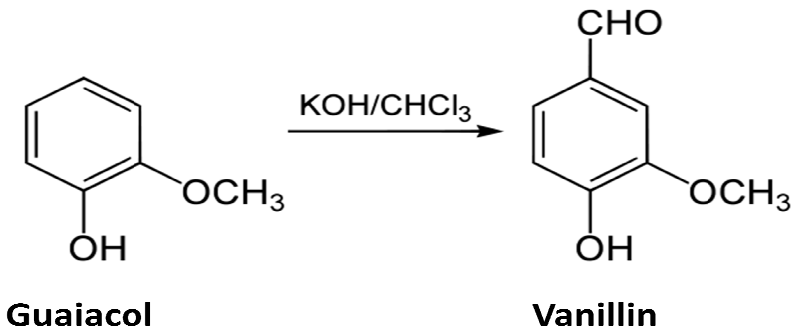


Figure 1.11 Production of vanillin from guaiacol



1.4.2 Biotechnological production of vanillin

The development of novel biocatalytic methods is a continuously growing area of chemistry, microbiology, and genetic engineering, and novel microorganisms and/or their enzymes are the subject of intensive screening due to the fact that biocatalytic reactions can be carried out under certain safety, health, environmental, and economical conditions (Borges at al., 2009).
Today, the main research is a mixture of scientific and economic considerations; for example some valuable flavours occur in traces in their plant sources rendering classical extraction impossible; than chemosynthesis offends the consumers superstition that 'natural' compounds are safer (Berger, 2009), while biotechnological process preferably accept natural, that is renewable substrates, and convert them to products in environmentally favorable processes. The advantages of biocatalysis over chemical catalysis are that these reactions are stereoselective and regioselective, and can be carried out at ambient temperature and atmospheric pressure (Borges at al., 2009).
However, the number and diversity of applications are still modest considering the great availability of useful microorganisms and the broad scope of reactions which they can trigger. Biotechnology are also an important route to appraise natural feedstock and food waste, allowing the conversion of low value compounds in products of great commercial interest.
As regards the biotechnological vanillin production several applications are attracting interest in recent years, mainly given the competitive cost of bio-vanillin compared to that obtained from natural source, and the growing consumers demand for natural flavours.
Natural vanillin can be obtained from cell or tissue culture for bioconversion of natural precursors to vanillin. Vanillin can be obtained from lignin by enzymatic degradation or using microbial or fungal cultures able to bioconvert, naturally or after genetic engineering, several substrates to vanillin.

Use of enzymes

The exact reactions during Vanilla beans curing are not known. It is known that curing process of green bean is needed to release the aglycons to set free aroma compounds and supposedly enzymes play an important role in flavour formation; β-glucosidase, which catalyzes the hydrolysis of vanillin from glucovanillin, being the most important enzyme (Dignum et al., 2001).

There are two main reasons to choose enzymes for bioconversion purpose. Firstly, these enzymes are generally able to catalyze the reactions stereospecifically resulting in chirally pure products, and secondly, they can perform regiospecific modifications that are not easily carried out by chemical synthesis or by microorganisms (Pras et al., 1995).

For this reasons several authors described the use of enzymatic preparations containing β-glucosidase to achieve vanillin release from Vanilla pods, as an alternative to conventional curing (Dignum et al., 2001; Ruiz-Ieràn et al., 2001). Gu et al., in 2012 used a pretreatment with cellulose and optimized the system with respect to: heating time, enzyme quantity and temperature; final vanillin extraction yield was 7.62 mg/g.

However vanillin is present in many natural matrices, and several enzymatic system to produce this molecule have been investigated from other plants. For example, enzymes from soybean are able to convert isougenol into vanillin after addition of powdered activated carbon and peroxide, were obtained 2.46 g/L of vanillin (Li et al., 2005). A soybean lipoxygenases can produce vanillin from esters of coniferyl alcohol (Markus et al., 1992).

In 2001 Van de Heuvel et al. presented two novel enzymatic routes for the biocatalytic production of natural vanillin; them used the flavoprotein vanillyl alcohol oxidase (VAO) , a broad-specificity *Penicillum flavoenzyme*, to convert both creosol and vanillylamine to vanillin with high yield.

Use of plant tissue culture

The production of fine chemicals by means of large-scale plant cell cultures is feasible. To set up cell cultures can be used Vanilla planifolia cells from different plant organs, such as stems, roots and leaves. But although *Vanilla planifolia* cell cultures have been studied extensively, no economically feasible vanillin production has resulted from this (Berger, 2007). The results obtained setting up cell cultures from *Capsicum frutescens* were not more encouraging (Rao & Ravishankar 2000a). Additional inherent problems with plant tissue culture are cell instability, slow growth rate and scale-up complexity. But, the insurmountable problem is the low production. Low or unreliable levels of production are a generic problem with plant cell cultures, and to a lesser extent with organ cultures. A great deal of effort has been expended in empirical approaches to increasing product yields in tissue culture systems, some of which have been applied, singly or in concert, to vanillin production (Walton et al., 2003). These have included the feeding of putative precursors (Funk and Brodelius, 1990a,b; Westcott et al., 1994), the use of elicitors or hormones (Funk and Brodelius 1990a, 1992), inhibition of

competing pathways (Funk and Brodelius, 1990a), cell immobilization (Rao & Ravishankar, 2000a; Westcott et al., 1994), adjustment of environmental culture conditions (Havin-Frenkel et al., 1996) and the use of an adsorbent to sequester vanillin (Westcott al., 1994). So far none of these approaches has thus delivered a commercial cell or organ-tissue culture system for vanillin production (Walton et al., 2003).

Plant cell-cultures can only be used effectively in systems in which the biochemical pathways of the target compounds are fully known, and this approach is therefore limited (Xu et al., 2007).

Use of microorganisms

Biotechnology employs the tools of genetic engineering to step up conventional methods of manufacturing important food ingredients, drugs and biopolymers (Kaur & Chakraborty, 2013). It broadens the range of possible substrates for biosynthesis of food flavouring and that of possible microorganisms for use as biocatalysts. When biotechnological processes are developed, the use of recombinant microorganisms as biocatalyst can increase the product yield of desired biologic products.

Biotechnological approaches have some advantages over other techniques, e.g. , mild reaction conditions, high substrate or product specificity which leads to only one product isomer and eco-friendly techniques that pose less harm to the environment (Schmid et al., 2001; Vandamme & Soetaert, 2002). Owing to these important advantages, research into biotechnological production of flavours has increased in recent years.

But biotransformation must jet overcome several drawbacks, such as the low water-solubility of the precursors, toxicity of precursors and products, and metabolic diversity which leads to unwanted by-products or further degradation of the target molecules (Schrader et al., 2004).

Developing a biosynthesis process the choice of the biocatalyst is the most important stage; there are two methods for selecting the appropriate microorganism: direct isolation from the wild, or research in microbial collections.

Taking advantage of the bacterial special features processes to obtain products of high added value have been developed; resorting to genetic engineering techniques, it was possible to modify metabolic properties of microorganisms for obtaining strains capable to produce high amounts of metabolites and recombinant proteins (Pras et al., 1995).

In view of use at industrial level, Hoeks lists the characteristics which must possess a good catalyst: a detectable catalytic activity, selectivity and substrate specificity, low production

costs, ability to ‘scale-up’, reproducibility and safety (Hoeks et al., 1995). In addition to this, a microorganism suitable for industrial use, should have other properties that facilitate and maximize the yield, which relate to: speed of growth, genetic stability, absence of toxic by-products, metabolic versatility, efficient substrate uptake and release of the product (Cheetham, 1998).

Microorganisms can be used in biotransformation processes as growing, resting or dead cell systems. Within resting cells processes growth and biotransformation occur in two successive phases. Thus optimum biotransformation conditions can be determined independently of the cellular growth. The product is obtained in a simple medium, which simplify and reduce the cost of the purification process. These advantages together to the use of recombinant strains can improve the production and yield of commercial interest products (Castellar et al., 2001).

Nevertheless, the time needed before novel biocatalysis strategies lead to industrially applied processes will not only depend on their scientific and technological feasibility but also, if not exclusively, on a less prejudiced and more balanced public perception of the use of genetic engineering for improved food quality and more environmentally friendly production processes.

1.5 Natural flavour production by microbial transformation

Biotransformation is an alternative tool with great potential, especially for the development of sustainable technologies for the production of additives and natural flavours. Currently, through stereoselective reactions, mediated by fungi and bacteria, a wide range of ingredients and food flavouring are produced.

Terpenes are used as substrate for hydroxylation reactions (oxidaction of C-H bond) to produce alcohols. Terpenes are a large and diverse class of organic compounds, used in food industry, because they are often strong smelling and thus may have had a protective function as insecticides and antimicrobial; they tend to be characterized by high structural complexity, meaning that chemical synthesis or structural modifications demand reactions with stereo- and enantioselectivity. Microbial transformations have proven to be an efficient alternative to chemical methods in the regio- and stereoselective functionalizations of terpenes, frequently giving rise to more biologically active products (Borges, 2009).

2-ethyl-3,(5/6) dimethylpyrazine is a characteristic compound of cooked and/or caramel foods; it has a characteristic smell of cocoa and tosted hezelnuts, and forms as a results of the

Maillard reaction. This molecule, commonly produced by chemical synthesis, can be produced using different strains of *Pseudomonas* (Blanco et al., 1994) and cultures of *Bacillus natto* (Ito et al., 1989).

An important flavour and fragrance compound with a rose-like odour is 2-Phenylethanol (2-PE). 2-PE occurs naturally in the essential oils of many flowers and plants, but in most cases, concentrations are too low to justify extraction, with exception of rose essential oil. So the vast majority of the 2-PE currently in use is produced chemically. Although microorganisms, especially yeasts, are capable of producing 2-PE by normal metabolism. *Ischnoderma benzoinum* and *Geotrichum penicillatum* are able to produce 2-PE; yields are relatively low, with 280 mg/l in 20 days for *I. benzoinum* and 0.0142 mg/l in 99 h in *G. penicillatum*. *Aspergillus niger* CMICC 298302 produced 1,375 mg 2-PE from 6 g/l L-Phe in 9 days .With regard to an industrial process, genetic engineering of yeast strains to stimulate de novo synthesis could be an option for increasing yields of 2-PE (Etschmann et al., 2002).

The most important lactone for flavour application is γ –decalactone, which has an oily-peachy aroma (Schrader et al., 2004). In the early eighties natural γ -decalactone was an extremely expensive, rare natural flavour (US$ 10000 per kg). The subsequent introduction and optimization of microbial processes has resulted in the price dropping to approx US$ 300 per kg.The commercial process uses ricinoleic acid, the main fatty acid of castor oil. Several microbial strains were used as biocatalyst; best results were obtained using *Yarrowia lipolytica* (9.5 g/L) (Pagot et al., 1997).

Acetic acid bacteria possess strong oxidative capabilities and are used for the oxidation of alcohols to the corresponding carboxylic acids (e.g.: propionic acid smells like raspberry, strawberry, cognac and butter; butyric acid smells like butter cheese ant nut fruit; 2-Methylbutyric acid smells like butter, cream and chocolate). With market prices minor than 100 Euros per kg, the carboxylic acids are relatively inexpensive natural flavours but of great importance to the flavour industry either because of their intense smell and sour taste or as substrates for the enzymatic production of flavour esters (Schrader et al., 2004).

The industrially most relevant compounds to obtain the ‘green’ organoleptic characteristic are C6 aldehydes and their corresponding alcohols. For example, 3(*Z*)-hexen-1-ol (‘leaf alcohol’) has a powerful odour of freshly cut grass and is an important flavour and fragrance material used for natural green top notes (Bauer et al., 2001). A novel production strategy based on genetically modified *Saccharomyces cerevisiae* coexpressing the hydroperoxide lyase gene from banana and the lipoxygenase gene, thus unifying all enzyme activities needed in one host, has been reported (Muheim et al., 1997, Häusler et al., 2001).

By functional expression in *E. coli*, a multifunctional *O*-methyltransferase, catalyzing the formation of 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF), an impact compound in strawberry flavour, has successfully been characterized (Wein et al., 2002).

These are only a few examples among many others to illustrate the progress currently being made by genetic engineering in flavour biosynthesis research.

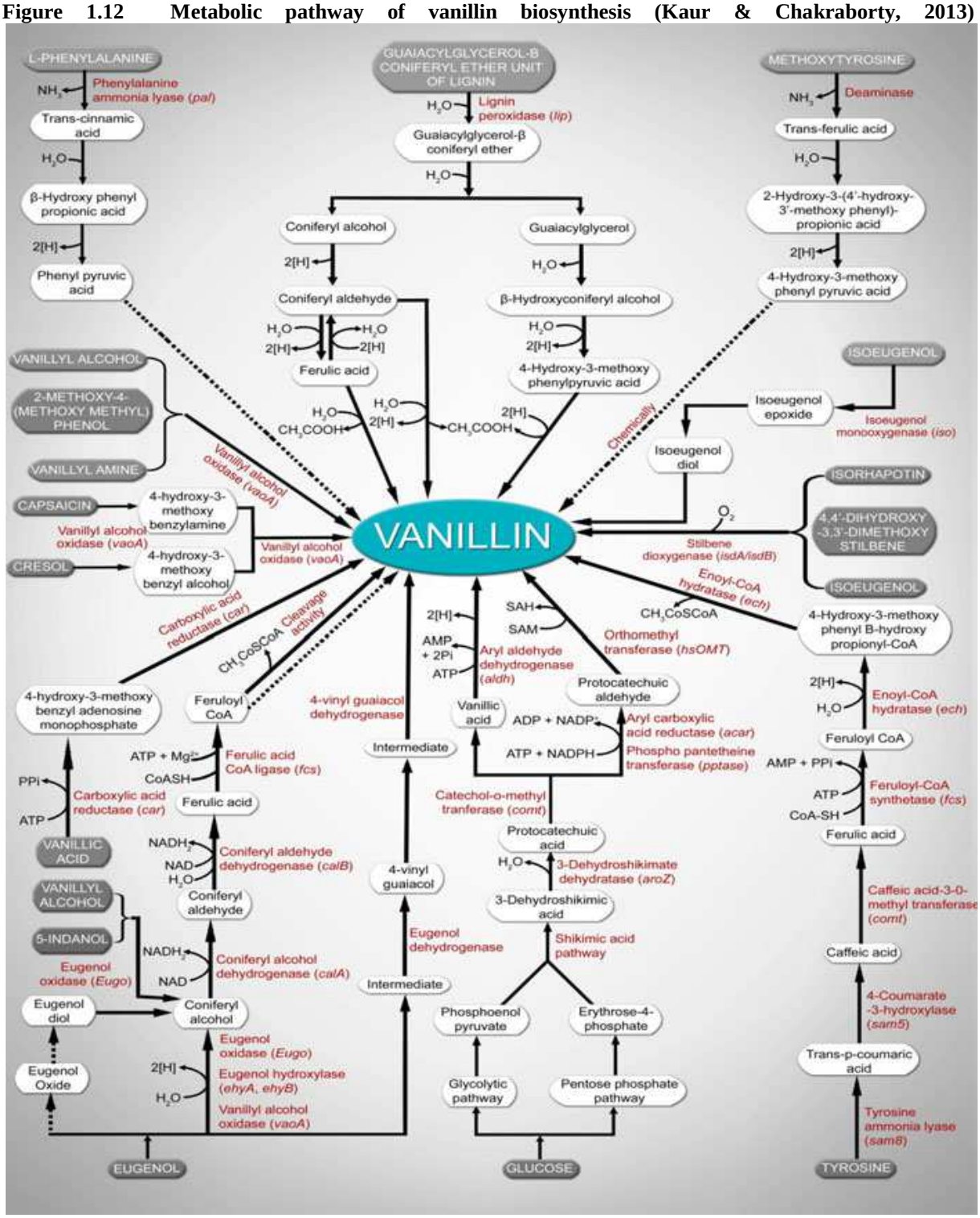
An increasing amount of research work is being published which involving the use of microbial biotechnology for flavourings production. Innovative strategies for biocatalytic flavour generation will certainly take advantage of the immense progress currently being made in the emerging fields of functional genomics, proteomics, protein and metabolic engineering.

1.5.1 Microbial biotechnology processes for vanillin production

Limited supply and high price of natural vanillin combined with the increasing customer-led demand for natural flavors, alternative natural sources of vanillin flavor have to be developed. Thus, production processes based on biotransformation have become more and more attractive. Several microbes such as bacteria, fungi and yeast have been used for the laboratory-scale up production of vanillin from different substrates. Unfortunately, many of these catalysts quickly oxidize or reduce vanillin into less toxic products such as vanillic acid and vanillyl alcohol (Andreoni et al., 1995; Overhage et al., 1999). As a consequence of the high chemical reactivity of aldehyde, this is often toxic and an accumulation is seldom observed (Lindahl, 1992). Frequently, vanillin is only a transitional intermediate (Sutherland et al 1983). To prevent these side reactions several research focused on metabolic engineering (inactivation of enzymes, such as vanillin dehydrogenase) and on optimization of biotransformation (addition of cofactors or adsorbent resin) to improve the yields of target products for scale-up and industrial use.

In particular , microbial biocatalysis can be used for the production of vanillin from phenolic stilbenes, lignin, eugenol, isoeugenol, ferulic acid, aromatic aminoacid, and glucose.

Figure 1.12 Metabolic pathway of vanillin biosynthesis (Kaur & Chakraborty, 2013)



Bioconversion of phenolic stilbenes to vanillin

Phenolic stilbenes are commonly found in spruce bark. Ligno stilbene-dioxygenases (*isd*A/*isd*B) extracted from *P. paucimobilis* strain TMY 1009 can oxidize phenolic stilbenes, to corresponding aromatic aldehydes. Cloning and expression of corresponding genes were carried out in *E. coli*. Strain was reported to oxidize naturally occurring isorhapotin to vanillin with a molar yield of up to 70 %. This process has also been patented (Yoshimoto et al., 1990a, b).

Bioconversion of aromatic amino acids

Phenylalanine ammonia lyase deaminates phenylalanine to trans-cinnamic acid, which is the key reaction involved in flavonoid, stilbene, and lignin biosynthesis in plants. This pathway follows with the formation of vanillin precursors like coniferyl alcohol, ferulic acid, and coniferyl aldehyde. Several white-rot fungi and *Proteus vulgaris* also possess phenylalanine ammonia lyase activity. According to *P. vulgaris* CMCC2840 deaminates methoxytyrosine to phenylpyruvic acid, which is then converted to vanillin by mild caustic treatment (Priefert et al., 2001).

Bioconversion of propenylbenzenes

Propenylbenzenes are aromatic compounds with various substitutions. They are physically extracted from plant essential oils. In the chemical industry, these are widely used as starting materials for synthesizing various products with applications as food preservatives and high-valued flavours. The production of many valuable aromatic compounds, including vanillin, has been widely reported in literature (Overhage et al., 2006; Hua et al., 2007; Yamada et al., 2007). Many microbes such as: *Aspergillus*, *Rhodococcus*, *Corynebacterium*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Serratia*, *Bacillus* and *Arthrobacter* species, have been reported to catalyse oxidation of the side-chain of propenylbenzenes. This is the first step in the biosynthesis of important products, such as vanillin, coniferyl alcohol, coniferyl aldehyde, and others (Kaur & Chakraborty, 2013). Although propenylbenzenes are usually toxic for most microbes, so most of the so far described processes showed rather vanillin yield.

The most common propenylbenzenes, eugenol and isoeugenol, are metabolized through an epoxide–diol pathway. This means that an epoxide structure and a diol structure exist in the

metabolic pathway. And only a little difference lies in the metabolic pathway between these two kinds of compounds: isoeugenol is always biotransformed through substituted benzoic acid, whereas eugenol is first metabolized to propenoic acid, producing the corresponding cinnamic acid derivates (Xu et al., 2007).

Eugenol is one of the most important raw materials for vanillin production and is the main constituent of oil extracted from clove tree *Syzygium aromaticum*. Washisu et al., in 1993 patented the production of vanillin from eugenol, using a strain of *Pseudomonas* spp TK2102, which accumulated vanillin up to 0.28 g/L, and other metabolites such as coniferyl alcohol, ferulic acid and vanillic acid. In most of the vanillin-producing bacterial species, vanillin is degraded through vanillic acid and protocatechuic acid (Kaur & Chakraborty, 2013).

To improve vanillin yield, metabolic engineering was introduced. A *vdh* mutant strain of *Pseudomonas* sp. HR199 has been constructed by inserting an omega element into vanillin dehydrogenase gene. Recombinant strain was able to convert 6.5 mM eugenol to 2.9 mM of vanillin (0.44 g/l) within 17 h (Overhage et al., 1999). Later on, the concept of metabolic engineering was exploited for production of biovanillin in *Rhodococcus* strain PD630 by coexpressing *vao*A from *P. simplicissimum* CBS 170.90 with *cal*A and *cal*B genes from *Pseudomonas* sp. HR199 and the metabolically engineered strain successfully converted eugenol to ferulic acid, which could be transformed to vanillin (Shimoni et al., 2003). Srivastava et al. (2010) for the first time established the eugenol bioconversion pathway in fungal systems that lead to hypothesize the metabolic pathway of eugenol in eukaryotic systems. Using vanillin biosynthetic pathway of *Pseudomonas fluorescens* as a case of study, they successfully identified the missing enzymes involved in the eugenol to vanillin bioconversion and then reconstructed the vanillin biosynthetic pathway in *Aspergillus niger*. Recently, a novel metabolic pathway for conversion of eugenol to vanillin was identified in *Bacillus cereus* strain PN24 (Kadakol & Kamanavalli, 2010). It can utilize eugenol, 4-vinyl guaiacol, vanillin, vanillic acid, and protocatechuic acid as growth substrates Eugenol dehydrogenase and 4-vinyl guaiacol dehydrogenase are important enzymes required for conversion of eugenol through 4-vinyl guaiacol to vanillin in *B. cereus* PN24. Vanillin was metabolized to protocatechuic acid which was further degraded by a β-ketoadipate pathway. More recently, another novel strain *Pseudomonas resinovorans* SPR1 was isolated whose resting cells were found to convert eugenol to 0.24 g/l of vanillin with 10 % molar yield at the end of the exponential growth phase after 30 h without further optimization (Ashengroph et al., 2011).

In 1988, Abraham et al., reported the first biotransformation of isougenol to vanillin by using the strain *Aspergillus niger* ATCC 9142. The resulted vanillin yield was very low, with a bioconversion efficiency of only 10%, due to further degradation of vanillin to vanillic acid and vanillyl alcohol. *Bacillus subtilis* HS8 isolated from soil is able to produce vanillin via isoeugenol-diol with molar yield of 14.7 and 1.36 g/l, vanillin was produced after 96 h (Zhao et al., 2005). Using *Bacillus fusiformis* SW-B9 60 % (v/v), isoeugenol to vanillin conversion is much faster and high yielding as 32.5 g/l vanillin is recovered after 72 h (Zhang et al., 2005). Under optimized reaction conditions, *Pseudomonas putida* IE27 showed highest vanillin-producing activity of 16.1 g/l vanillin from 150 mM isoeugenol, with a molar conversion yield of 71 % at 20 °C after 24 h incubation in the presence of 10 % (v/v) dimethyl sulfoxide (Yamada et al., 2007). There are few more reports where moderate levels of vanillin were generated using biotransformation capabilities of various bacterial and fungal strains. *Pseudomonas chlororaphis* CDAE5 was grown on 10 g/l isoeugenol, and 1.2 g/l vanillin was obtained after 24 h reaction at 25 °C and 180 rpm (Kasana et al., 2007). In *B. subtilis*, vanillin recovery from isoeugenol substrate is comparatively poor (0.61–0.9 g/l) due to end product toxicity (Shimoni et al., 2000). Isolated *Candida galli* PGO6 can produce vanillin and vanillic acid in concentrations of 0.59 ± 5.7 g/l (molar yield 48 %) and 0.18 ± 1.7 g/l (molar yield 19 %), respectively, after 30 h of initiation of bioconversion by this strain (Ashengroph et al., 2010). Recently, a halobacterium *Psychrobacter* sp. CSW4 was screened, capable of converting isoeugenol to vanillin (Ashengroph et al., 2012). Vanillin yield was improved under resting cell conditions with substrate optimization, and maximal vanillin concentration 1.28 g/L was achieved from 10 g/L concentration of isoeugenol after a 48-h reaction.

Bioconversion of lignin

Lignin, an aromatic polymer, is one of the most abundant natural sources of aromatic compounds. Although nature-identical vanillin is produced from lignin by chemical oxidation, few reports have been published on microbial vanillin production from lignin (Priefert et al., 2001). Analytical pyrolysis has been used to investigate lignin degradation by several white-rot fungi including *Pleurotus eryngii* and lignin depolymerization in *Phanerochaete chrysosporium* (Martinez et al., 2001; Tien & Kirk 1983), but vanillin has been detected only in trace amounts with other metabolites like dehydrodivanillin, vanillic acid, and ferulic acid.

Biotransformation of vanillic acid

Vanillic acid is either oxidatively decarboxylated to methoxyhydroquinone or it is reduced to vanillin and vanillyl alcohol. Vanillic acid decarboxylation yields vanillin in low amounts only. This drawback can be overcome by addition of cellobiose prior to vanillic acid supplementation that channels vanillic acid metabolism via reductive pathway, leading to a molar yield of 51% (Bonnin et al., 1999); by using high-density cultures (Oddou et al 1999); by using different types of bioreactors (Stentelaire et al., 2000); employing XAD-2 resin as an adsorbant (Priefert et al., 2001). A gene encoding carboxylic acid reductase from *Nocardia* sp. was expressed in *E. coli* BL21 (DE3) that reduced vanillic acid into vanillin. *Car* is the first example of a new gene family encoding oxidoreductases with remote acyl adenylation and reductase sites (Kaur & Chakraborty, 2013).

1.5.2 Bioconversion of ferulic acid

The only commercial biocatalytic route for vanillin production is based on the bioconversion of ferulic acid, a hydroxycinnamic acid. Ferulic acid is an abundant phenolic phytochemical found in plant cell wall components such as arabinoxylans as covalent side chains. It is related to trans-cinnamic acid. As a component of lignin, ferulic acid is a precursor in the manufacture of other aromatic compounds. The etymology is from the genus *Ferula*, referring to the giant fennel (*Ferula communis*), from which it was isolated for the first time in 1866. This compound is present in several plants and cereals and can be released by treatment with strong alkali or using cinnamoyl esterases or ferulic acid esterases together with plant cell wall glycosyl hydrolases. In addition, several mechanical, physical, thermo-physical and enzymatic pretreatment, can be combined with enzymatic hydrolysis to improve enzymatic action. It is found in abundance in corn hulls (31.0 g/kg), maize bran (30 g/kg), sugarbeet (5–10 g/kg), rice endosperm cell wall (9 g/kg), wheat (6.6 g/kg), and barley grains (1.4 g/kg) (Kaur & Chakraborty, 2013). In these sources ferulic acid is mainly found in the form of insoluble bound.

By virtue of effectively scavenging deleterious radicals and suppressing radiation-induced oxidative reactions, ferulic acid may serve an important antioxidant function in preserving physiological integrity of cells exposed to both air and impinging UV radiation. Similar photoprotection is afforded to skin by ferulic acid dissolved in cosmetic lotions. Its addition to foods inhibits lipid peroxidation and subsequent oxidaive spoilage. By the same mechanism

ferulic acid may protect against various inflammatory diseases. A number of other industrial applications are based on the antioxidant potential of ferulic acid (Graff et al., 1992).

Biotransformation of ferulate into vanillin is present in aerobic and anaerobic bacteria, yeast and fungi. Ferulic acid to vanillin conversions may occur through non-oxidative decarboxylation, side chain reduction, coenzyme-A-independent deacetylation, and coenzyme-A-dependent deacetylation. Finally, vanillic acid produced from vanillin either enters protocatechuic acid pathway or guiacyl pathway for further degradation through TCA cycle (Kaur & Chakraborty, 2013) as summarized in Figure 1.12.

Streptomyces sp. and *Amycolatopsis* sp. are more important from an industrial point of view as their ferulic acid biotransformation efficiencies are excellent and highest among all the tested vanillin producers (Muller et al., 1998; Muheim et al., 1999; Achterholt et al., 2000). Vanillin yields in *P. fluorescens* AN103 were improved by disrupting *vdh* gene (Martinez-Cuesta et al., 2005). Vanillin production was raised to 19.2 g/l in *Streptomyces* sp. V- 1 using adsorbent DM11 resin (Hua et al., 2006). Ferulic acid was transformed into vanillin by *Pseudomonas* isolate with production rate of 8.5 mg/l (Agrawal et al., 2003). It was also converted to vanillin (0.10–0.15 g/l) and vanillic acid using *Streptomyces halstedii* GE107678 (Brunati et al., 2004). Resting cells of *E. coli* strain JM109 pBB1 were used for biotransformation of ferulic acid to vanillin with a yield of 0.851 mol/L at a dilution rate of 0.022 h−1(Torre et al., 2004). *Enterobacter* sp. Px6-4, isolated from Vanilla roots, converted ferulic acid via 4-vinylguaiacol to vanillin (Li et al., 2008). *Staphylococcus aureus* also has an ability to consume ferulic acid with accumulation of 45.7 mg/l vanillin on the second day of incubation (Sarangu et al., 2005). However, it was quickly degraded there by reducing vanillin yield to 9.8 mg/mL after 7 days of incubation. In a one-step biotransformation process, *P. innabarinus* biotransformed ferulic acid to 126 mg/l vanillin with a molar yield of 54 % under statistically optimum condition in the presence of glucose as carbon source and corn steep liquor and ammonium chloride as organic and inorganic nitrogen source, respectively (Tilay et al., 2010).

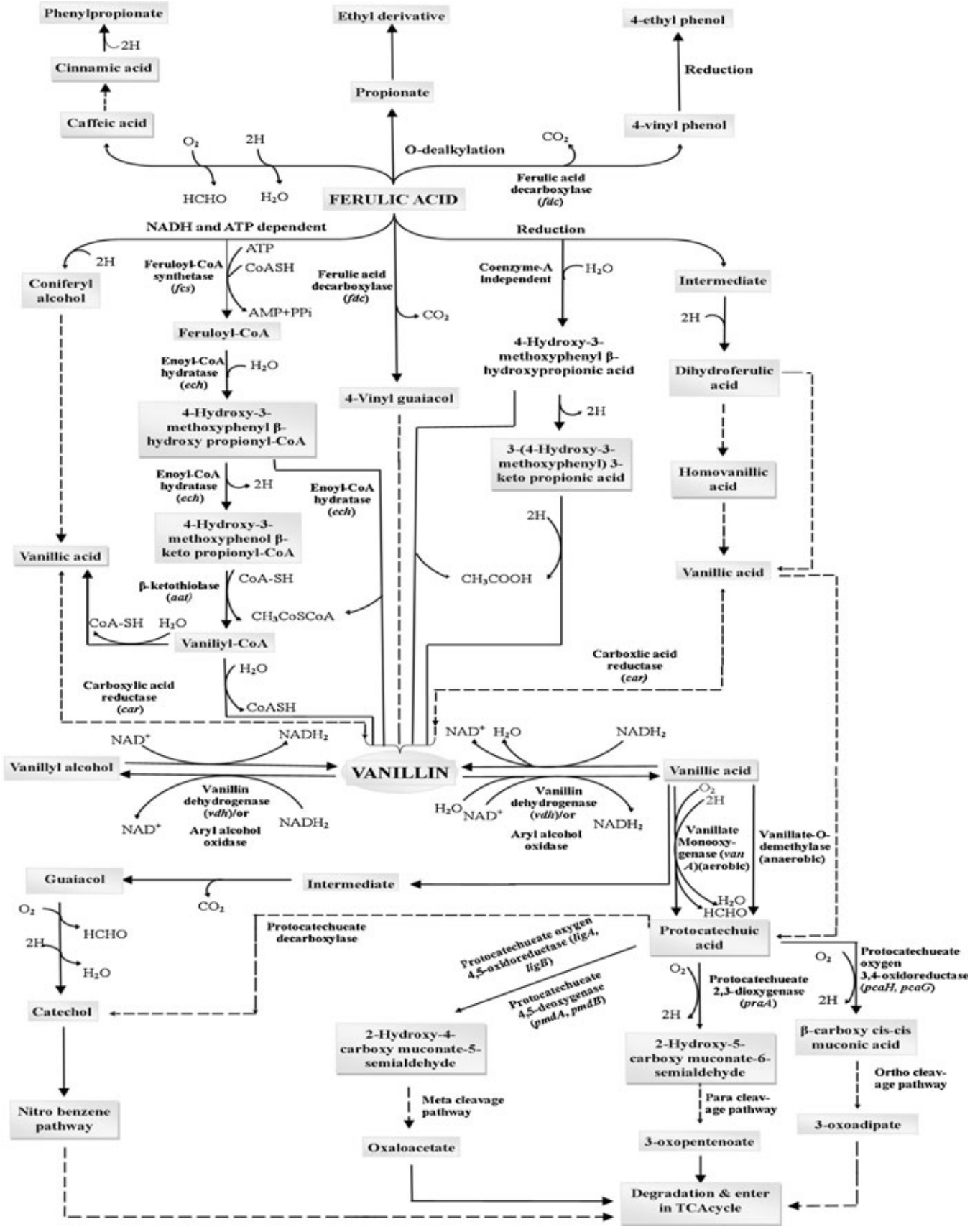
In this kind of biotransformation, greater problems are linked to the efficiency of the system. Vanillin is a highly reactive molecule which degrades quickly, and, at high concentration, is toxic to many microorganism which tend to turn it into other compounds. To overcome this problem, different strategies can be adopted. Some authors had proposed genetic constructs able to follow new metabolic pathways such as the bioconversion of glucose or glycoside to vanillin (Kaur & Chakraborty, 2013), the choice of the most productive mutant, genetically more stable and not able to degrade vanillin.

Recently were used *E. coli* recombinant strains inside of which genes coding for key enzyme were included (*fsc* for feruloil Co-A synthetase and *ech* for feruloil Co-A hydratase/aldolase). f*cs* and *ech* genes of *Amycolatopsis* sp. were expressed in metabolically engineered *E. coli* to produce vanillin under control of IPTG-inducible *trc* promoter. Vanillin production (1.1 g/L) was obtained with cultivation in 0.2 % (w/v) ferulic acid. Barghini engineered *E. coli* strain JM109 with a low-copy number vector pBB1 carrying genes *fcs* and *ech* from *P. fluorescens* BF13 (Barghini et al., 2007). The final concentration of vanillin was 3.5 mM after 6 h incubation by sequential induction with 1.1 mM ferulic acid. Vanillin (2.52 g/L) was obtained under resting cell conditions. Using integrative vector pFR2, *Pseudomonas* genes *fcs* and *ech* were integrated into lacZ gene of *E.coli*. The resultant strain was very stable and more efficiently produce vanillin than strains expressing ferulic acid catabolic genes from a low copy vector and 6.6 kg per kg biomass vanillin was reported using resting cells of metabolically engineered *E. coli* (Ruzzi et al., 2008). *E. coli* DH5α (pTAHEF-gltA) engineered with *glt*A (encoding citrate synthase, gene required for conversion of acetyl-CoA) yielded 1.98 g/L vanillin in 48 h of culturing on 3 g/L ferulic acid (Kaur & Chakraborty, 2013). An *icdA* mutant (with disrupted isocitrate dehydrogenase activity) was found to be more efficient in converting acetyl-CoA to CoA. Use of XAD-2 resin reduced toxicity of vanillin and vanillin accumulation was enhanced by 2.6 times. The real synergistic effect of vanillin of 5.14 g/L was produced in 24 h of the culture with molar conversion yield of 86.6 % (Lee et al., 2009). *vdh* gene of *P. fluorescens* BF13 strain was inactivated via targeted mutagenesis, and results demonstrated that engineered strain BF13 accumulated vanillin with concurrent expression of structural genes (*fcs*) and (*ech*) from a low-copy plasmid. The developed strain produced up to 8.41 mM vanillin, which is the highest final titer of vanillin produced by a *Pseudomonas* strain from agro-industrial wastes which contain ferulic acid (Di Gioia et al., 2011).

Bioconversion of ferulic acid into vanillin using *E coli resting* cells is linked to both product and substrate toxicity. Working on this biological system has been shown that to increase vanillin accumulation high amounts of ferulic acid most be provided (Barghini et al., 2007). Since bioconversion efficiency depends on both the amount of substrate that the way in which this is provided, it could adopt a fed-batch mode to conduct the process. In order to maintain substrate concentration as a constant in the bioconversion medium, it is possible to use a two-phase system. Its basic principle is to partition the toxic substrate between the aqueous phase containing the micro-organisms and a solid phase which has typically been comprised of a gel matrix (Daugulis, 2001). Large amounts of substrates can be dissolved in the second phase,

resulting in substrate partitioning to a reduced equilibrium concentration in the aqueous phase. Only substrate in the aqueous phase is available to the biocatalysts and degradation will result in a disequilibrium, which in turn will result in partitioning of additional substrate from the solid phase into the aqueous phase. The substrate delivery into the aqueous phase is controlled by the microbial degradation rate if the mass transfer rate is significantly larger than the microbial consumption rate. Solid–liquid two-phase system has several advantages over liquid– liquid system in terms of biological compatibility of the second phase. Solid polymeric substances which are typically used are biologically inert, being neither toxic to the organism of choice nor can they substitute as an alternative carbon source and divert biological activity away from the target substrate (Pripch et al., 2006; Tomei et al., 2008). A further advantage is the large interfacial area available for mass-transfer between aqueous and delivery phase. Typically employed delivery phases are spherical or cylindrical beads with diameter of 2–4 mm resulting in a total specific interfacial area of 0.07 m^2/dm^3 (m^2 interfacial area per dm^3 fermentation broth at 50 g solid phase per L aqueous phase). Other geometrical shapes with even smaller surface to mass ratios such as sheets are rods might hold operational advantages, but mass transfer limitations are likely to occur under such conditions (Rehmann et al., 2007).

Figure 1.13 Biochemical reactions involved in the conversion of ferulic acid to vanillin, vanillyl alcohol, vanillic acid, and degradation of protocatechuic acid (Kaur & Chakraborty, 2013)



1.6 Regulations

Community directives are flanked by numerous national rules on production and trade in food, requiring harmonisation, very difficult to achieve due to the unanimity required by the system for approving directives of approximation. The impasses was overcome thanks to introduction of mutual recognition principle, which guarantees free movement of goods and services without the need to harmonise national legislations; goods and services which are lawfully produced in one Member State cannot be banned from sale on the territory of another Member State, even if they are produced to technical or quality specifications different from those applied to its own products. In general, the rules of the Member State of origin prevail. This guarantees compliance with the principle of subsidiarity by avoiding the creation of detailed rules at European level and by ensuring greater observance of local, regional and national traditions and makes it possible to maintain the diversity of products and services.

Following these principles, the European Commission has stopped introducing laws systematically regulating the minimum requirements that each foodstuff should have, in order to take horizontal directives aimed at regulating the products in general in relation to some of their possible specific contents (Costato, 2007). So in order to ensure the free movement of goods and to build the single market, several directives on the approximation of laws in regard to additives and flavourings used in food were adopted (Germanò, 2008).

A series of regulations (Regg. CE 1331/2008, 1332/2008, 1333/2008 and 1334/2008 all dated 16 December 2008) have recently made some simplifications and updates to the legislation on food additives, enzymes and flavourings. The adoption of this set of regulation intended: to simplify the legislation on additives, creating a single instrument for principles, procedures and authorizations, to give the Commission the jurisdiction to update the Community list of substances at issue, to consult the EFSA (European Food Safety Authority) and establish a revaluation programme for existing food additives, to provide for a specific authorization of additives which are composed of, or produced from or contain genetically modified organisms (Costato & Albisinni, 2012).

The common authorization procedure for food additives, food enzymes and food flavouring is subject of Reg. CE 1331/2008, which defines procedural arrangements for updating the lists for each category of substances. Regulation is aimed to simplify the system, with a significant reduction, compared to the past, of the administrative burdens.

The regulating framework on additives has always tried to balance conflicting interest: the need to protect the health of the consumers by ensuring proper and adequate information and

the need of the agro-food industry to maintain production for long time (Costato, 2008). Article 3 of Reg. CE 1333/2008 gives additives definition: substances not normally consumed as food itself and not normally used as a characteristic ingredient of food, whether or not they have nutritive value, but which are intentionally added to food for a specified technological purpose, becoming directly or indirectly a component of such foods.

A food additives may only be approved if it does not pose a safety concern to the health of consumers, if there is a reasonable technological need that cannot be otherwise achieved; and if its use does not mislead the consumer (Reg. CE 1333/2008 art. 6). Before incorporating food additives in the list of the approved additives, the Commission must examine all existing authorizations with regard to quantities absorbed, technological need and the potential to mislead the consumer. Once approved, the additive continues to be kept under constant observation (Costato & Albisinni 2012).

Regg. CE 1331/2008 and 1332/2008 are the first to regulate the use of enzymes in food, because, traditionally, enzymes are considered non-toxic and there is no Community procedure for the assessment of their safety or authorization; only some Member States have had procedures for a mandatory or voluntary authorization, resulting an unequal treatment in the different states, while it is necessary to harmonise legislation at Community level.

A food enzyme is a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms containing one o more enzymes capable of catalyzing a specific biochemical reaction and which is added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods (Reg. CE 1332/2008 art. 3). Regulation 1332/2008 provides for the establishment of a list of authorized enzymes, their conditions of use and to define the rules governing their labelling. Only food enzymes included in the list may be placed on market.

1.6.1 The discipline of flavourings

The new Regulation (EC) No 1334 of the European Parliament and of the Council on flavourings and certain food ingredients with flavouring properties for use in and on foods was adopted on 16 December 2008; it repeals Council Directive 88/388/EEC and Commission Directive 91/71/EEC as from 20 January 2011 on. This Regulation establishes the flavourings and source materials approved for use in and on foods, the conditions of use

of flavourings and food ingredients with flavouring properties and the rules on the labelling of flavourings; it applies to flavourings used to impart odour and or taste to food, but it does not apply to: raw foods; smoke flavourings; mixtures of spices and or fresh, dried or frozen herbs, mixtures of teas and mixtures for infusion, as long as they have not been used as food ingredients.

Flavourings are products which are not intended to be consumed as such, which are added to food in order to impart or modify odour and or taste. They are made or consist of the following categories: flavouring substances, flavouring preparations, thermal process flavourings, smoke flavourings, flavour precursors or other flavourings or mixtures thereof. (Reg. CE 1334/2008 art.3).

The field of food flavourings, unlike that of enzymes, has always been the subject of attention by Community legislation; Directive 88/388/EEC has been the reference normative text on flavouring for a long time.

This Regulation replaces the existing legislation on food flavourings to take into account scientific and technological developments; at Community level, it was aimed at harmonising the use of food flavourings and ingredients with flavouring properties in and on foods. The Regulation sets out flavourings and source materials for which an evaluation and approval is required. The Union list of flavourings and source materials will be laid down in Annex I. In particular, it prohibits the addition of certain substances as such to food and lays down maximum levels for certain substances, which are naturally present in flavourings and in food ingredients with flavourings properties, but which may raise concern for human health.

With regard to labelling, classification of aromatic substances has been retouched, with specific requirements for use of the term ‘natural’. The old directive provides for three categories: natural flavouring substances, ‘identical to natural’ and artificial flavourings. Now, the statement “identical to natural flavourings” has been removed and introduced a new specification: *natural flavourings* correspond to substances that are naturally present and have been identified in nature; *natural flavouring substance* is flavouring substance obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes.

Labelling of food flavourings must comply with the general labelling conditions defined in Directive 2000/13/EC.

Before 20th January 2011 (date of entry into force) the only vanillin that could be labelled as ‘natural vanillin’ is vanillin that comes from vanilla beans. vanillin obtained from a natural source and by a natural process, can be classified as ‘natural flavouring’ in a non-vanilla product. Now, under the new legislation, vanillin produced in biotechnological processes starting from natural substrates can be classified as natural flavouring on condition that the natural starting material is specified; thus, vanillin produced in biotechnological processes starting ferulic acid can be classified as natural flavouring, satisfying the increasing interest of the consumers in natural products.

On the 1st of October 2012 the Commission adopted two new Regulations on flavourings. Consumers and companies still face divergent national rules despite of the long history of safe use of flavourings. The new legislation harmonises and clarifies the rules for the use of these substances in the single market.

Regulation EU 872/2012 contains a new EU list of flavouring substances which can be used in food. It enters into force on 22 October 2012 and will apply as of 22 April 2013.The EU food industry will only be able to use flavouring substances that are on the EU list; flavouring substances not on the list will be banned after an 18-months phasing-out period.

Regulation EU 873/2012 introduces transitional measures for other flavourings e.g. flavourings made from non-food sources to be evaluated and authorised later.

1.7 Response Surface Methodology

The most important stages in a biological process are modeling and optimization to improve a system and increase the efficiency of the process without increasing the cost (Bas et al., 2007). Optimizing refers to improve the performance of a system, a process, or a product in order to obtain the maximum benefit from it. Traditionally, optimization in biological analyses is carried out by monitoring the influence of one factor at a time; this technique, called *one-variable-at-a-time*, is not only time-consuming and tedious but also does not depict the complete effects of the parameters in the process, ignores the combined interactions between physicochemical parameters (Ebrahimpour et al., 2008). Another drawback is the large number of experiments to conduct the research, which leads to an increase in the consumption of reagents and materials.

In order to overcome this problem, the optimization of analytical procedures has been carried out by using multivariate statistic techniques. Among the most relevant multivariate technique used in analytical optimization is Response Surface Methodology (RSM) (Bezerra et al., 2008). Response surface methodology (RSM) was developed by Box and his collaborators in 1951; this term was originated from the graphical perspective generated after fitness of the mathematical model. RSM is a collection of mathematical and statistical techniques for empirical model building based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical previsions. By careful design of experiments, RSM was aimed at optimizing a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response..

Biological processes are the result of a synergistic combination of effective parameters interactions, the change of one parameter can be compensated by changes of other parameters. During recent years, the RSM has been used extensively for optimization in many areas of industrial and biological research and process development in chemistry and biochemistry. Its use for optimization and analysis of biotechnological process with microorganisms and enzymatic engineering has given good results (Ryad et al., 2010). For example, Dash and Gummadi optimizing using RSM managed to increase the caffeine degradation rate of 45% (Dash & Gummadi, 2007). Using a 22 full factorial design to optimize media and growth conditions, Ries et Macedo obtained a 10-fould improvement in the production of phytase by

S. cereviasiae; in the work of Fenice (2013) chitinolytic enzymes production by *Lecanicillium muscarium* CCFEE 5003 was enanched by 23% (Fenice et al., 2013).

Before applying the RSM methodology, it is first necessary to choose an experimental design that will define which experiments should be carried out in the experimental region being studied. There are some experimental matrices for this purpose. Experimental designs for first-order models (e.g., factorial designs) can be used when the data set does not present curvature. However, to approximate a response function to experimental data that cannot be described by linear functions, experimental designs for quadratic response surfaces should be used, such as threelevel factorial, Box–Behnken, central composite, and Doehlert designs.

Some stages in the application of RSM as an optimization technique are as follows: (1) the selection of independent variables of major effects on the system; (2) the choice of the experimental design and carrying out the experiments according to the selected experimental matrix; (3) the mathematic–statistical treatment of the obtained experimental data through the fit of a polynomial function; (4) the evaluation of the model's fitness; (5) the verification of the necessity and possibility of performing a displacement in direction to the optimal region; and (6) obtaining the optimum values for each studied variable (Bezerra et al., 2008).

1.7.1 Full three-level factorial design

Full three-level factorial design is an experimental matrix that has limited application in RSM when the factor number is higher than 2 because the number of experiments required for this design (calculated by expression $N=3k$, where N is experiment number and k is factor number) is very large, thereby losing its efficiency in the modeling of quadratic functions. However, for two variables, the efficiency is comparable with designs such as central composite. Figure 1.13 shows the representation of the three-level factorial designs for the optimization of two variables. Table 1.5 shows the experimental matrix for the optimization of two variables using this design. The majority of applications of three-level factorial designs are in the area of chromatography. Table 1.6 shows some works in which this experimental design was used.

Figure 1.14 Experimental designs based on the study of all variables in three levels: optimization of two variables

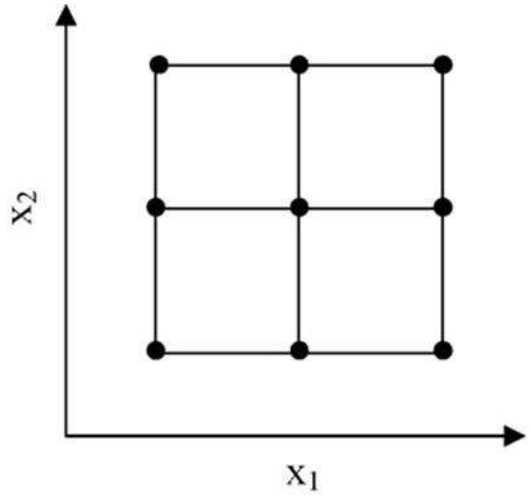


Table 1.5 A experimental matrix for designs based on variables study in three level factorial design for two variables

x1	x2
-1	-1
-1	0
-1	1
0	-1
0	0
0	1
1	-1
1	0
1	1

Table 1.6 Some applications of three level factorial design in analytical chemistry (Bezerra et al., 2008)

Analytes	Samples	Analytical technique	Objective of study
Caffeine, theobromine and theophylline	Coffee, tea and human urine	Reversed-phase HPLC	Improving the chromatographic resolution among these three substances
Niacin	Fresh and dry-cured pork products	Ion chromatography	Optimizing the mobile phase composition
Anionic, cationic, and neutral drugs	Pharmaceutical formulations	Electrokinetic chromatography	Stabilising the effects of the sodium dodecyl sulfate and 2-propanol concentration in the separation of these analytes
Clothiapine, clozapine, olanzapine, and quetiapine	Pharmaceutical formulations	Capillary zone electrophoresis	Development of a method for separation of these four atypical antipsychotics
Sulfonamides	Foodstuffs	HPLC	Developing a molecularly imprinted polymer for separation of the analytes
Candesartan, eprosartan, irbesartan, losartan potassium, telmisartan, and valsartan	Pharmaceutical formulations	Capillary zone electrophoresis	Optimizing the separation of these angiotensin-II-receptor antagonists
Underivatized phenol and cresols	Soil samples with a high content of carbon	GC	Optimizing the supercritical fluid extraction of these analytes
Copper	Petroleum condensate	GF AAS	Developing a method for the direct determination of analyte using detergentless microemulsions

2. State of the art

In recent years, there has been a growing interest in natural and healthy foods, especially with regards to flavouring agents and preservatives. Amongst the variety of natural flavours used in food industry, vanilla occupies a prominent market place and finds extensive application for the production of food, beverages and pharmaceuticals (Sinha et al., 2008). Natural vanilla is a complex mixture of flavour components extracted from the cured pods of orchids of the genus *Vanilla.* The flavour profile of vanilla contains more than 200 components, of which only 26 occur in concentration greater than 1 mg/kg. The aroma and flavour of vanilla extract is attributed mainly to the presence of vanillin (4-hydroxy-3-methoxybenzaldehyde), which occurs in a concentration of 1.0 – 2.0 % (w/w) in cured vanilla pods (Bettazzi et al., 2006).

Vanillin has an annual consumption in the global flavour market of 12000 tons per annum (Sinha et al., 2008). The limited supply and, consequently, high price of vanilla has led to the usage of a large amount of synthetic equivalents (i.e. vanillin and ethyl vanillin). At present about 99% of vanilla flavourings sold in the market are made with vanillin produced by chemical synthesis using coniferin, eugenol, guaiacol and lignin as a precursor. Although synthetic vanillin is able to meet the global annual demand of vanilla flavourings, it suffers from serious drawbacks linked to the use of hazardous chemicals and increasing interest of consumers in natural products.

Recently, there has been a great interest for exploration of more eco-friendly procedures for biosynthesis of vanillin. Biotechnological production of vanillin through the use of a biocatalyst (fungi, bacteria, plant cells or isolated enzymes) and natural precursors (lignin, eugenol, phenolic stilbenes and ferulic acid) is a valid alternative to the production of the aroma by chemical synthesis or extractive means (Priefert et al., 2001; Walton et al., 2003). However, at present, production of vanillin in large volume by biological means appears to be limited due to its higher production cost compared to chemical synthesis.

Ferulic acid, the most abundant hydroxycinnamic acid in the plant world (Clifford, 2000), is a suitable substrate for microbial synthesis of natural vanillin (Benz and Muheim, 1996; Sinha et al., 2008); it is a cheap raw material and can be easily obtained from agricultural waste residues such as cereal bran and sugar beet pulp.

Interestingly, the biotechnological flavor may have a lower price compared to vanilla and can be still considered natural, according to EC Regulation no 1334/2008 (December 16th 2008), if it is obtained from a natural substrate (i.e. ferulic acid). However, high-titer production of vanillin by biological means is limited due to toxicity of both substrate (ferulic acid) and product (vanillin) for whole-cells.

A number of microorganisms was proposed as a catalyst for production of vanillin from ferulic acid, but they usually have a limited application at industrial scale due to their low tolerance to high levels of vanillin. *Escherichia coli* is considered as a promising candidate for cost-competitive production of vanillin because it is known to survive in high concentrations of organic solvents and has not evolved a catabolic pathway that allows vanillin degradation (Lee at al., 2008). *E. coli* is a non-native vanillin producer and need to be transformed with catabolic genes encoding key enzymes responsible for conversion of ferulic acid to vanillin (feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase). Moreover, the bioconversion of ferulic acid to vanillin by metabolically engineered *E. coli* cells is influenced by several factors, such as: physiological state of the biomass; number of catabolic genes per cell; initial biomass and substrate concentration.

Barghini et al. (2007) demonstrated that *E. coli* JM109(pBB1), a recombinant strain carrying ferulic acid-catabolic genes (*fcs* and *ech)* from *Pseudomonas fluorescens* BF13, was a good catalyst for vanillin production, but the ability of *E. coli* cells to convert ferulic acid to vanillin was negatively affected at high substrate concentration. These Authors demonstrated that *resting* cells of JM109(pBB1) could produce up to 2.52 g/L of vanillin, under conditions of batch operation, with repeated addition of ferulic acid. Unfortunately, low concentration of substrate led to low final product concentrations which determine increase in the product recovery cost from aqueous solutions and additional costs for wastewater treatment. A viable solution to maintain the substrate concentration below the inhibitory values and prevent dilution of the final product is the use of hydrogels for the controlled release of the substrate in the bioconversion medium (Rehmann, 2007). Agarose gels provide a well-defined, controllable scaffold to entrap ferulic acid and can be beneficial in elucidating effects of modulated release of the substrate on the bioconversion process.

Luziatelli et al. (2008), recently, developed a single-copy integrative vector to study expression of ferulic catabolic genes in *E. coli* cells. Using this vector Authors demonstrated that *E. coli* strain (named FR13) carrying a single copy of *fcs a*nd *ech* genes from *P. fluorescens* BF13 was more efficient in vanillin production than congenic strains with multiple copies of ferulic catabolic genes.

A significant increase in vanillin production with *E. coli* cells was achieved by Yoon et al. (2007) combining the use of a vanillin-resistant mutant with a vanillin adsorbent resin (XAD-2®). Lee et al. (2008) demonstrated that the addition of macroporous resins to the bioconversion buffer is a valuable tool to limit vanillin toxicity and can allow to obtain a final vanillin titre of 5.14 g/L.

Nevertheless, to be competitive with other biological systems (such as *Amycolatopsis* strains), the final concentration of vanillin obtained from *E. coli* cells should be increased up to 10 g/L, which requires the identification of bottlenecks allowing for further optimization of the bioconversion process.

3. Aims

Aim of the thesis

Biotechnological production of vanillin from natural feedstocks is a viable alternative to its production by chemical synthesis and offers potential opportunities for economical utilization of agro-food wastes and residues. In recent years, several studies have focused on the production of vanillin using engineered *E. coli* strains and ferulic acid, as a substrate (Yoon et al. 2005; Barghini et al. 2007; De Faveri et al. 2007). Evidence from published data indicates that vanillin production is dependent on many parameters that are not yet well understood. These parameters include pH and temperature, as observed for the conversion of isoeugenol to vanillin by *Brevibacillus agr*i 13 (Wangrangsimagul et al. 2012), or agitation speed and initial concentration of ferulic acid that significantly affects the ability of *E. coli* cells to convert ferulic acid to vanillin (Barghini et al., 2007; De Faveri et al., 2007; Lee et al., 2008 & 2009; Luziatelli et al., 2008).

This PhD thesis aims to optimize the yield and selectivity of *E. coli*-based vanillin production process. For this reason it has been evaluated, the multiple effect of pH and composition of the reaction buffer on system productivity, and, using a Response Surface Methodology approach, substrate concentration and stirring speed on the conversion of ferulic acid to vanillin and vanillyl alcohol. Another aim of this study was to develop an efficient two-phase (solid-liquid) system for controlled release of ferulic acid in the bioconversion medium which minimizes substrate toxicity and increases final product concentration.

4. Materials and Methods

4.1 Bacterial strains, plasmids, culture conditions and cells storage

Bioconversion of ferulic acid into vanillin was carried out using resting cells of *E. coli* strains carrying ferulic catabolic genes from *Pseudomonas fluorescens* BF13. The bacterial strains and plasmids used in this study are listed in Table 4.1.

Table 4.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
P. fluorescens BF13	Wild type, ferulate plus	Ruzzi et al., 1997
E. coli JM109	recA1 endA1 gyr A96 thi-1 hsdR17 rk- mK+ supE44 relA1 λ- Δlac-proAB F' traD36 proAB+ lacIq ZΔM15	Promega
E. coli JM109(pBB1)	*E . coli* JM109 derivate carrying plasmid pBB1	Barghini et al., 2007
E. coli JM109(pFS)	*E. coli* JM109 derivate carrying plasmid pFS	Barghini et al., 2007
E. coli FR13	*E. coli* JM109 derivate carrying plasmid pFR2 integrated into lacZ locus	Luziatelli (2008)
Plasmids		
pLOI2227	Integration vector containing FRT-KmR-FRT fragment, pSC101 origin, Km^r, 3443 kb	Martinez-Morales et al., 1999
pBB1	Low copy plasmid, RK2 origin containing a 5098 bp *EcoR*I-*BamH*I fragment with the promoter (P_{fer}), *ech*, Δ*vdh* and *fcs* genes from *P. fluorescens* BF13, Ap^r; Tc^r; 12.064 kb	Barghini et al., 2007
pFS	High copy plasmid, ColE1 origin containing a 5098 bp *EcoR*I-*BamH*I fragment with the promoter (P_{fer}), *ech*, Δ*vdh* and *fcs* genes from *P. fluorescens* BF13, Ap^r; 8.194 kb	Barghini et al., 2007
pFR2	Integration vector; pLOI2227 derivative containing a 6591-bp fragment with the promoter (P_{fer}), *ech*, Δ*vdh* and *fcs* genes from *P. fluorescens* BF13 and 1036 - bp fragment with a portion of *lacZ* gene; Km^r; 10034 kb	Luziatelli (2008)

Ap^r, ampicillin resistance; Tc^r , tetracycline resistance; Km^r, kanamycin resistance

Pre-cultures were prepared from a stock culture, frozen in liquid nitrogen and kept at -80°C. all bioconversion experiments were carried out using cells grown on LB medium in Erlanmeyer shaken flasks (relationship between flask volume and liquid was equal to 10:1) composition (per liter) as follows: tryptone 10 g; NaCl 5 g; yeast extract 5g. Antibiotics and incubation temperatures used for each strains are reported in Table 4.2.

For short period, cells were maintained at 4°C on LB agar plates supplemented with bacterial agar (15 g/L) and antibiotics as required; for longer periods cells were stocked at -80°C in LB medium containing glycerol 40% (v/v).

Table 4.2 Growth conditions for biomass production

E. coli strains	Antibiotics	Incubation temperature
JM 109 pBB1	Ap*	30°C
JM109 pFS	Ap*	30°C
FR13	Km**	44°C

Incubation was performed in orbital shaker (Unitron, Infors-ht, Bottmingen, Switzerland).
*Ampicillin (50 µg/mL). **Kanamicin (25 µg/mL)

4.2 Bioconversions experiments by *E. coli resting* cells

Cells were cultivated in shaken flasks and, when the desired optical density was achieved, biomass was collected by centrifugation (4000 rpm for 10 minutes at 4 °C), washed twice in appropriate buffer and resuspended in the bioconversion buffer, in order to obtain a final concentration of biomass in a range of 4.5-7.5 mg (wet weight)/mL. In a standard bioconversion process, cell concentration was maintained at 4.5 mg (wet weight)/mL.
Bioconversions were performed in 100 mL flasks containing 15 mL of cell suspension amended with yeast extract (2 mg/L) and ferulic acid (7.7 to 23.2 mM). Samples were incubated in an orbital shaker (120-180 rpm) at 30°C. During the bioconversion, 0.5 mL samples were periodically withdrawn and centrifugated at 4000 rpm, and the supernatant analyzed by high-pressure liquid chromatography to determine substrate and metabolite concentrations in the liquid phase.

4.3 Buffer composition

Reaction conditions for vanillin production were optimized with respect to phosphate buffer composition (presence/absence of NaCl and NH_4Cl), phosphate molarity, sodium-to-potassium ratio, pH of buffer solution (pH 6.8-9.0). In brief, the following saline phosphate buffers were used (Tab.4.3): 64mM sodium-potassium phosphate buffer at different pH values (buffer A, pH 6.8; buffer B, pH 8.0; buffer C and D, pH 9.0) (composition per litre: Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g) and similar [Na]/[K] ratios (between 4.2 and

4.68); sodium-potassium phosphate buffer, at pH 9.0, with [PO_4] molarity 46.9 mM and [Na]/[K] 1.13 (buffer E), or with 70 mM phosphate concentration and [Na]/[K] 0.26 (buffer F), 0.0125 (buffer K), 88.9 (buffer L), 0.93 (buffer M); buffer G had 100mM phosphate and [Na]/[K] 140.84; a potassium phosphate buffer (H) and a sodium phosphate buffer (J) both with [PO_4] 200mM.

In order to evaluate the application of a two-phase system, based on the use of microspheres of calcium alginate-ferulic acid, buffers without phosphate were also tested (Tab. 4.4).

Table 4.3 Phosphate buffers used in bioconversion experiments

	Bioconversion buffer											
Molar concentration (mM)	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**J**	**K**	**L**	**M**
[PO_4]	64	64	64	64	46.92	70	100	200	200	70	70	70
[Na]	92.60	98	103	94	53	30.31	200	410	0	1.75	140	65.10
[K]	22	22	22	22	46.92	114	1.42	0	420	140	1.57	70
[Na]/[K]	4.02	4.45	4.68	4.27	1.13	0.26	140.84			0.0125	88.9	0.93
pH	6.8	8.0					9.0					

Table 4.4 Alternative bioconversion buffers used for vanillin production

Buffers*	**Concentration**	**pH**
TRIS-HCl	0.1 M	9.0
Sodium borate buffer	0.15 M	9.0
Glycine buffer	0.1 M	9.0

* All buffers were amended with NaCl and KCl to obtain specific molar concentration for sodium and potassium of 84 mM and 22 mM, respectively.

4.4 Preparation and use of ferulic acid loaded agarose rods

In this work a solid-liquid two-phase system for the modulated release of ferulic acid in the liquid phase was developed. For this purpose ferulic acid (1.5%) was entrapped into agarose (1.75% w/v) gel cylinders (Figure 4.1 and 4.2). Agarose solutions were cooled at 50°C prior to the addition of an isovolume of ferulic acid 3% (w/v). Solutions were poured in the wells of a 96-well microplate (Sarstedt, Newton USA) and cooled at room temperature for 3 hours in

static conditions. Each cylinder had a diameter of 0.6 ± 0.001 cm and a height of 1 ± 0.001 cm, and contained 0.034 ± 0.02 mmoles of ferulic acid. Bioconversions were carried out in shaken flasks with different cylinder-to-unit of volume ratios (three to twelve cylinders per 15 mL volume).

Figure 4.1 Agarose monomer

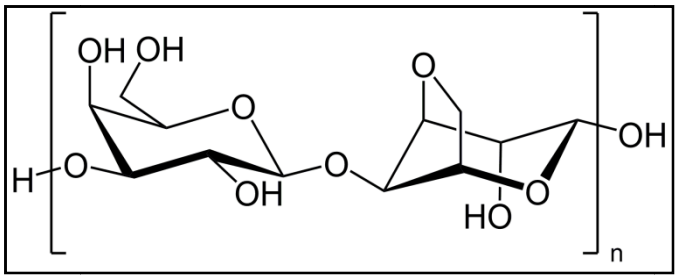
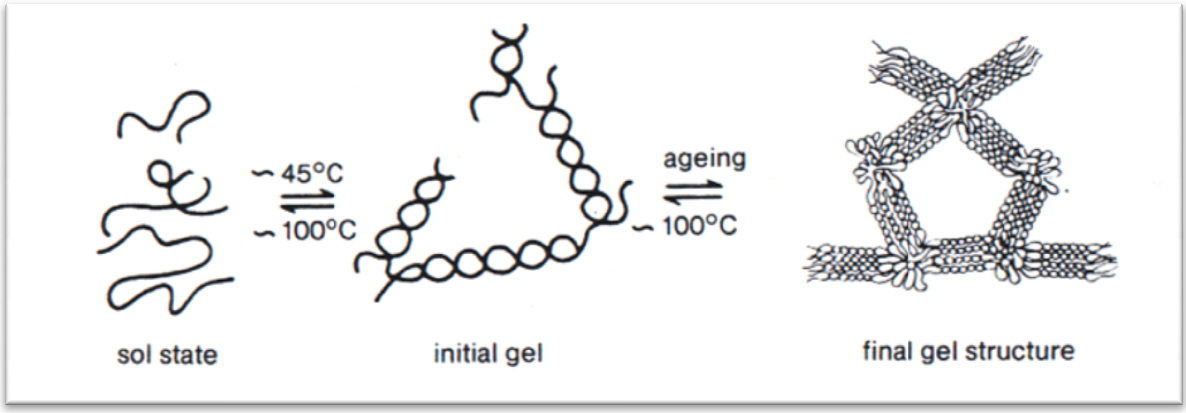


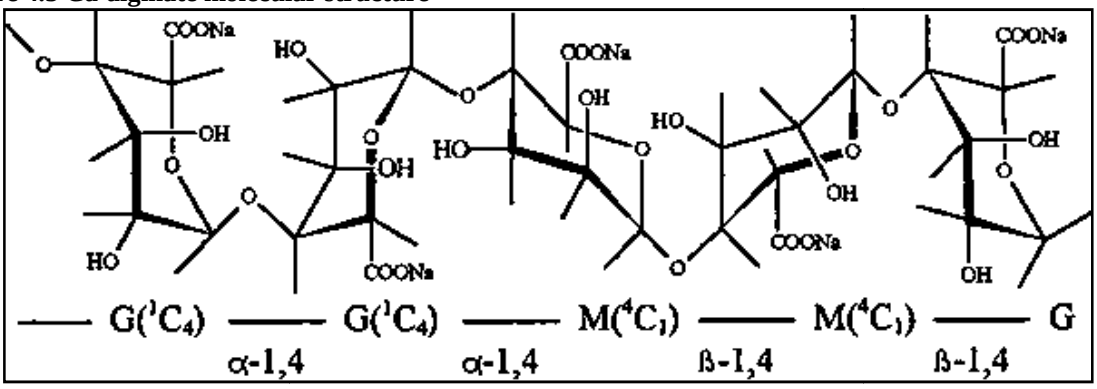
Figure 4.2 Agarose gel structure.



4.5 Preparation and use of calcium alginate-ferulic acid microspheres

Sodium alginate, in presence of divalent cations as Ca^{2+}, is able to form gels. This trait allows to trap organic molecules, such as ferulic acid, within the gel (Fig. 4.3). For this purpose a stock solution of sodium alginate (6% w/v) was mixed to a isovolume of ferulic acid (3% w/v). This solution was dropped into a beaker containing a mixture of calcium chloride (1% w/v) and ferulic acid (1.5% w/v), to allow the formation of microspheres (0.15 mL volume and 0.012 mmoles of ferulic acid each one) and prevent the early release of the substrate.

Figure 4.3 Ca-alginate molecular structure



4.6 Use of copolimeric resin XAD-4®

XAD-4® macroporous adsorption resin with crosslinked styrene-divinylbenzene framework was purchased from Sigma Aldrich® (Fig. 4.4). The physical properties of the resin were listed in Table 4.5. The resin was first soaked with ethanol for 24 h, and subsequently with 2% hydrochloric acid (v/v) and 2% (w/v) sodium hydroxide for 2 hours. Finally, it was washed to neutral with deionized water and dried at 80 °C. XAD-4® was directly used in bioconversion liquor to capture vanillin (final concentration 0.1g/mL).

Figure 4.4 XAD-4® resin particles

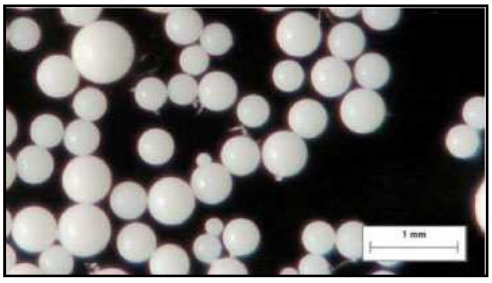


Table 4.5 XAD-4® Resin properties

Autoignition temp.	800 °F
Matrix	styrene-divinylbenzene
Particle size	20-60 mesh
Pore size	~0.98 mL/g pore volume 40 Å mean pore size
Surface area	725 m^2/g
Density	1.02 g/mL (true wet)(L) 1.08 g/mL (skeletal)(L)

4.7 Experimental design for optimization of bioconversion parameters

Effects of agitation speed and substrate concentration on vanillin and vanillyl alcohol production were evaluated by Response Surface Methodology using a Central Composite Design (Bezerra et al., 2008). Statistical examination of results and generation of response surfaces were performed by the software package Modde 5.0 (Umetrics AB, Umea, Sweden). Data were subjected to the analysis of variance (ANOVA) and fitted according to a second order polynomial model shown by equation:

$$Y = \beta 0 + \Sigma \beta i Xi + \Sigma \beta ii Xi^{2} + \Sigma \beta ij XiXj$$

where Y is the predicted response variable, β0 is the intercept, βi and βii the linear coefficient and quadratic coefficients, respectively, βij the interaction coefficient and Xi and Xj the coded forms of the input variables. The variables and their levels selected in order to optimize the system were: 180-120 rpm for agitation speed and 23.1-7.7 mM for ferulic acid concentration. The correspondence between coded and uncoded variables was established by linear equations deduced from their respective variation limits (Tab. 4.6).

Table 4.6 Dimensionless, coded independent variables used in this study

Variable	Nomenclature	Definition	Variation range
dimensionless stirring speed	X_1	Stirring speed 180-120 (rpm)	(-1,1)
dimensionless ferulic acid concentration	X_2	Ferulic acid 23.1-7.7 (mM)	(-1,1)

Maximum vanillin concentration (Y_1), minimum vanillyl alcohol production (Y_2) were chosen as the dependent variables.

To estimate the impact of single independent variables on the responses, regardless of the presence of the other factors, main effects were calculated by the equation:

$$Y = \beta 0 + \beta i Xi + \beta ii Xi^{2}$$

The design was expanded to a circumscribed central composite design, being 12 the total number of experiments required for this experimental design. The determination coefficient

(R^2) and the absolute average deviation (AAD) were determined. R^2 is a measure of the amount of the reduction in the variability of response obtained by using the repressor variables in the model. Because R^2 alone is not a measure of the model's accuracy, it is necessary to use absolute average deviation analysis, which is a directed method for describing the deviations. R^2 must be close to 1.0 and the AAD between the predicted and observed data must be as small as possible.

All bioconversion experiments were carried out in phosphate saline M9 buffer (pH 9.0) using *E. coli* FR13 *resting* cells, in a final bioconversion volume of 15 mL. Duplicates were performed at all design points in randomized order. Vanillin and vanillyl alcohol formation were experimentally determined by HPLC quantitative analysis.

4.8 Analytical methods

Biomass concentration was determined by optical density (OD) measurements at 600 nm using a spectrophotometer Cary 50 Bio Uv-vis (Varian, Palo Alto CA, USA). A calibration curve was used to relate OD with cell wet weigh determined by scientific scale (E42S, Gibertini, Italia).

Substrate and metabolites occurring in culture supernatant were analyzed by liquid chromatography using a Varian ProStar (Varian, Palo Alto CA, USA) high pressure liquid chromatography system. The HPLC reverse phase system was equipped with a C-18 column (150 x 4.6 mm) and UV detection. The mobile phase was composed of water : methanol (49:49), containing 2% acetic acid and, the flow rate was 0.6 mL/min. Data acquisition and processing were controlled by the System Control Varian Star software (Varian, Palo Alto CA, USA). Sample detection was achieved at two wavelengths, 260 and 320 mm. Compounds were identified comparing their retention time with those of authentic samples. Retention time for vanillyl alcohol, vanillin and ferulic acid are listed in Table 4.7.

Preparation of crude extracts of *E. coli* JM109 pBB1and spectrophotometric determination of feruloyl-CoA synthetase (*fcs*) activity in phosphate buffer at pH range from 7.0 to 10.0, were carried out as described by Calisti et al. (2008). The concentration of soluble protein was determined using the BCA Protein Assay Kit (Pierce, Rockford IL), with bovine serum albumin as a standard.

Table 4.7 HPLC calibration curves for vanillyl alcohol, vanillin and ferulic acid

Compound	Equation	R^2	Retention time (min)
Vanillyl alcohol	Y = 6.388x – 0.0015	0.999	5.93
Vanillin	Y = 65.150x + 0.1095	0.996	8.75
Ferulic acid	Y = 95.596x + 0.268	0.994	9.44

4.9 Statistical analysis

The analysis were performed in triplicate, and the results are presented as the mean ± standard deviation. The Tukey test was used to determine significant differences between the mean values for the different components.

4.10 Chemicals

All chemicals and HPLC solvents were of the highest purity commercially available. Ferulic acid, vanillin and XAD-4® were purchased from Sigma Aldrich (Spruce St. St. Louis, MO). Salts were purchased from Carlo Erba (Milan, Italy). Microbiological media and agar were purchased from Difco (Detriot, USA) and OXOID (England).

5. Results and discussion

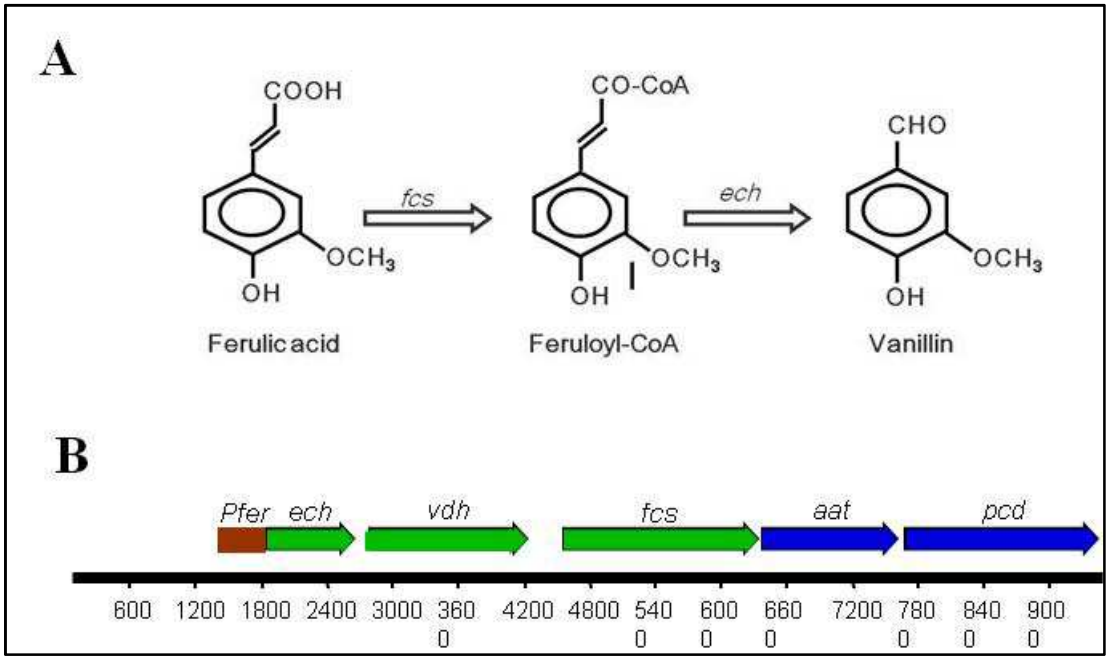
5.1 Optimization of bioconversion buffer

Bioconversion experiments described in this thesis were carried out using resting cells of *E. coli.* These *E. coli* strains express both feruloyl-SCoA synthetase (*fcs*) and feruloyl-SCoA hydratase/aldolase (*ech*).

Escherichia coli cells are not able to utilize ferulic acid or vanillin as a carbon source, however it has been demonstrated that concerted expression in *E. coli* of genes encoding degradation (such as *Pseudomonas fluorescens* strain BF13) confers the ability to convert ferulic acid to vanillin to the recombinant strain (Overhage et al., 1999).

In our laboratory we used several molecular tools to introduce ferulic catabolic genes into *E. coli* cells (low copy, high copy and integrative plasmid vectors) and we demonstrated that the vanillin production yield could be increased using *E. coli* JM109 containing a low copy plasmid (pBB1, Barghini et al., 2007) or a single copy of the catabolic cassette integrated into the *E. coli* chromosome (FR13; Luziatelli et al., 2008)

Figure 5.1 Enzymatic reactions involved in the conversion of ferulic acid to vanillin (A) and organization of the ferulic catabolic operon in *P. fluorescens* strain BF13 (B)



fcs: feruloyl-CoA synthetase; *ech*: feruloyl-CoA hydratase/aldolase; *vdh*:vanillin deidrogenase

5.1.1 Effect of buffer pH on the conversion of ferulic acid to vanillin

Quantitative determination of enzymes activity with cell extracts from *E. coli* JM109 pBB1 expressing *fcs* from *Pseudomonas fluorescens* BF13 indicated that feruloyl-CoA synthetase activity at pH 7.0 and 9.0 were similar, maximum activity was determined at pH 8.0 and it is a significantly decreased at pH higher than 9.5 (Tab. 5.1).

E. coli, during optimal growth conditions maintains its intracellular pH within a range of pH 7.4-7.8 over a external pH range of 5.0-9.0 (Slonczewski et al., 1981; Ziberstein et al., 1984; Slonczewski et al., 2007). In alkaline shift of *E. coli* from 7.2 to 8.3 results in a rapid and transient alkalinization during which the intracellular pH briefly reaches the new external pH. Growth arrests after the shift and does not resume until the cytoplasmic pH is about 7.9, about 15 minutes after the shift, the complete restoration of the pre-shock intracellular pH is observed about 30 minutes after the shock (Ziberstein et al., 1984). The possibility of microorganisms to remain viable during conditions not permitting growth is essential for persistence in a charging environment. Many neutralophiles (including *E. coli*) posses inducible means of maintaining limited pH homeaostasis for several hours under “extreme” pH conditions, enabling survival below pH 3 or above pH 10 (Sloncezewski et al., 2009).

To evaluate the effect of buffer pH on the production of vanillin, bioconversion experiments were carried out incubating *resting* cells in phosphate buffer (64 mM) at pH 6.8, 8.0 and 9.0. results reported in Table 5.2 indicated that increasing pH of the bioconversion buffer from 6.8 (condition A) to 9.0 (condition C) led to an increase of approximately 2-fold in the vanillin accumulation (from 3.10 ± 0.04 mM to 5.92 ± 0.09 mM) and a significant decrease in the production of vanillyl alcohol (from 3.36 ± 0.14 mM to 1.89 ± 0.06 mM), with an increase in the selectivity of the process (mmoles of vanillin/mmoles of vanillyl alcohol), from 0.92 ± 0.09 to 3.13 ± 0.07. Interestingly, growth in the pH of the bioconversion buffer also determined a significant increase (2.35-fold) in the substrate consumption rate which varied from 0.40 ± 0.01 mmoles of ferulic acid/h to 0.94 ± 0.01 mmoles/h.

The increase in the vanillin yield and in the selectivity of the bioconversion process could be related to the predicted deprotonation of this molecule at pH 9.0 which could affect the reduction of vanillin to the corresponding alcohol. A simile effect was observed by Wangrangsimagul et al. (2012) on *Brevibacillus agri* 13. In addition, the increase in the ferulic acid consumption rate, in response to pH change, could be related to variations in membrane permeability and feruloyl-CoA synthetase activity that, as we showed in data reported in Table 5.1, is higher at moderately alkaline pH.

Based on the observation that in *resting* cells the intracellular pH is dependent from extracellular pH (Sloncezewski et al., 2009), we expected that an increase in the intracellular pH should determine an increase in the *fcs* activity and facilitate the transport of ferulic acid (in the cell) and vanillin (out of the cell), limiting the reduction of vanillin to vanillyl alcohol.
Similar results were obtained using resting cells of *E. coli* strains carrying ferulic catabolic genes on a high-copy (JM109 pFS; Barghini et al. 2007) or integrative (FR13; Luziatelli et al., 2008) plasmid vector (Tab. 5.3). with all strains the highest vanillin production was obtained incubating cells at pH 9.0 with a 0.9-fold increase in yield, from 5.32 ± 0.00 to 6.00 ± 0.06 mM, with FR13 strain and a 2.4-fold increase, from 0.90 ± 0.03 to 2.2 ± 0.01 mM, using JM109 pFS cells as a catalyst. In all cases, using buffer C, the selectivity of the process increased significantly up to a value of 5.00 ± 0.04 (FR13 strain Tab. 5.3).
In conclusion, our results indicated that, regardless of the recombinant strain, the use of alkaline bioconversion buffer enhanced the vanillin productivity and selectivity of *E. coli* cells.

Table 5.1 Effect of pH on feruloyl-CoA synthetase activity (*fcs*)

Buffer pH	7.0	7.5	8.0	8.5	9.0	9.5	10.0
***fcs* activity (unit/mL)**	0.0225	0.0335	0.0412	0.0383	0.0257	0.0162	0.0005

Experiments were carried out with 10 µL of crude extract (6 µg of total proteins) from *E. coli* JM109 pBB1 in phosphate buffer at pH range from 7.0 to 10.0.
The results showed are the average of three separate tests.

Table 5.2 Effect of the pH of the bioconversion buffer on the system

	Bioconversion buffer			
	A¥	**B¥**	**C¥**	**D°**
pH	**6.8**	**8.0**	**9.0**	
Vanillin (mM)*	3.10 ± 0.04[a]	5.80 ± 0.04[b]	5.92 ± 0.09[c]	5.50 ± 0.00[d]
Vanillyl alcohol (mM)*	3.36 ± 0.14[a]	2.20 ± 0.02[b]	1.89 ± 0.06[c]	1.65 ± 0.01[d]
Vanillin/vanillyl alcohol	0.92 ± 0.09[a]	2.63 ± 0.03[b]	3.13 ± 0.07[c]	3.33 ± 0.01[d]
Ferulic acid consuption rate (mmoles/h)**	0.40 ± 0.01[a]	0.84 ± 0.01[b]	0.94 ± 0.09[b]	0.91 ± 0.03[b]

Results were obtained using *E. coli* JM109 pBB1 resting cells at 30 °C. All buffers have [PO_4] 64 mM and [Na]/[K] between 4.2 and 4.68. The results were the average of three separate tests.
¥ buffer with NaCl and NH_4Cl. °buffer without NaCl and NH_4Cl
* values calculated after 24h of bioconversion. **values calculated from the second to fifth hour.

Table 5.3 Effect of alkaline pH buffer on several biological systems

E. coli strains	Buffers	Vanillin (mM)*	Vanillyl alcohol (mM)*	Vanillin/Vanillyl alcohol
High-copy plasmid transformant (JM 109 pFS)	**A£**	0.90 ± 0.03[a]	1.4 ± 0.02_a	0.64 ± 0.02[1]
	C¥	2.20 ± 0.01[b]	2.4 ± 0.03_b	0.91 ± 0.01[2]
Single copy integrant (FR13)	**A£**	5.32 ± 0.00[A]	2.60 ± 0.02_A	2.04 ± 0.01_1
	C¥	6.00 ± 0.06[B]	1.20 ± 0.04_B	5.00 ± 0.04_2

Bioconversions were carried out at 30 °C using resting cells. All buffers have [PO_4] 64 mM. The results were the average of three separate tests.
£buffer at pH 6.8 and [Na]/[K] ratio is 4.02. ¥ buffer at pH 9.0 and [Na]/[K] ratio is 4.68. * values calculated after 24h of bioconversion.

5.1.2 Effect of phosphate molarity on the system productivity

Concentration of intracellular solutes change significantly in response to changes in medium osmolarity, therefore, in a second set of experiments we investigated the effect of different [Na]/[K] concentration ratios and phosphate molarity on the productivity and selectivity of the ferulic acid to vanillin bioconversion process. All experiments were carried out at pH 9.0 and in buffers depleted of NH_4Cl. Results reported in Table 5.4 indicated that increasing phosphate concentration, from 46.9 mM (buffer E) to 70 mM (buffer F) determined an increase in the vanillin concentration up to 5.72 ± 0.12; at phosphate concentration higher that 70 mM the molar concentration of vanillin decreased up to 4.71 ± 0.00 (buffer H). Moreover, the use of a sodium depleted buffer (buffer J) led to a lower substrate consumption rate (0.74 ± 0.02); while, with a potassium depleted buffer (buffer H) the selectivity of the process was significantly lower (2.35 ± 0.01). Extreme values of [Na]/[K] ratio (buffer F: 0.26 and buffer G: 140.84) allowed us to obtain a higher vanillin productivity (vanillin concentration up to 5.72 mM).

Results also indicated that [Na]/[K] ratio affected ferulic acid consumption rate and process selectivity, i. e. buffer F compared to buffer G, the substrate consumption rate was about 12% lower while the product selectivity was 16% higher.

Table 5.4 Effect of phosphate concentration on the bioconversion process

	Bioconversion buffer					
Molar concentration (mM)	**E**	**D**	**F**	**G**	**H**	**J**
[PO_4]	**46.9**	**64**	**70**	**100**	**200**	**200**
[Na]/[K]	**1.13**	**4.27**	**0.26**	**140.84**	**no K**	**no Na**
Vanillin (mM)*	5.42 ± 0.04^{a}	5.50 ± 0.00^{a}	5.72 ± 0.12^{b}	5.61 ± 0.09^{b}	4.71 ± 0.00^{c}	5.41 ± 0.02^{d}
Vanillyl alcohol (mM)*	1.26 ± 0.01^{a}	1.65 ± 0.01^{b}	1.44 ± 0.05^{c}	1.64 ± 0.02^{b}	2.00 ± 0.01^{d}	1.62 ± 0.01^{b}
Vanillin/vanillyl alcohol	4.31 ± 0.02^{a}	3.33 ± 0.01^{b}	3.97 ± 0.08^{a}	3.42 ± 0.11^{b}	2.35 ± 0.01^{c}	3.33 ± 0.01^{b}
Ferulic acid consuption rate (mmoles/h)**	0.90 ± 0.01^{a}	0.91 ± 0.03^{a}	0.86 ± 0.01^{b}	0.98 ± 0.06^{a}	0.96 ± 0.02^{a}	0.74 ± 0.02^{c}

All buffers have the same pH (9.00) and are NaCl and NH_4Cl depleted. The results were the average of three separate tests.
* values calculated after 24h of bioconversion. **values calculated from the second to fifth hour.

5.1.3 Effect of [Na]/[K] concentration ratio on biovanillin production

In a subsequent series of experiments we evaluated, in more detail, the effect of changes in the [Na]/[K] ratio on the production of vanillin. Experiments were carried out in buffers at pH 9.0, phosphate molarity of 70 mM and [Na]/[K] ratio of 0.0125 (buffer K), 88.9 (buffer L), 0.93 (buffer M) (Tab. 5.5).

$[Na^+]$ and $[K^+]$ play a primary role in the regulation of osmolarity in *E. coli* cells, and are involved in the regulation/activation of many cellular enzymes. Active transport is responsible for cells containing relatively high concentrations of potassium ions but low concentrations of sodium ions. The mechanism responsible for this is the sodium-potassium pump, discovered in 1957 by Jens Christian Skou, which moves these two ions in opposite directions across the membrane. It is known that the carrier is an ATP-ase and that it pumps three sodium ions out of the cell for every two potassium ions pumped in. The difference between the internal and the external osmolarity, known as the turgor pressure, is required for growth and bioconversion. In *E. coli* the turgor pressure is maintained at approximately 3 atm (Laimins et al. 1980) by the accumulation of K^+ and expulsion of Na^+.

It should be noted that [Na]/[K] significantly affects several parameters such as the maximum amount of vanillin and the substrate consumption rate. Conversely no significant difference is observed on selectivity of the system. Increasing the ratio of Na and K a decrease, from 0.86 ± 0.01 to 0.52 ± 0.02 (mmoles/h), in the consumption rate was observed; if this value is approximately 1 there is also a decrease in selectivity. Studies conducted on this system have shown that if the concentration of sodium increases from 1.75 mM (Buffer K) to 30 mM (buffer F), there is an increase in the speed of catalysis that descends back if this value achieves 65-70 mM (buffer results not shown). The same tests were carried out adding NaCl (1M) and NH_4Cl (1M) to buffer K, L and M. The addition of this salts resulted in an increase from 15 to 45% in the ferulic acid consumption rate, but not in an increase of final vanillin accumulation (results not show). In this system vanillin yield decreased from 7.69 ± 0.05 to 6.08 ± 0.06 using buffer K and from 7.56 ± 0.02 to 5.29 ± 0.05 using buffer L.

Table7 5.5 Effect of [Na]/[K] concentration ratios on the bioconversion process

	Bioconversion buffer		
	K	**L**	**M**
[Na]/[K]	0.0125	88.9	0.93
Vanillin (mM)*	7.69 ± 0.00[a]	7.56 ± 0.02[a]	6.84 ± 0.04[b]
Vanillyl alcohol (mM)*	1.23 ± 0.02[a]	1.23 ± 0.01[a]	1.16 ± 0.01[b]
Vanillin/vanillyl alcohol	6.25 ± 0.02[a]	6.14 ± 0.01[a]	5.7 ± 0.01[b]
Ferulic acid consuption rate (mmoles/h)**	0.86 ± 0.01[a]	0.52 ± 0.02[b]	0.66 ± 0.03[b]

All buffers have [PO_4] 70 mM and the same pH value (9.00). The results were the average of three separate tests.
* values calculated after 24h of bioconversion. **values calculated from the second to fifth hour.

5.1.4 Bioconversion experiments carried out in buffers without phosphate

In order to assess the potential of calcium alginate gel for the entrapment and controlled release of ferulic acid, the identification of a buffer, without phosphate, was necessary. This phosphate-free buffer could be used, as an alternative to optimized buffer (Section 5.1.3), to efficiently perform the bioconversion of ferulic acid into vanillin. The buffers used were TRIS-HCl, sodium borate buffer and glycine all at pH 9.0. As shown in Table 5.6, the vanillin accumulation after 24 hours of bioconversion, consumption and production rate significantly varied in the different conditions examined. Using glycine buffer, although the production rate during the first hours is less than 50% compared to that obtained in TRIS-HCl, in the same timeframe this buffer has the highest substrate consumption rate and after 24 hours of incubation the amount of aldehyde produced is double (2.76 ± 0.06 mM compared to 1.25 ± 0.07 mM in TRIS-HCl).

Based on our results we can conclude that among buffers without phosphate, glycine buffer allowed us to obtain the best results, in terms of quantity of produced vanillin. For this reason the glycine buffer can represent a valid alternative to phosphate buffer in processes that involve the use of microspheres of calcium alginate – ferulic acid.

Table8 5.6 Effect of bioconversion buffer on vanillin production: buffers devoid of phosphate

Buffer	Vanillin (mM)*	Vanillin production rate (mmoles/h)**	Ferulic acid consumption rate (mmoles/h)**
TRIS-HCl	1.25 ± 0.07^{a}	0.64 ± 0.01^{A}	$0.515 \pm 0.02_{a}$
Sodium borate buffer	1.05 ± 0.04^{a}	0.07 ± 0.01^{B}	$0.378 \pm 0.01_{b}$
Glycine	2.76 ± 0.06^{b}	0.33 ± 0.03^{C}	$0.807 \pm 0.04_{c}$

Bioconversions were carried out at 30°C, 150 rpm using *resting* cell of *E. coli* FR13 as biocatalyst [4.5 mg (wet weight)/mL] and 7.7 mM ferulic acid. The results were the average of three separate tests.
*values calculated after 24 hours of bioconversion. **values calculated from second to fifth hours.

5.2 Optimization of bioconversion conditions to improve vanillin production using the RSM methodology

In recent years the Response Surface Methodology (RSM) has become the most popular method for the optimization of experimental conditions. It is an effective mathematical and statistical technique, requiring minimal experimental trials, where interactive factors may be involved (Ryad et al., 2010).

After optimization of the composition of the bioconversion buffer by a one-variable-at-a-time technique, we evaluated, by RSM, the effect of stirring speed (X_1, 120-180 rpm) and substrate initial concentration (X_2, 7.7-23 mM) on the production of vanillin and unwanted products such as vanillyl alcohol. According to a full three-level factorial design, the whole design consisted in a total of 12 experiments with three coded levels for all the two factors and three replicates at the center of the design. Experiments were carried out in duplicate and replicates at the center point were used to estimate the experimental error employed.

The range of variables, experimental replicates and results for vanillin yield are presented in Table 5.7.

Table 5.7 3^2 full factorial design matrix and responses of the dependent variables

Run n°	x_1	x_2	Stirring speed (rpm)	Ferulic acid (mM)	Y_1	
					Exp.	Pred.
1	-1	-1	120	7.7	4.34	4.48
2	0	-1	150	7.7	6.51	6.44
3	1	-1	180	7.7	4.73	4.84
4	-1	0	120	15.4	5.72	5.84
5	0	0	150	15.4	7.00	7.80
6	1	0	180	15.4	5.90	6.19
7	-1	1	120	23.1	4.30	4.09
8	0	1	150	23.1	5.98	6.05
9	1	1	180	23.1	4.60	4.44
10	0	0	150	15.4	7.82	7.80
11	0	0	150	15.4	8.54	7.80
12	0	0	150	15.4	8.22	7.80
13	-1	-1	120	7.7	4.50	4.48
14	0	-1	150	7.7	6.51	6.44
15	1	-1	180	7.7	4.93	4.84
16	-1	0	120	15.4	5.85	5.84
17	0	0	150	15.4	7.20	7.80
18	1	0	180	15.4	6.10	6.19
19	-1	1	120	23.1	4.10	4.09
20	0	1	150	23.1	5.63	6.05
21	1	1	180	23.1	4.60	4.44
22	0	0	150	15.4	7.80	7.80
23	0	0	150	15.4	8.15	7.80
24	0	0	150	15.4	8.10	7.80

Bioconversions were carried out in phosphate saline M9 buffer (pH 9.0) using *E. coli* FR13 as biocatalyst. Experimental values are average of duplicates within ± 5% standard error. x_1 = coded value of stirring speed (X_1); x_2 = coded value for ferulic acid initial concentration (X_2); Y_1 = vanillin yield (mM).

5.2.1 Optimization of vanillin production

Global predictivity of the model

On the bases of the experimental results we calculated the coefficient of determination R^2 which measures the variability explained by the factors and their interactions in the observed responses (Ryad et al., 2010). This value corresponds to 0.951 for the model, which means that 95.1% of the vanillin production is attributed to independent variables and only 5% of the total variability is not explained by the model. R^2_{Adj} (adjusted determination coefficient) value is the correlation measure for testing the goodness-of-fit of the regression equation (Liu et al. 2010). The R^2_{Adj} value of this model is 0.937, indicating only 1.7% of the total variations

were not explained by the model. The Absolute Average Deviation (AAD) is low (1.2). Thus the model can be considered as globally predictive, since it explains the global variability.

Analysis of the quadratic model

As shown in Table 5.8, the analysis of variance (ANOVA) of vanillin yield indicated that the model revealed a high reliability and a good statistical performance. For the analyzed response, the probability for the regression was significant at 95% and there was no lack of fit. The results indicate that the model used to fit response variables was significant ($p < 0.0001$) and adequate to represent the relationship between the response and the independent variables. F-test suggests that the model had a very high F-value (F=69.88), indicating that such model was highly significant.

Table 5.8 ANOVA table for the quadratic model for vanillin production (results in bold are significant)

Source	SS	DF	MS	F-value	*p*-value
Model	45.42	5	9.08	69.88	**0**
Residual	2.34	18	0.13		
Lack of fit	0.31	3	0.10	0.76	0.534
Pure error	2.03	15	0.13		
Total	949.133	24			

SS: sum of square; DF: degree of freedom; MS: mean square

Concerning our findings, the RSM applied to Y_1 showed that this parameter is highly correlated to the model; the regression analysis (Tab. 5.8) demonstrate that the quadratic parameters were significant at the level of $p < 0.0001$. It was evident that linear and interaction terms were insignificant at the level of $p > 0.1$.

Table 5.9 Estimated regression model of relationship between vanillin production and independent variables (results in bold are significant)

Term	Coefficient	Standard error	*p*-Value
Y_1			
Intercept	**7,7956**	0,1164	**0,0000**
x_1	0,1271	0,0752	0,1082
x_2	-0,1432	0,0752	0,7298
x_1^2	**-0,9302**	0,0815	**0,0000**
x_2^2	**-0,8095**	0,0815	**0,0000**
$x_1 x_2$	-0,0013	0,0665	0,9841

Response for the vanillin production could be expressed by the following equation in terms of coded values:

$$Y_1 = 7.7956 - 0.9302x_1^2 - 0.8095x_2^2$$

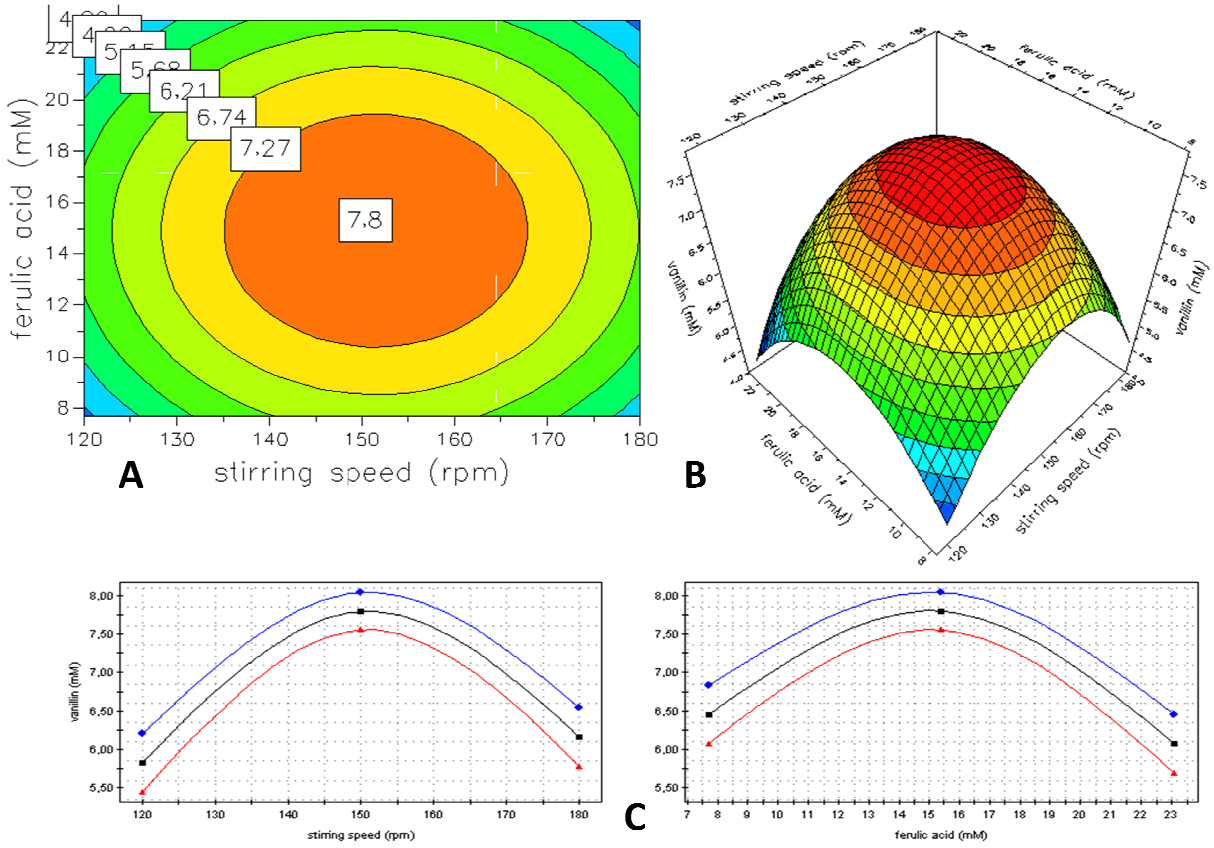
Where Y_1 is the amount of vanillin (mM) while x_1 and x_2 are the coded variables for stirring speed and ferulic acid concentration.

Analysis of response surface

The regression equation was graphically represented by 3D response surface and 2D contour plot (Fig. 5.2). Figure 5.2B shows vanillin accumulation after 24 hours of incubation measured at various combinations of stirrer speed and ferulic acid concentration as suggested by the model and the effect of the dependent variables on the vanillin accumulation can be observed. As expected, both stirring speed and substrate concentration strongly affected catalytic activity of *E. coli* FR13 cells. However, positive effects were only observed until a threshold level is attained, after which negative effects started. This was evident either when the variables were considered alone or in combination. Figure 5.2C shows the single effect of independent variables including maximum, minimum and average predicted activities (confidence level, 95%). The circular contour plot (Fig. 5.2A) indicates that interaction between variables was negligible, as it can also be appreciated from the data recorded in Table 5.8. Accordingly to the model, when ferulic acid concentration and stirring speed were maintained in the range of 12-18 mM and 135-165 rpm, production of vanillin increased from 7.49 ± 0.05 to 7.80 ± 0.06. Beyond these ranges, and regardless to the combination that

was chosen, vanillin yield always significantly decreased, in certain conditions, the decrease was dramatic (i.e. ferulic acid > 20mM). The response surface results indicated that the highest vanillin concentration (7.8 mM) could be obtained incubating cells at 150 rpm in the presence of 15.5 mM of ferulic acid.

Figure 5.2 Contour plot (A), Response Surface (B) and single effect of stirring speed and ferulic acid concentration on vanillin production (C)



Bioconversion experiments were carried out using *E. coli* FR13 *resting* cells and M9 phosphate buffer at pH 9.0 (15 mL).

5.2.2 Reduction in the formation of unwanted by-products

Vanillyl alcohol is a product formed by the reduction of vanillin; the formation of this by-product can account for reduction in vanillin molar yield and system product selectivity. Monitoring and limiting the formation of vanillyl alcohol is essential in order to maintain a high productivity. Therefore, Response Surface Methodology was used with the aim of identifying stirring speed and ferulic acid concentration conditions leading to a minor vanillyl alcohol production.

Global predictivity of the model

The goodness of the model was checked by the statistical parameters for correlation and significance (R^2, R^2_{adj} and AAN), calculated on the bases of the experimental results. The determination coefficient (R^2) was higher than 0.927, indicating that 92.7% of the variability in the response could be explained by the second order polynomial equation given down, allowing a close reproduction of experimental data, meanwhile the values of the adjusted determination coefficients (R^2_{adj}) was higher than 0.903, indicating that the model-predicted values are in perfect agreement with the experimental values. AAD coefficient, representing the average of the absolute deviations, a summary statistic of variability, is low (0.20), indicating the global predictivity of the model. The whole design is listed in Table 5.10 that shows experimental and predicted data obtained.

Table 5.10 3^2 full factorial design matrix and responses of the dependent variables

Operational conditions						
Run n°	x_1	x_2	Stirring speed (rpm)	Ferulic acid (mM)	Y_2	
					Exp.	Pred.
1	-1	-1	120	7.7	1.56	1.58
2	0	-1	150	7.7	1.36	1.29
3	1	-1	180	7.7	1.10	1.12
4	-1	0	120	15.4	1.23	1.15
5	0	0	150	15.4	1.04	0.90
6	1	0	180	15.4	0.84	0.77
7	-1	1	120	23.1	0.97	1.02
8	0	1	150	23.1	0.91	0.81
9	1	1	180	23.1	0.65	0.73
10	0	0	150	15.4	0.78	0.90
11	0	0	150	15.4	0.84	0.90
12	0	0	150	15.4	0.78	0.90
13	-1	-1	120	7.7	1.56	1.58
14	0	-1	150	7.7	1.30	1.29
15	1	-1	180	7.7	1.10	1.12
16	-1	0	120	15.4	1.23	1.15
17	0	0	150	15.4	0.97	0.90
18	1	0	180	15.4	0.84	0.77
19	-1	1	120	23.1	0.97	1.02
20	0	1	150	23.1	0.91	0.81
21	1	1	180	23.1	0.71	0.73
22	0	0	150	15.4	0.84	0.90
23	0	0	150	15.4	0.84	0.90
24	0	0	150	15.4	0.78	0.90

Bioconversions were carried out in phosphate saline M9 buffer (pH 9.0) using *E. coli* FR13 as biocatalyst. Experimental values are average of duplicate within ± 5% standard error. x_1 = coded value of stirring speed (X_1); x_2 = coded value for ferulic acid initial concentration (X_2); Y_2 = vanillyl alcohol concentration(mM).

Analysis of the quadratic model

As shown in Table 5.11, the model revealed a high reliability and a good statistical performance; the probability for the regression was significant at 95%. The analysis of variance reveals a very high significance for the regression equation ($p < 0.0001$) and confirms the suitability of the model. F-test and the value of probability show that the model is statistically significant, while the lack of fit is not significant, this indicating that the model fits well the experimental data.

Table 5.11 ANOVA table for the quadratic model for vanillyl alcohol production (results in bold are significant)

Source	SS	DF	MS	F-value	*p*-value
Model	1.335	5	0.267	35.018	**<0.0001**
Residual	0.137	18	0.008		
Lack of fit	0.068	3	0.023	5.020	0.013
Pure error	0.068	15	0.005		
Total	25.692	24	1.071		

SS: sum of square; DF: degree of freedom; MS: mean square

For vanillyl alcohol concentration, the application of RSM yielded the following regression equation:

$Y_2 = 0{,}8952 - 0{,}1900x_1 - 0{,}2383x_2 + 0{,}1519x_2^2$

In this case, in opposition to Y_1, the effects of the linear terms of the two independent variables are statistically significant at a probability level lower than 5% (Tab. 5.12). The coefficients of both variables are negative, confirming the inhibitory effect of a high initial substrate concentration, and indicating that alcohol formation is favoured by agitation at low speed (Fig. 5.3). The regression analysis (Tab. 5.12) demonstrate that the linear parameters were significant at the level of p <0.0001 while quadratic term linked to ferulic acid concentration was evident at the level of p <0.0008. Interaction and quadratic term linked to stirring speed were not significant (p >0.1).

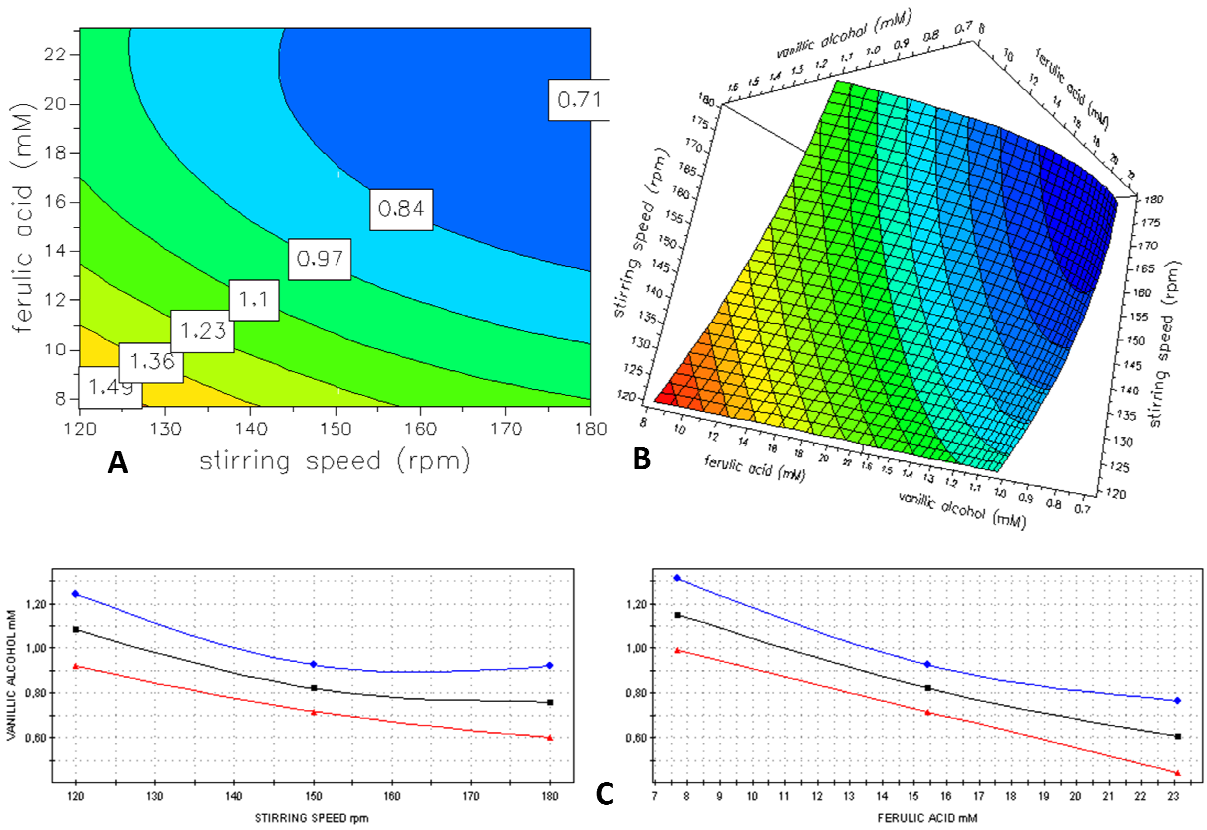
Table 5.12 Estimated regression model of relationship between vanillyl alcohol production and independent variables (results in bold are significant)

Term	Coefficient	Standard error	*p*-Value
Y_2			
Intercept	0,8952	0,0282	**0,0000**
x_1	-0,1900	0,0252	**0,0000**
x_2	-0,2383	0,0252	**0,0000**
x_1^2	0,0669	0,0378	0,3987
x_2^2	0,1519	0,0378	**0,0008**
$x_1 x_2$	0,0425	0,0309	0,1855

Analysis of response surface

From regression equation, 3D response surface and 2D contour plot were obtained (Fig. 5.3). The three-dimensional figure shows the effects of the independent variables and their mutual interaction on vanillyl alcohol production. At high values of the two variables (180 rpm and 23 mM, respectively), a decrease in the production of vanillyl alcohol was observed, while the decrease of both substrate concentration and stirring speed, in all cases, led an increase in vanillyl alcohol concentration. It is worth noting that the highest vanillyl alcohol concentration (1.55 ± 0.07 mM) was obtained at the lowest values of both parameters: of 7.7 ferulic acid and 120 rpm. This results can be ascribed to a strong reductive activity, stimulated by low aeration and long bioconversion time. When ferulic acid concentration values were low (7.7 mM), vanillyl alcohol production increased with stirring speed. However, when substrate concentration was kept at its higher level (23 mM), a lower by-product yield was always achieved (Fig. 5.3). Thus, stirring speed is the most important factor in controlling vanillyl alcohol production. This was even more evident considering the single parameter effect: agitation was much more effective than substrate concentration, being its relative curve much steeper (Fig. 5.3C). The experimental results indicated a minimum vanillyl alcohol concentration of 0.71 mM which is in good agreement with the model prediction (0.73 mM).

Figure 5.3 Contour plot (A), Response Surface (B) and single effect of stirring speed and ferulic acid concentration on vanillyl alcohol production (C)



Bioconversion experiments were carried out using *E. coli* FR13 *resting* cells and M9 phosphate buffer at pH 9.0 (15 mL).

5.2.3 Bioconversion under optimized conditions

Since the general aim of this project is to improve vanillin yield and minimize formation of vanillyl alcohol we used the model to predict the best experimental conditions to obtain this goal. The analysis indicated that the optimum stirring speed and ferulic acid concentration were 151 rpm and 14.94 mM, respectively. The model-predicted value of vanillin and vanillyl alcohol amount at optimal values were found to be 8.24 mM and 0.90 mM, respectively. To confer this prediction three sets of experiments were carried out at the optimal values of stirring speed and substrate concentration. The experimental values of vanillin and vanillyl alcohol were observed to be 8.51 ± 0.02 mM and 1.15 ± 0.02 mM respectively, in agreement with the model prediction, this indicating that the optimization was reliable in the present study.

We finally performed experiments in the optimized conditions of stirring speed and ferulic acid concentration using the best buffer formulation identified in Section 5.1 (buffer K).

Table 5.13 Bioconversions carried out at optimal condition of stirring speed and initial ferulic acid concentration (151 rpm and 14.94 mM), using optimal and standard composition buffer

	Optimal buffer[¥]	**Standard Buffer[£]**
[PO_4]	**70**	**64**
[Na]/[K]	**0.0125**	**4.7**
Molar concentration (mM)		
Vanillin*	11.63 ± 0.1^{a}	8.51 ± 0.02^{b}
Vanillyl alcohol *	1.73 ± 0.08^{a}	1.15 ± 0.01^{b}
Vanillin/vanillyl alcohol	6.72 ± 0.09^{a}	7.4 ± 0.02^{b}

Bioconversion experiments were carried out using *E. coli* FR13 resting cells. All buffers have pH 9.0. (15 mL) [¥] Buffer K (Section 5.1). [£] Buffer C (Section 5.1)
*values calculated after 24 hours of bioconversion.

Data shown in Table 5.13 indicated that, modifying the bioconversion buffer it was possible to finally increase the vanillin yield by 26% (from 8.51 ± 0.02 to 11.63 ± 0.1), but, unfortunately, a similar increase (plus 33%) was also observed in the production of vanillyl alcohol (Tab. 5.13). We are evaluating different strategies to avoid this problem (i.e. continuous removal of vanillin).

5.2.4 Adaptation to substrate

Substrate toxicity is one of the major problems that affects conversion of ferulic acid into vanillin by *E. coli* cells. We know, from our previous results (Section 5.2.1), that high amount of ferulic acid negatively affect the production of vanillin whose yield can decrease as much as 25%. Since working with dilute substrate solutions, waste-water treatment and product recovery costs can be very high, it is essential to found a compromise between product yield and final vanillin concentration to make the process sustainable. For this purpose we carried out bioconversion experiments, using a substrate concentration up to 19.50 mM.

Table 5.14 Effect of low and high initial ferulic acid concentration on vanillin production

	Low substrate concentration			High substrate concentration		
time (h)	**Ferulic acid (mM)**	**Vanillin (mM)**	**Molar yield (%)**	**Ferulic acid (mM)**	**Vanillin (mM)**	**Molar yield (%)**
0	13.90 ± 0.00	0.00		19.56 ± 0.00	0.00	
4	11.43 ± 0.00	2.63 ± 0.02		16.73 ± 0.07	2.03 ± 0.02	
24	3.96 ± 0.00	8.15 ± 0.03[a]	58	9.68 ± 0.03	6.24 ± 0.03[b]	31

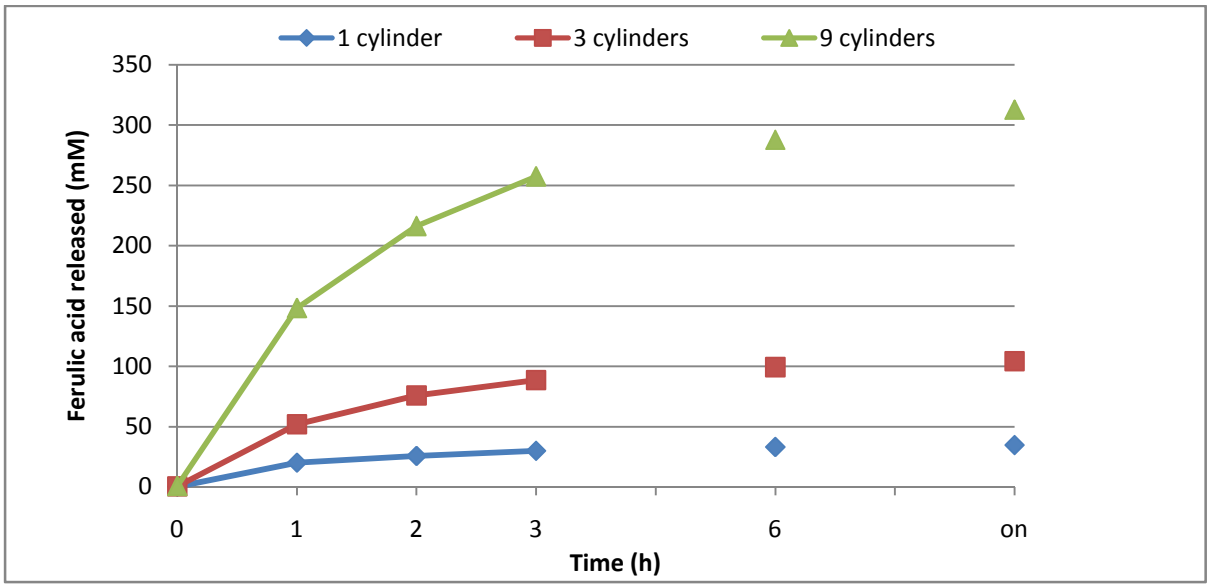
Results reported in Table 5.14 indicated that increasing the initial substrate concentration from 14.00 to 19.50 mM a decrease of almost 20% in vanillin accumulation was observed (from 8.15 ± 0.03 to 6.24 ± 0.03 mM). The ability of the cells to utilize ferulic acid was not affected by the increase in the initial ferulic acid concentration in both conditions, a 10 mM decrease in ferulic acid concentration was achieved. Interestingly, in experiments carried out providing ferulic acid in two steps (14 mM initial concentration + 5mM after two hours of incubation), we observed that reduction in final vanillin concentration can be partially overcome adapting cells to ferulic acid (under this condition vanillin final concentration is: 7.92 ± 0.05 mM and molar yield of 42%).

5.3 Development of efficient two-phase (solid-liquid) system for the controlled release of ferulic acid

5.3.1 Preliminary tests aimed at evaluating the potential of a two – phase (solid – liquid) system

To overcome problems associated with toxicity from high concentration of the substrate (> 20 mM), we evaluate the possibility to carry out bioconversion experiments using cells adapted to ferulic acid. For this purpose we used a sol-gel technology to encapsulate ferulic acid into an agarose-gel matrix and modulate the release in the liquid medium containing *E. coli* cells. Bioconversion experiments were carried out in phosphate saline buffer at pH 9.00, with *resting* cells of *E. coli* FR13, and a number of cylinders between 3 and 6. Release of ferulic acid in the liquid phase was optimized controlling composition (agarose and ferulic acid concentration) and geometry (cylinder size) of gel matrix as well as solid density (number of cylinders per unit volume). Better results were obtained with agarose (1.75% w/v) cylinders 0.6 cm x 1 cm in size, containing 6.75 mg of ferulic acid (data not shown). Diffusion experiments, carried out with 1, 3 and 9 cylinders (Fig. 5.4) indicated that after one hour between 60% and 70% of the ferulic acid contained in the matrix were released into the liquid phase; 80% of the substrate was released at the third hour, and already the sixth almost 100% of the ferulic acid has leaked from agarose cylinders. Bioconversion experiments (Tab. 5.15**)** show that the use of agarose-ferulic acid cylinders was compatible with the bioconversion system. The amount of vanillin accumulated after 24 hours of incubation and production rate were proportional to the amount of ferulic acid provided through the cylinders. In all conditions the amount of residual substrate in the liquid phase allows to estimate a consumption more high than 90%. Therefore two-phase system developed in this work could be a useful tool to overcome the substrate toxicity.

Figure 5.4 Release of ferulic acid from agarose cylinders



Diffusion experiments were carried out at 30°C in 15 mL of saline phosphate buffer (pH 9.0).
1, 3 and 9 cylinders of agarose gel contain 0.034 mmoles, 0.102 mmoles, and 0.306 mmoles, of ferulic acid respectively.

Table 5.15 Effect of the solid density on production of vanillin in bioconversion experiments carried out using the use of a two-phase (solid-liquid) system

Solid density (n°cylinders/15 mL)	Total amount of ferulic acid		Residual ferulic acid (mM)*	Vanillin (mM)*	Vanillin production rate (mM/h)**
	(mmoles)	(mg)			
3	0.104	20.25	0.47 ± 0.03	6.84 ± 0.06	0.77 ± 0.03
4	0.139	27.00	0.76 ± 0.06	7.67 ± 0.04	0.80 ± 0.04
5	0.173	33.75	0.94 ± 0.05	8.12 ± 0.02	0.88 ± 0.05
6	0.208	40.50	1.09 ± 0.04	9.96 ± 0.02	0.93 ± 0.05

Experiments were carried out in triplicate at 30°C, with *E. coli* FR13 cells, in 15 mL of phosphate saline buffer (pH 9.0).
*value calculated after 24 hours of incubation. ** value calculated between 3 and 6 hours.

5.3.2 Optimization of agarose- ferulic acid two phase system

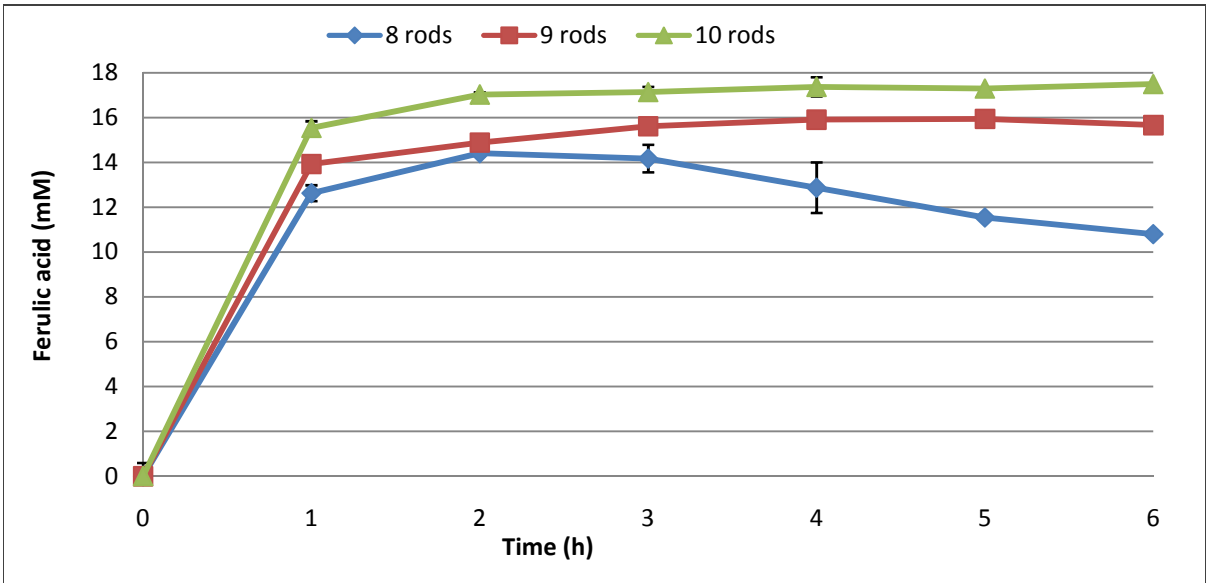
The encouraging results of preliminary experiments with agarose-ferulic acid complex led us to optimize the method. Taking advantage of the two- phase system, the maximum ferulic acid concentration that could be tolerated was investigated. In order to increase the ferulic acid concentration in the bioconversion medium until critical limit points out RSM (Section 5.2), bioconversions experiments were carried out adding 8 (54 mg), 9 (60.75 mg) and 10 (67,5 mg) cylinders to the liquid phase (15 mL). Threshold ferulic acid concentrations has been reached using 9 and 10 cylinders. Using 8 cylinders substrate concentration was always maintained within tolerated limits (Fig. 5.5). Critical concentration of ferulic acid (> 20mM) was never reached. In none of the conditions ferulic acid was completely utilized over 24 hours period (Tab. 5.16). Vanillin yield (13.51 – 15.79 mM), conversion rate (0.72 – 0.87 mM/h) and ferulic acid consumption (> 70%) significantly changed in the different conditions examined. Best results were obtained by providing 9 cylinders. Under this condition vanillin molar yield was higher than 75% and final vanillin concentration was 15.74 ± 0.01 mM; the latter value is 12% higher compared to the 8-cylinders condition, and about 10-fold higher than that reported by De Faveri et al. (2007) using *E. coli* JM109 pBB1. Adding more cylinders determined a decrease in the conversion rate from 0.79 ± 0.04 to 0.72 ± 0.05 mM/h, which is probably due to an excessive accumulation of substrate in the bioconversion buffer. Although the two-phase system was able to attenuate substrate toxicity effect, and avoid reduction in the vanillin production rate; these effect could not be completely abolished using this approach.

Table 5.16 Vanillin production in solid-liquid in experiments carried out increasing the number of agarose-ferulic acid cylinders from 8 to 10

Solid density (n°cylinders/15 mL)	Total amount of ferulic acid		Vanillin (mM)*	Vanillin production rate (mM/h)**	Residual ferulic acid (%)
	mmoles	mg			
8	0.274	54.00	13.51 ± 0.42[a]	0.87 ± 0.03	25
9	0.313	60.75	15.74 ± 0.01[b]	0.79 ± 0.04	28
10	0.348	67.50	14.81 ± 0.16[c]	0.72 ± 0.05	33

Experiments were carried out in triplicate in saline phosphate buffer at pH 9.0 (15 mL), with *E coli* FR13 cells [4.5 mg(wet weight)/mL).
*value calculated after 24 hours of incubation. **value calculated between 3 and 6 hours.

Figure 5.5 Ferulic acid concentration in the liquid phase during the first six hours of bioconversion



Bioconversion were carried out using *E. coli* FR13 cells [4.5 mg (wet weight)/ml], in M9 saline phosphate buffer (pH 9.0). Error bars were standard deviations.

5.3.3 Optimization biomass concentration

To achieve a reduction in the residual concentration of ferulic acid which remained unchanged in the bioconversion medium after 24 hours of incubation, and at the same time to increase the yield of the bioconversion, some experiments were carried out increasing the biomass concentration from 4.5 to 7.5 (wet weight)/mL. Data reported in Table 5.17 indicated that increase in the biomass concentration from 4.5 to 6.0 mg (wet/weight)/mL determined a 2-fold reduction in the residual ferulic acid concentration. A further increase in the biomass concentration [up to 7.5 mg(wet weight)/mL] was not followed by a further reduction in residual substrate concentration. However, increase in the final vanillin concentration and in the vanillin production rate (calculated between 3 to 6 hours) were not directly proportional to the increase in biomass concentration (Tab. 5.17). Furthermore, increase in the biomass concentration up to 7.5 mg(wet weight)/mL determined a significant increase in the formation of vanillyl alcohol which increased from 1.94 ± 0.03 to 2.40 ± 0.03 mM. In summary, these results indicated that increasing the biomass concentration from 4.5 to 6.0 mg(wet weight)/mL it was possible to increase the vanillin production up to 12.52 ± 0.03 mM without the formation of additional amount of secondary metabolites such as vanillyl alcohol.

Table 5.17 Effect of biomass concentration on the production of vanillin in two phase solid-liquid system

Biomass Concentration [mg(wet weight)/mL]	Residual ferulic acid (mM)*	Vanillin (mM)*	Vanillin production rate (mM/h)**	Vanillyl alcohol (mM)*
4.5	$1.78 \pm 0.02_{a}$	11.60 ± 0.04^{a}	0.78 ± 0.04	1.94 ± 0.05^{A}
6.0	$0.85 \pm 0.02_{b}$	12.52 ± 0.02^{b}	0.88 ± 0.03	1.94 ± 0.03^{B}
7.5	$0.85 \pm 0.05_{b}$	12.68 ± 0.01^{b}	0.89 ± 0.04	2.40 ± 0.03^{B}

Experiments were carried out in triplicate in saline phosphate buffer at pH 9.0 (15 mL), using *E. coli* FR13 cells. All bioconversions were carried out using 9 agarose-ferulic acid cylinders (0.313 mmoles of ferulic acid).
*value calculated after 24 hours of bioconversion. **value calculated from third to sixth hours

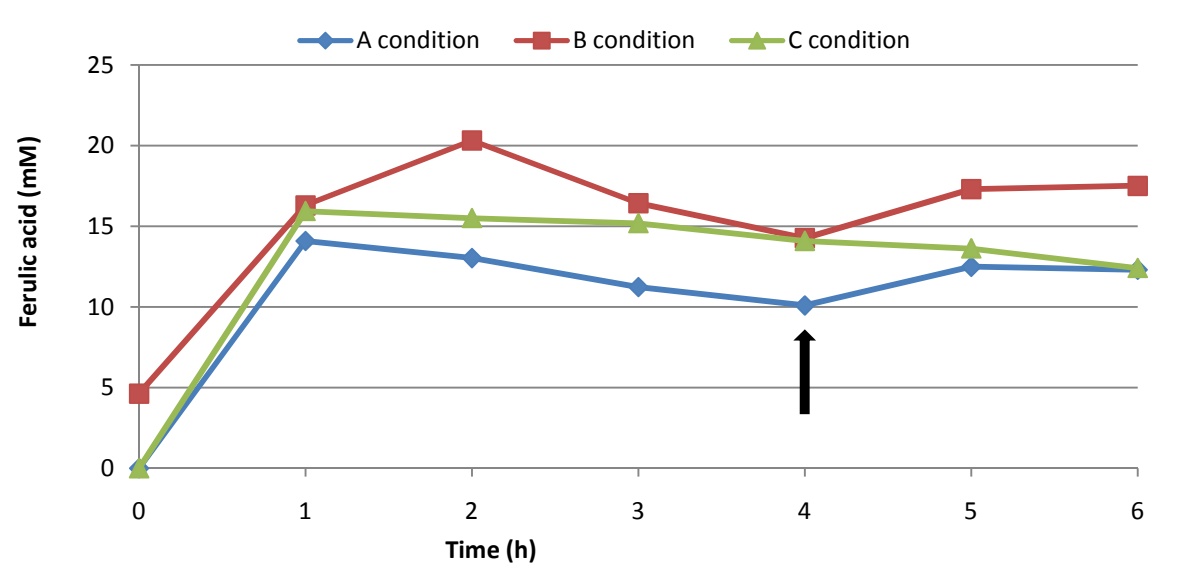
5.3.4 Sequential addition of agarose-ferulic acid cylinders to the bioconversion

Optimization of biomass concentration allowed us to reduce a 50% the residual concentration of ferulic acid (from 1.78 ± 0.02 to 0.85 ± 0.02) and increase the final vanillin concentration up to 12 mM. To further improve these results we carried out experiments in which solid density (number of cylinders per volume unit) was increased during the bioconversion process. The experimental procedure is reported in Table 5.18. In all experiments we added 12 cylinders to the liquid phase (15 mL): 10 + 2 (condition A), 9 + 3 (condition B), 12 + 0 (condition C). In condition B, to obtain an initial ferulic acid concentration above 15 mM (after 1 hour of incubation) phosphate buffer was amended directly with 5 mM ferulic acid (Tab. 5.18). Results indicated that when during the process the ferulic acid concentration reaches about 20 mM, there was a reduction in final vanillin concentration, molar yield and substrate consumption rate (Tab. 5.19). When the ferulic acid concentration in the liquid phase was maintained below 15 mM (condition A and C) no significant differences were observed in both yield and production; instead, under A condition a 2-fold increase in the substrate consumption rate, with respect to C condition, was observed. In brief, this set of experiments indicated that using a fed-batch strategy in two phase solid-liquid system and maintaining ferulic acid concentration under 20 mM during the first hours of bioconversion, it was possible to improve the substrate consumption rate (from 0.62 ± 0.17 to 1.33 ± 0.12). Results demonstrated that two-phase system allows us to obtain the advantages of a fed-batch mode without solution dilution and dispersion of the product. Confirming results obtained with Response Surface Methodology, concentration of ferulic acid up 20 mM negatively affect the performance of the system.

Table 5.18 Experimental procedure for fed-batch experiment in solid-liquid system

	Initial cylinders	Cylinders added after 4 hours	Total Cylinders	Ferulic acid added to the liquid phase
A	10	2	12	0 mM
B	9	3	12	5 mM
C	12	0	12	0 mM

Figure 5.6 Concentration of ferulic acid in the liquid phase during first six hours of bioconversion



Bioconversion were carried out in triplicate, using *E. coli* FR13 cells [6.0 mg (wet weight)/ml], in 15 mL saline phosphate buffer (pH 9.0). Error bars were standard deviations.
Black arrow indicates the addition of cylinders carried out in A and B conditions

Table 5.19 Bioconversions experiments carried out by sequential addition of agarose-ferulic acid cylinders

	Vanillin (mM)*	Ferulic acid consuption rate (mM/h)**	Molar yield (%)
A	17.88 ± 0.01^{a}	1.33 ± 0.12^{A}	65
B	16.55 ± 0.01^{b}	0.68 ± 0.13^{B}	59
C	17.69 ± 0.02^{a}	0.62 ± 0.17^{B}	63

Experimental conditions are shown in Tab.5.17. *value calculated after 24 hours of bioconversion. **value calculated between 2 and 4 hours.

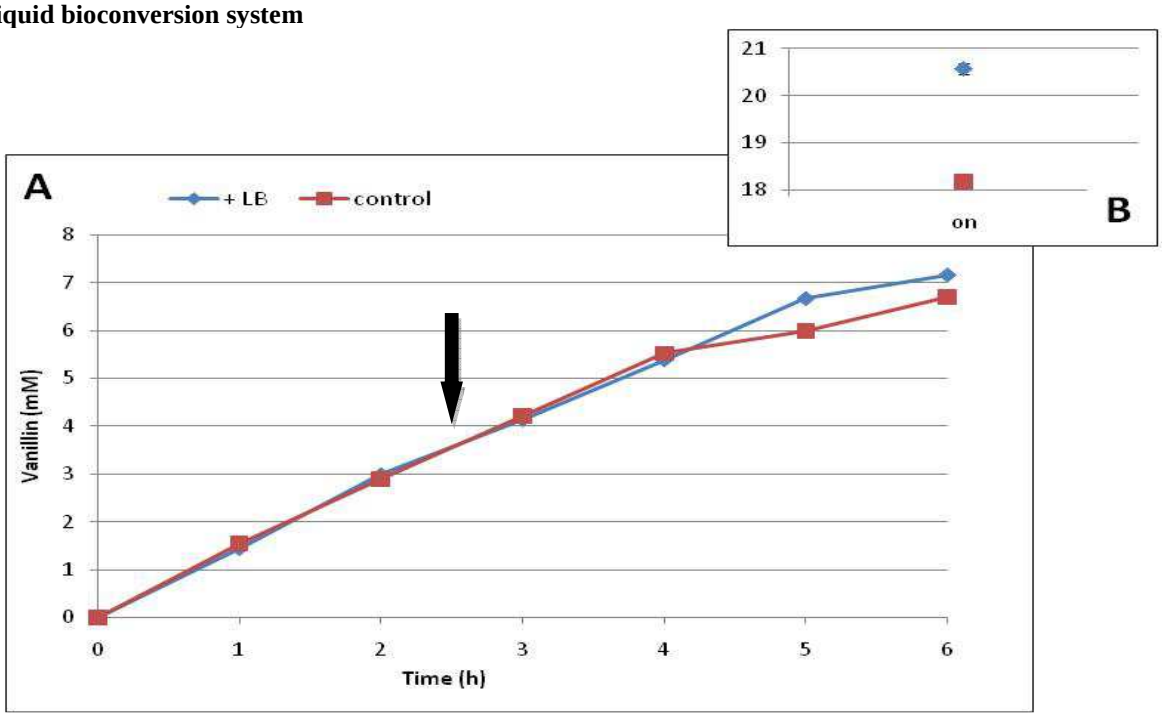
5.3.5 Influence of addition of nutrient on bioconversion process

In all bioconversion experiments described so far a slowdown in the vanillin production rate, regardless of biomass concentration or the number of rods used in the two-phase system, was observed. Bioconversion of ferulic acid to vanillin is a cofactor-dependent process, and a slowdown in the production rate could be linked to cofactor availability into the *resting* cells including the NAD^+/NADH ratio. To stimulate cellular activity and maintain a constant production rate over the time, were carried out some experiments amending the liquid phase with LB medium (final concentration 0.067-fold) as a source of nutritional factors. Vanillin production and ferulic acid consumption were constantly monitored over the first six hours. Results reported in Figure 5.7 showed that the addition of LB medium to the liquid phase allowed us to maintain the high initial production rate for a longer time, suggesting that the addition of nutritional factors to the two-phase bioconversion system has a positive effect on the vanillin production. This positive effect persisted over time and determined, after a overnight of incubation, a 6% increase in vanillin production compared to the control; as reported in Figure 5.7 (panel B) the final vanillin concentration increased from 18.17 ± 0.05 mM up to 20.57 ± 0.11 mM. In agreement with other authors (Berrios-Rivera et al., 2002; Lee et al., 2008) our results indicated that the presence of cofactors plays an important role in the metabolic flux and can mitigate the stress caused by high concentration of the substrate. In conclusion in the presence of nutritional factors the catalyst could be active for a longer period with a greater efficiency.

The repeated addition of LB medium to bioconversion buffer did not determined a further increase in vanillin production (Fig. 5.8).

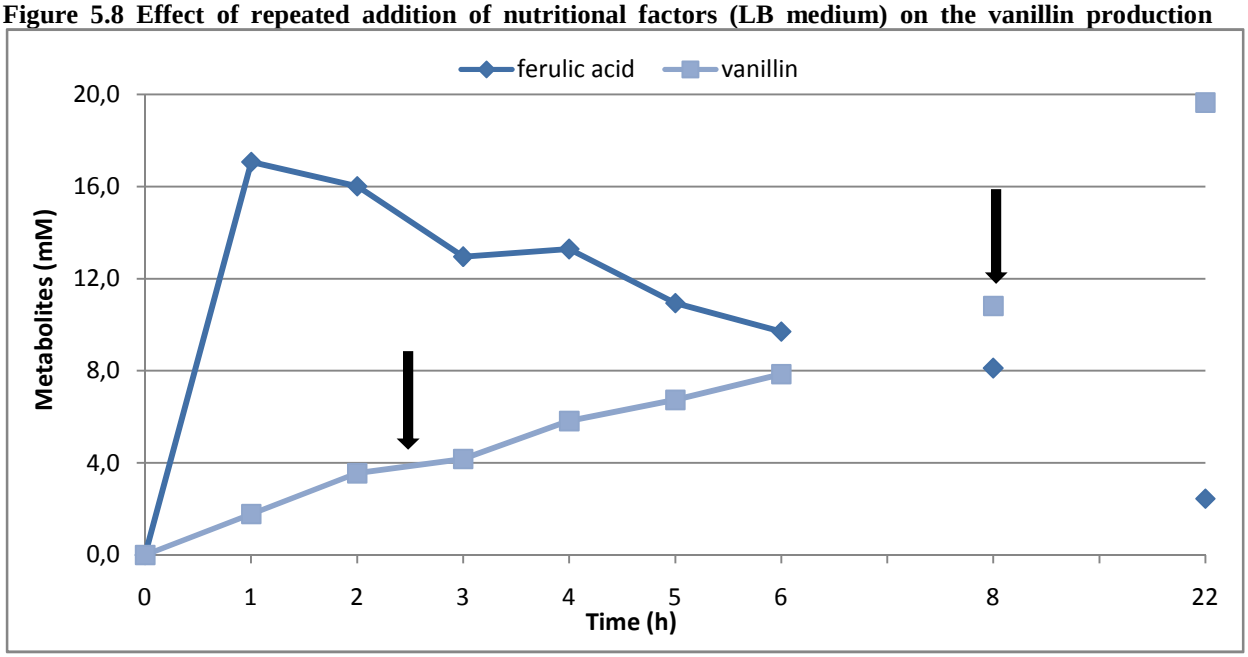
The combine use of the two-phase bioconversion system and the addition of nutrient factors to the bioconversion medium allowed us to increase the final vanillin concentration in the liquid phase of 5-fold and obtain up 20.57 mmoles/L of vanillin in a overnight incubation which is 20% more of the total amount of vanillin obtained by Barghini et al. (2007) recycling *E. coli* biomass for four cycles (in a 96-hours process).

Figure 5.7 Effect of the addition of nutrient factors (LB medium) in the production of vanillin in solid liquid bioconversion system



Panel A: vanillin concentration in the liquid phase during the first six hours of bioconversion. Panel B: vanillin final concentration after 24 hours of incubation. Bioconversions were carried out in triplicate, with *E. coli* FR13 cells [6.0 mg (wet weight)/ml], in 15 mL of saline phosphate buffer (pH 9.0), using the solid liquid system (12 cylinders which contained 1.25 mg of ferulic acid). Error bars were standard deviations. Black arrow indicates the addition of LB medium after 2.5 hours

Figure 5.8 Effect of repeated addition of nutritional factors (LB medium) on the vanillin production

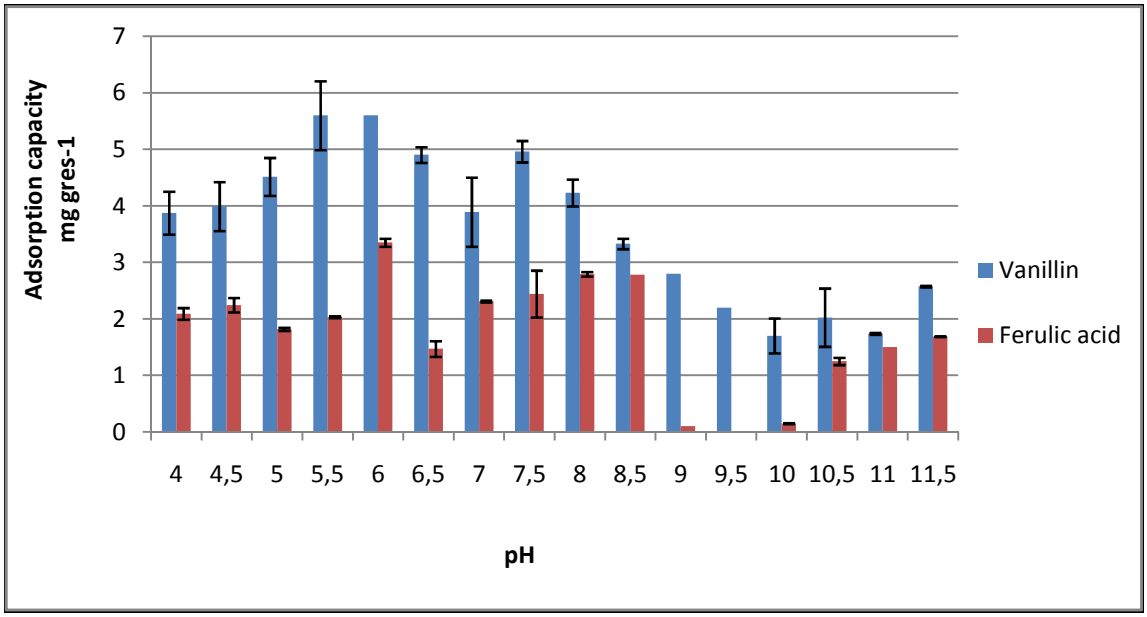


Bioconversions were carried out in triplicate, using *E. coli* FR13 cells [6.0 mg (wet weight)/ml] as biocatalyst, in 15 mL of saline phosphate buffer (pH 9.0), using the solid liquid system (12 cylinders which contained 1.25 mg of ferulic acid). Error bars were standard deviations. Black arrows indicate the addition of LB medium after 2.5 and 8 hours.

5.3.6 Use of macroporous resin XAD-4® in bioconversion process

Reduction in the vanillin production rate can be also related to the accumulation of vanillin in the liquid phase (Priefert et al. 2001; Hua et al. 2006). Several studies showed that the use of macroporous resins can avoid accumulation of vanillin and allow recovering of the product from the liquid phase during the bioconversion process (Zhang et al. 2006; Lee et al. 2008).
In previous experiments we demonstrated that both vanillin and ferulic acid can be absorbed by XAD-4® resin but that the selectivity of the resin was dependent by the pH of the liquid phase (Fig. 5.9). At moderate acidic conditions (pH 5.5-6.0) the resin had the best capacity to absorb vanillin (5.98 mg vanillin/g resin), while the highest selectivity (absorbed vanillin/absorbed ferulic acid) was achieved at alkaline pH (9.0-9.5) (Fig.5.9).
We tried to combine the two phase solid-liquid system described before with the use of XAD-4® resin. Bioconversion experiments were performed as described in Table 5.20 with multiple additions of cylinders to the liquid phase and (condition B) of nutritional factors.
The graphs shown in the Figure 5.10 refer to two treatments (black and grey) that have in common the addition of three cylinders after eight hours and 0.1 g/ml of resin after ten hours; treatment in black also includes the addition of LB medium after 2.5 hours. In this figure is quite evident the shifting of the inflection point as a result of the addition of nutrients to the bioconversion buffer (black line) and the undeniable efficiency of the resin which is able to capture more than 90% of dissolved vanillin. Separation of aldehyde from the resin consists of two moments: flushing in phosphate buffer to remove the cells and the actual separation by ethanol. The process of separation and recovery of the product is very complicated and yet to be optimized. Vanillin estimated by summing the aldehyde residual in the middle and that recovered by ethanol amounts to 12.42 ± 0.03 and 11.99 ± 0.19, for tests with and without LB, respectively. Also in this case, the tests that take advantage of the positive effect of the cofactors addition gave better results: even the inflection point in the first six hours doesn't seem to be present. Unfortunately, as demonstrated by previous studies carried out in our laboratory , the presence of the resin inside the bioconversion buffer, though at a later time, has an inhibitory effect on cellular activity. This effect persists with the addition of LB, even if it is attenuated by the presence of nutrients for the cells.

Figure 5.9 Adsorption capacity of XAD-4® resin at 30°C after 2 hours of bioconversion

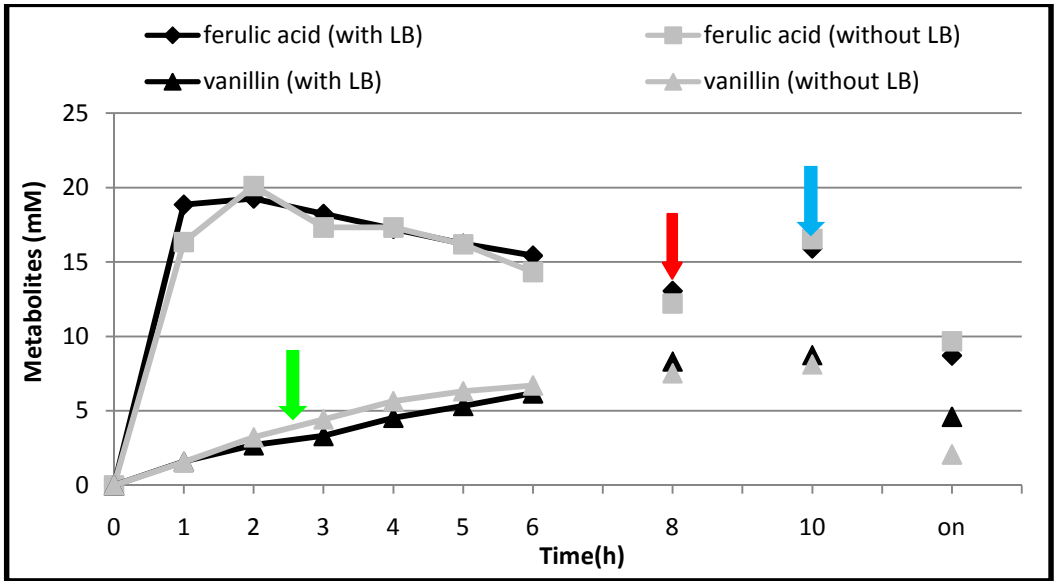


Adsorption experiments using mixture of vanillin and ferulic acid were carried out at different pH, in triplicate. Adsorption capacity was expressed as mg of metabolites adsorbed per gram of resin. Error bars are standard deviations.

Table 5.20 Experimental conditions for the use of XAD-4® resin

	Total cylinders	Initial cylinders	Cylinders added after eight hours	LB 4X added after 2.5 hours	XAD-4® added at ten hours (g/mL)
A	12	9	3	0	0.1
B	12	9	3	1.6% v/v	0.1

Figure 5.10 Bioconversion experiments carried out using the solid-liquid system in the presence of macroporous resin (XAD-4®) and nutritional factors (LB medium)



Green arrow indicates LB medium addition. Red arrow indicates agarose – ferulic acid addition. Blu arrow indicates resin addition

5.3.7 Bioconversions carried out using calcium alginate entrapped ferulic acid

We also evaluate the possibility to use microspheres of calcium alginate for controlled release of ferulic acid in the liquid phase. Bioconversions were carried out in glycine buffer (pH 9.0) using resting cells of *E. coli* FR13 as biocatalyst [4.5 mg(wet weight)/ml] and a variable number of microspheres of calcium alginate – ferulic acid (12 to 48 microspheres, average unit volume of 0.15 mL; Tab. 5.20). As shown in Table 5.21, in none of the three conditions the substrate released from the complex was completely consumed within 24 hours. Furthermore, since ferulic acid is released very quickly from the gel, by increasing the number of microspheres, the concentration of ferulic acid in the medium rapidly becomes critical (27 mM using 24 microspheres and 50 mM using 48 microspheres), thus affecting vanillin production rate. After 24 hours of incubation the most significant differences were observed; with 24 microspheres the accumulation of vanillin was double compared to that obtained with 48 spheres, and greater than 30% compared to that achieved with 12 microspheres.

Table 5.21 Effect of the number of microspheres used on the release of ferulic acid and the formation of vanillin

	Total ferulic acid (mmoles)	Residual ferulic acid (mM)*	Vanillin (mM)*	Vanillin production rate (mM/h)**
12 microspheres	226	10.65 ± 0.11	3.22 ± 0.09^{a}	0.15 ± 0.01^{A}
24 microspheres	453	23.58 ± 0.09	4.73 ± 0.10^{b}	0.13 ± 0.01^{A}
48 microspheres	679	47.79 ± 0.12	2.43 ± 0.03^{c}	0.043 ± 0.00^{B}

*value calculated after 24 hours of bioconversion. **value calculated from second to fifth hours.

6. Conclusions

Results presented in this thesis demonstrated that the ability of *E. coli* cells to produce vanillin can be significantly increased modifying some process parameters and utilizing a two-phase bioconversion system.
Evaluating the effect of pH and composition of the bioconversion buffer on the vanillin production, we could demonstrate that:

- at alkaline pH (9.0), vanillin accumulation was increased 2-fold and vanillyl alcohol formation had a significant decrease;
- vanillin production rate and bioconversion selectivity were affected by the concentration of phosphate buffer;
- changes in [Na]/[K] ratio affected the substrate consumption rate and maximum amount of vanillin which accumulated in the medium.

The highest productivity and selectivity were obtained carrying out the bioconversion experiments in 70 mM sodium-potassium phosphate buffer (pH 9.0), with a [Na]/[K] ratio of 0.0125.

Optimization of the bioconversion conditions using Response Surface Methodology allowed us to demonstrate that vanillin production is dependent on the simultaneous effect of initial concentration of ferulic acid and stirring speed. The same results also indicated that stirring speed is the most important variable in controlling vanillyl alcohol formation during the bioconversion process.

Using *E. coli* FR13 *resting* cells under optimized working conditions, vanillin yield was increased from 8.51 ± 0.02 to 11.63 ± 0.1 mM.

Finally, we developed and tested a new two-phase (solid-liquid) system for controlled release of ferulic acid in the bioconversion medium which allowed us to set up experiments in the presence of large amounts of substrate, maintaining the concentration of ferulic acid in the liquid phase below the inhibitory level. Using the two-phase system is possible to increase the productivity of 20% compared to Barghini et al. (2007), reduce the bioconversion time from four to one day, and increase the final vanillin concentration in the liquid phase of 5-fold. The maximum amount of vanillin that accumulated in the liquid phase under optimized conditions was 20.57 ± 0.05 mM, the highest found in the literature for recombinant *E. coli* strains.
The knowledge gained from this project can be useful for a scale-up of the process from shaking-flask to bench-top fermenter. Moreover, since solid-liquid two phase systems have

not been used for biotechnological production of vanillin and aromatic aldehydes, this study could also serve as a guide for employing *E. coli* resting cells in high-level biosynthesis of aromatic molecules of interest to the food industry.

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