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TITOLO TESI

Vanillin production from ferulic acid with *Pseudomonas fluorescens* BF13-1p4

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ABSTRACT

Bioconversion of ferulic acid to vanillin represents an attractive opportunity for replacing synthetic vanillin with a bio-based product, that can be label "natural", according to current food regulations. Ferulic acid is an abundant phenolic compound in cereals processing by-products, such as wheat bran, where it is linked to the cell wall constituents. In this work, the possibility of producing vanillin from ferulic acid released enzymatically from wheat bran was investigated by using resting cells of *Pseudomonas fluorescens* strain BF13-1p4 carrying an insertional inactivation of *vdh* gene and *ech* and *fcs* BF13 genes on a low copy number plasmid. Process parameters were optimized both for the biomass production phase and the bioconversion phase using food-grade ferulic acid as substrate and the approach of changing one variable while fixing the others at a certain level followed by the response surface methodology (RSM). Under optimized conditions, vanillin up to 8.46 mM (1.4 g/L) was achieved, whereas highest productivity was 0.53 mmoles vanillin L^{-1} h^{-1}). Cocktails of a number of commercial enzyme (amylases, xylanases, proteases, feruloyl esterases) combined with bran pre-treatment with steam explosion and instant controlled pressure drop technology were then tested for the release of ferulic acid from wheat bran. The highest ferulic acid release was limited to 15-20 % of the ferulic acid occurring in bran, depending on the treatment conditions. Ferulic acid 1 mM in enzymatic hydrolyzates could be bioconverted into vanillin with molar yield (55.1%) and selectivity (68%) comparable to those obtained with food-grade ferulic acid after purification from reducing sugars with a non polar adsorption resin. Further improvement of ferulic acid recovery from wheat bran is however required to make more attractive the production of natural vanillin from this by-product.

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1 INTRODUCTION

1.1 Potential of biotechnological routes for the production of natural flavors

The world market demand for flavors and fragrances, which are widely used in the food and feed as well as cosmetic and pharmaceutical industries, is continuously increasing. Most available flavoring compounds are now produced *via* chemical synthesis, with a very small contribution by the production of "natural" flavors, that is extracted from botanic sources or tissue culture.

In the last decades there has been an increasing consumer trend towards "green" and "eco-friendly" as well as "healthy" processes, which are associated to "bio" or "natural" products.

Despite its satisfactory yields, the flavors production by chemical processes suffers of several disadvantages such the high environmental impact and the low quality of the end product. Moreover, the compounds chemically produced are labeled as "nature identical" (EC Flavor Directive 88/388/EEC) or "artificial" (US Code of Federal Regulations 21 CFR 101.22), decreasing their economic interest.

Therefore, natural flavors are favored in the worldwide market despite their considerably higher prices. However, natural flavor production by direct extraction from botanic sources can no longer satisfy the large market demand because of low concentrations of desired product in plants, which increase the extraction and purification procedures, as well as dependency of the harvest on seasonal, climatic and political features.

Alternative natural sources for flavors production are needed and biotechnology is, by far, the most attractive field of exploration. In general, the advantages of biotechnological approaches are mild reaction conditions, high regio- and enantio-selectivity leading to only one product isomer, no formation of toxic wastes and thus fewer environmental problems.

Moreover, the label of "natural" is no longer limited to flavors extracted from botanic materials, according to the recent US Food and European legislation. The European regulation on flavors (EEC No 1334/2008) defines in article 3 (2) c): ''*Natural flavoring substance shall mean a flavoring 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"*. Within the US Food regulation, the definition of ''natural'' is similar, and, thus, new flavors are entered into the GRAS list (generally recognized as safe).

In this respect, thus, the industrial attraction for biotechnological production of natural flavors, so-called bioflavors, is increasing constantly in order to replace the traditional chemical processes. In consequence, extensive research studies have been carried out mainly in the field of flavors production from various natural precursors by using several microorganism or single enzymes. Among the flavors, vanillin is, by far, the most important for biotechnological applications.

1.2 Vanillin: general features

Vanillin is a plant secondary metabolite and the main constituent of natural *Vanilla*, which acts as an important flavoring and aromatic component used worldwide. Vanillin is very versatile flavor and most people enjoy the aroma, making it the world's principal and most popular flavor (Schrader *et al.*, 2004).

Vanillin is mainly used for the preparation of food (i.e. ice-cream, various other dairy products, chocolates and cakes), confectionary and beverages (cola-type drinks), and as a fragrance ingredient in perfumes and cosmetics (Ranadive, 1994). Besides its flavor and fragrance qualities, vanillin is very useful as an intermediate in the synthesis of chemical and pharmaceutical industries for the production of herbicides, antifoaming agents or drugs such as papaverine, lL-dopa, lL-methyldopa and the antimicrobial agent,

trimethoprim (Hocking, 1997). Vanillin displays also antioxidant and antimicrobial properties and hence has the potential for use as a food preservative (Sinha *et al.*, 2008).

The main botanical source of vanillin are the pods of the tropical *Vanilla* orchid (principally *V. planifolia*) but it occurs in trace amounts also in other plants, including commercial products such as tobacco (Makkar & Beeker, 1994). However, the pods of the *Vanilla* orchid still remain the only commercial source of natural vanillin (Ramachandra Rao & Ravishankar, 2000a).

More than 12.000 tons of vanillin are annually produced and the market demand, estimated as higher than 15.000 tons for the year 2010, is currently increasing, as reported in the GPS Safety Summary revision by Rhodia (2011). However, only less than 0.5 % of the total vanillin production derives from *Vanilla*; the remainder main portion is synthesized much more cheaply *via* chemical processes. Vanillin extracted from *Vanilla* pods has in fact a variable and high price in market, which is in between $1200 and $4000 per kg, compared to the price of the synthetic product, which is less than $15 per kg (Lomascolo *et al.*, 1999; Muheim & Lerch, 1999). Many factors contribute to the variable and high cost of natural vanillin, i.e. the limited availability of *Vanilla* pods depending on climate-associated fluctuations of harvest yields, economical and political decisions, and also the labor-intensive cultivation, pollination, harvesting and curing of vanilla pods. The difference between the prices of natural and synthetic vanillin combined with the increasing consumer trend towards "green" and "eco-friendly" as well as "healthy" processes, which are associated to "bio" or "natural" products, has led to a growing interest of the flavor industry to produce vanillin from other natural sources (Priefert *et al.*, 2001). Biotechnological applications are enjoying increasing interest in recent years, since the label "natural" can be attributed when a product is derived from natural raw materials by biosynthesis, according to the current ECC and US Food legislation. Following this trend, a large number of studies have been recently developed on the field of biotechnological processes for vanillin production (Dubal *et al.*, 2008; Bicas *et al.*, 2010).

The following paragraphs review the biosynthesis of natural vanillin and its extraction from *V. planifolia*, as well as the production of vanillin by chemical and biotechnological processes. Moreover, the possibility of valorising wheat bran as a natural feedstock for biovanillin production is described.

1.3 Vanillin structure and properties

Isolated vanillin appears as white crystalline powder with a pleasant, sweet aroma, and a characteristic vanilla-like flavor. Chemically, it is an aromatic aldehyde (3-methoxy-4-hydroxybenzaldehyde), belonging to the group of simple phenolic compounds. Its formula is $C_8H_8O_3$ and structurally, its functional groups include aldehyde, ether and phenol (Converti *et al.*, 2010). The chemical structure and geometry of vanillin are shown in Figure 1. Some relevant physico-chemical properties are summarized in Table 1.

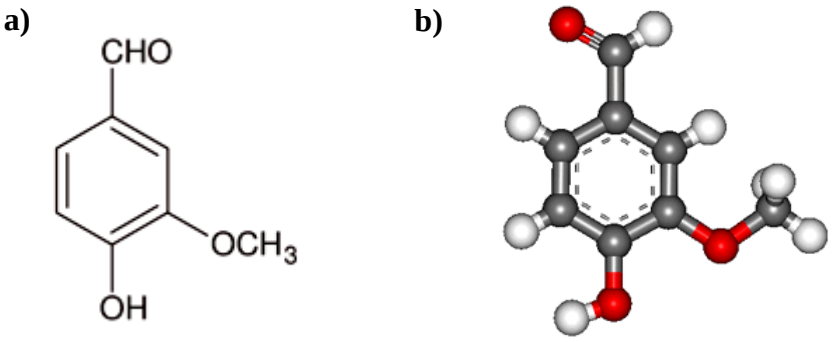

Figure 1. Chemical structure (a) and geometry (b) of vanillin molecule.

Property	Value
Solubility in water at 20° (g/L)	10
Molecular weight (g/mol)	152.15
Melting point (°C)	80/83
Boiling point (°C)	~ 285

Table 1. Physico-chemical properties of vanillin.

According to Gildemeister and Hoffmann (1899), vanillin crystallizes from hot water in the form of colorless needles at 81‑82°C. It possesses the strong and intensely sweet odor characteristic of vanilla. On careful heating, vanillin can be sublimated without

decomposition; by prolonged heating at 105°C, vanillin decomposes with the formation of non-volatile products. Vanillin is readily soluble in alcohol, ether, chloroform and hot water; relatively insoluble in cold water, for which reason vanillin can be recrystallized from water.

1.4 Biosynthesis of vanillin in *V. planifolia*

The flavor profile of vanilla extract contains more than 200 components, of which vanillin is the most abundant aromatic compound responsible of the characteristic vanilla aroma. Vanillin occurs in a concentration of 1.0-2.0% w/w of dry weight in cured *Vanilla* pods (Westcott *et al.* 1994), where it is accumulated exclusively in conjugated form, principally as the β-$_D$-glucoside. The green *Vanilla* beans are harvested approximately six to eight months after pollination and at this stage they display no trace of the vanilla flavor (Walton *et al.*, 2003). The pleasant aroma is released only by fermentation, called "curing", when the glucoside of vanillin, glucovanillin, and related β-$_D$-glucosides are hydrolyzed by β-$_D$-glucosidase, with the result that free vanillin and related substances (notably 4-hydroxybenzaldehyde) are released (Odoux *et al.*, 2003).

The appearance of vanillin during curing is in principle simple, unlike the pathway by which vanillin β-$_D$-glucoside is initially synthesized. Several biosynthetic routes have been proposed, but the complete pathway of vanillin formation is still unresolved. There is general agreement in the literature that vanillin is a product of the shikimic acid route. In this pathway, phenylalanine or tyrosine undergo deamination to a C_6–C_3 phenylpropanoid, which then serves as a precursor for the biosynthesis of vanillin. Although it is generally agreed that vanillin originates from a phenylpropanoid C_6–C_3 compound, much uncertainty remains concerning the chain-shortening and other reactions leading from the putative hydroxycinnamic acid precursor to vanillin β-$_D$-glucoside.

There are two major views as to how a phenylpropanoid precursor is converted to vanillin (Havkin-Frenkel & Belanger, 2008). One school of thought, proposed by Zenk (1965) suggested that the aromatic ring on C_6 –C_3 compounds (trans-cinnamic,p-

coumaric acids) undergoes hydroxylation and methylation giving rise to ferulic acid. The latter then undergoes chain shortening to vanillin by β-oxidation. This scheme is termed the 'ferulate pathway' (Figure 2A). Another view argues that chain shortening of a phenylpropanoid is the first metabolic event, followed by hydroxylation and methylation of the aromatic ring to yield vanillin. This is termed the 'benzoate pathway' (Figure 2B) (Podstolski *et al.*, 2002).

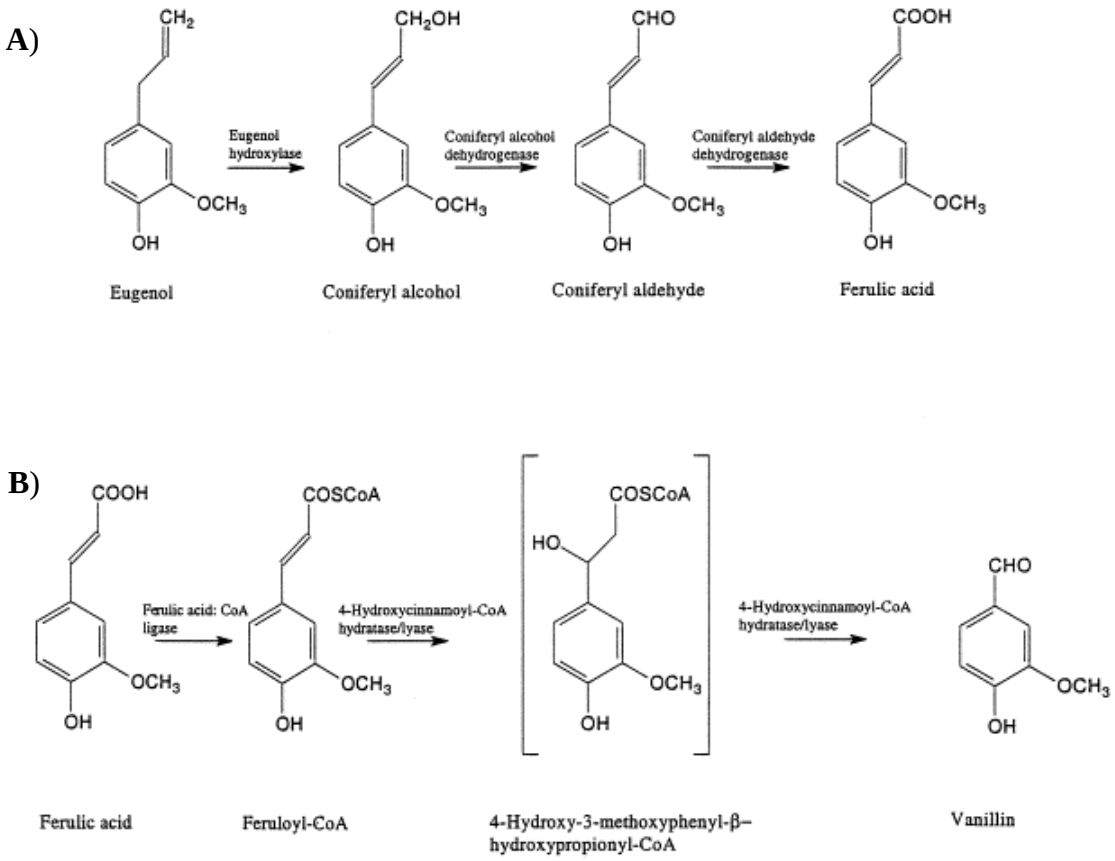

Figure 2. Overview of metabolic pathways leading to vanillin: (A) ferulate pathway and (B) benzoate pathway (Walton *et al.*, 2003).

It is also possible that an early intermediate in the shikimic acid pathway gives rise directly to the benzoate pool, bypassing the production of phenylpropanoids and their degradation to benzoate-pathway intermediates (Wildermuth *et al.*, 2001).

Recently, Negishi *et al.* (2009) carried out experiments with ^{14}C-labeled compounds in disks of green vanilla pods. Preliminary results showed that vanillin is synthesized via ferulic acid from 4-coumaric acid and glucosylated to form glucovanillin in mature

Vanilla beans. However, the investigation to purify, isolate and identify the key enzyme responsible for shortening of the phenylpropanoid side chain is still in progress.

1.5 Production of natural vanillin from *V. planifolia*

Natural vanillin is mainly obtained from the bean or pod of the tropical orchid *Vanilla planifolia*, and in less extent of *V. tahitiensis* and *V. pompona*, which are commonly denominated as "vanilla". Among these, *V. planifolia* is the most valued for its flavoring qualities and is therefore predominantly cultivated for production of vanillin (Anandaraj *et al.*, 2005). *Vanilla* is a native plant of Mexico, known and used already at the time of the Aztecs and further brought into Europe by Spanish conquistadors (Sinha *et al.*, 2008). Currently, *Vanilla* is cultivated mainly in Indonesia, which is the largest producer of vanillin, Madagascar and China, as summarized in Table 2 (FAO-STAT, 2013). *Vanilla* is a perennial climbing orchid with sessile leaves and succulent green stems producing aerial roots at the nodes and it is cultivated by vegetative propagation (Ranadive, 1994).

Country or Continent	Production (tons)
Indonesia	2600
Madagascar	1946
China	1300
Mexico	395
Tonga	202
Comoros	66
Asia	4170
Africa	2107
America	407
Oceania	259
European Union	-
World	6943

Table 2. World production of vanillin in 2010 (FAO-STAT, 2013).

Owing to the closed structure of the flowers, self-pollination is almost impossible (Figure 3). It is observed that the vanilla flower stays in bloom for less than 24h and pollination at just the right time (8-11 AM) is necessary for fertilization and fruit development. Artificial pollination is thus necessary and carried out by hand using a bamboo stick to get a good yield (Figure 3).

After fertilization has taken place, it requires about 10-12 months for the beans to fully mature. The harvesting time varies from one region to another, usually around six to eight months after fertilization. The processing of *Vanilla* to produce vanillin begins with the curing of freshly harvested pods. The aim of the curing process is to stop the natural vegetative process and to accelerate changes responsible for the formation of aromatic flavor constituents. The curing method differs from one production area to the other, and this can have a major influence on variation in quality and aromatic profile of pods that are traded.

Although there are several ways of curing *Vanilla*, mainly the Mexican process (sun method) and the Madagascar process (Bourbon method), they all share four main phases, i.e. killing, sweating, drying and packaging.

Figure 3. *Vanilla* plant at flowering stage and by-hand pollination.

The killing process avoids post-harvest vegetative growth and promotes the enzymatic reactions responsible for the production of aroma and flavor. The process is called "killing" because it disrupts the plant cell structure by different ways, such as by hot water scalding, sun or oven wilting, and freezing. The next sweating is the most crucial step, since vanillin and many related compounds are released from their glucosides at this phase. The temperature is thus raised to promote the enzymatic reactions and rapid

initial drying so as to prevent harmful fermentation. At the end of the sweating period, which may last from 7 to 10 days, the cured beans need further drying to reduce their moisture content and thus to protect them from microbial spoilage. The drying process is performed at room temperature until the pods reach a third of their initial weight.

In the last step of packaging, the pods are stored in closed boxes for one to several months. Various chemical and biochemical reactions such as esterification, etherification, oxidative degradation, etc. take place during this period to reach the aroma and flavor desired (Ramachandra Rao & Ravishankar, 2000a; Converti *et al.*, 2010; Exley, 2010).

The production of natural vanillin from botanical source is not only very laborious and expensive, but is also limited by fluctuations in crop yields associated with the agro-climatic conditions, and the political and economic decisions. Since the natural vanillin production covers less than 0.5% of the world market demand, the remainder is fulfilled by chemically synthesized vanillin derived from lignin or fossil hydrocarbons like guaiacol (Zamzuri & Abd-Aziz, 2013).

1.6 Production of vanillin by chemical synthesis

The first chemical process to obtain vanillin was developed by Tiemann in 1876. This process was based on eugenol, found in oil of clove, as raw material and was commercially used until the 1920s. Later vanillin was synthesized from lignin-rich spent sulphite liquor, a byproduct of the wood pulp industry. The lignin process consists on treating an aqueous solution of lignin with oxidants, at very alkaline pH, high temperatures and pressures. These oxidants can be air, oxygen, nitrobenzene or metallic oxides, with or without the help of catalysts. Lignin is degraded and oxidized, producing vanillin along with other by-products (Bjørsvik & Liguori, 2002; Mathias, 1993). The presence of these contaminants with chemical structure close to vanillin requires the use of intensive purification procedures, making nowadays the lignin process no longer popular also because of environmental concerns. Furthermore, even if the precursor input is from a natural source, vanillin chemically synthesized from lignin

is labeled as synthetic vanillin by food legislation, due to the extensive chemical modifications required to obtain the final product (Rabenhorst & Hopp, 2000).

Today most synthetic vanillin is produced from the petrochemical raw material guaiacol. There are several routes for synthesizing vanillin from guaiacol. At present, the most competitive and significant of these technologies consist on using guaiacol and glyoxylic acid (Esposito *et al.*, 1997; Kumar *et al.*, 2012). This is a two-step process that starts with a condensation reaction, in alkaline media, between guaiacol and glyoxylic acid. The resulting vanillylmandelic acid is then converted to vanillin by oxidative decarboxylation. The sequence of reactions is shown in Figure 4.

The vanillin obtained from guaiacol by this technology is nearly absent of by-products, simplifying the role of the subsequent procedure of product purification. Although this cleaner process has reduced the environmental impact compared with lignin-derived production, guaiacol is a petrochemical product and, as with vanillin synthesized from lignin, extensive chemical alterations render a product that is considered synthetic (Xu *et al.*, 2007).

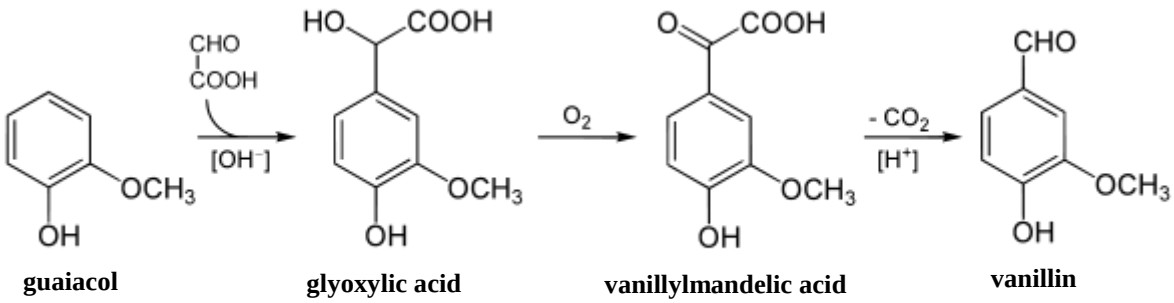

Figure 4. Chemical reaction sequence for producing vanillin from guaiacol (Kirk-Othmer Encyclopedia, 2005).

1.7 Production of vanillin *via* biotechnological approaches

Biotechnological applications are enjoying increasing interest in recent years, since biotechnology offers a feasible opportunity for replacing synthetic vanillin with a bio-based product, that can be label "natural", according to current food regulations. In addition, biotechnological processes are cheaper, produce less unwanted side products, and mainly are "greener", that is, can be performed under milder, less dangerous conditions, utilize less energy, and produce less greenhouse gas emissions.

Biotechnological approaches for biovanillin production include use of enzymes to release or generate vanillin from *Vanilla* and other plant material, development of tissue cultures, use of microorganisms to bioconvert several substrates and, finally, genetic engineering.

1.7.1 Use of enzymes

Knowledge of the vanillin biosynthetic pathway and the involved enzymes, which catalyze successive steps in the process, might furnish an in vitro enzyme-based system for the production of vanillin. Biotechnology could potentially be applied to clone genes for relevant enzymes that could be used for the production of vanillin or vanillin intermediates, offering control over defined steps in the production process.

Several authors described the use of enzyme preparations containing β-glucosidase, which catalyzes the hydrolysis of vanillin from glucovanillin, to achieve vanillin release from *Vanilla* pods, as an alternative to conventional curing (Dignum *et al.*, 2001a; Ruiz-Terán *et al.*, 2001; Odoux & Havkin-Frenkel, 2005).

Enzymes can also be used in principle to generate vanillin from other plant-derived materials by biotransformation. For example, Kamoda *et al.* (1989) investigated the use of lignostilbene αβ-dioxygenase isolated from *Pseudomonas* sp. TMY1009, to catalyze the oxidative release of vanillin from stilbenes, commonly found in wood bark. Synthetic enzymes, produced by DNA cloning of soybean lipoxygenase and used in transformed microorganisms, were also exploited for vanillin production vanillin from esters of coniferyl alcohol (Markus *et al.* 1992). Vanillin can also be released from

creosol (a major component of creosote obtained from heating wood or coal tar) and vanillylamine (obtainable by the hydrolysis of capsaicin, the main pungent principle of chili peppers). Van den Heuvel *et al.* (2001) used the flavoprotein vanillyl alcohol oxidase (VAO), a broad-specificity *Penicillium* flavoenzyme, to convert both creosol and vanillylamine to vanillin with high yield. The VAO-mediated conversion of creosol proceeds via a two-step process in which the initially formed vanillyl alcohol is further oxidized to vanillin. This route to vanillin has questionable biotechnological potential because of the low amount of capsaicin in pepper or other plant sources. On the other hand, creosol may not be considered a natural precursor, because of extensive chemical alterations of wood or coal tar processing. Such approaches are in principle attractive, since the technologies should be reproducible, predictable and acceptable and, given adequate demand, scale-up and stability, the cost of the enzymes may not be prohibitive (Walton *et al.*, 2003).

1.7.2 Use of plant tissue culture

For some years, several studies explored the metabolic potential of plants to produce a wide range of flavors for the synthesis of vanillin in cultured cells and organs, such as leaves and stems, of *Vanilla planifolia*, and, more recently, also in cells of *Capsicum frutescens* (Ramachandra Rao & Ravishankar, 2000b; Dignum *et al.*, 2001b). The strategies with cultured plant cells include the feeding of putative precursors, the use of elicitors or hormones inhibition of competing pathways, cell immobilization, adjustment of environmental culture conditions and the use of an adsorbant, such as charcoal and resins, to sequester the vanillin produced (Walton *et al.*, 2003).

Knuth and Sahai (1991) found that the nature and the concentration of vanilla component precursors added to the medium were a factor influencing flavor production in *V. fragrans* cultures. Phenylalanine and ferulic acid resulted in little enhancement of vanillin production, whereas addition of vanillyl alcohol resulted in a significant increase in vanillin content.

Havkin-Frenkel and Pederson (2000) reported that feeding *Vanilla* plant tissue culture with vanillin precursors, such as 3,4-dihydroxybenzaldehyde, resulted in complete

uptake of applied compounds from the media and close to complete conversion to vanillyl alcohol. They hypothesized to couple the plant system to methano-bacteria such as *Methylosinus trichosporium* OB3b, which could convert the vanillyl alcohol to vanillin. However, the precursor, 3,4-dihydroxybenzaldehyde, is not readily accessible in a natural form. In addition, coupling to yet another system (microbial) is a further complexity, which might make the working concept economically prohibitive.

Westcott *et al.* (1993) developed a process for producing natural vanillin flavor from ferulic acid using vanilla plant aerial roots as the biocatalyst. The charcoal used in the process acts as a product reservoir for the vanillin produced, thus relieving possible product inhibition and/or further metabolism. The aerial root tissue can be reused several times, but its activity gradually declines with reuse. The concentration of produced vanillin is approximately 35-fold greater than those originally present in the aerial root tissue and is about 40% of that present in matured vanilla beans. Using aerial roots supplied with ferulic acid, vanillin is produced five to ten times faster than its normal synthesis in vanilla beans, or in aerial roots not supplied with precursor. In addition, the composition of the vanilla flavor produced using the aerial root method is comparatively close to that of vanilla beans.

Suspended and immobilized cell cultures of *C. frutescens* (chili pepper) accumulated vanillin flavor metabolites when fed with isoeugenol. The addition of *b*-cyclodextrin and isoeugenol increased the accumulation of vanillin. Isoeugenol-treated immobilized cells, when challenged with aqueous mycelial extract of *Aspergillus niger*, yielded maximum vanillin concentrations, whereas the addition of a medium filtrate of *A. niger* led to a marginal increase in the vanillin (Ramachandra Rao & Ravishankar, 1999).

Another attractive and possible strategy would be to introduce an enzyme or pathway *de novo* to generate or enhance vanillin from a mainstream intermediate of the *Vanilla* plant phenylpropanoid pathway. As described extensively by Walton *et al.* (2003), the isolation of the gene encoding the vanillin-forming enzyme HCHL (4-hydroxycinnamoyl-CoA hydratase/lyase), from a soil bacterium (*Pseudomonas fluorescen*s strain AN103) raised this possibility (Narbad & Gasson, 1998; Gasson *et al.*,1998). Enzyme HCHL is involved in the mechanism of ferulic acid chain shortening in plants and precisely catalyses the hydration and retro-aldol cleavage of feruloyl-CoA to produce vanillin, together with acetyl-CoA. However, attempts to produce vanillin in

plant systems via HCHL expression were unsuccessful, as observed by Mitra and coworkers (1999).

Although these studies demonstrated the possibility to accumulate vanillin in cell- or organ-tissue culture successfully, the yields of vanillin were not high enough for processes to be viable economically. Additional inherent problems with plant tissue culture are cell instability, slow growth rate and scale-up complexities, making *Vanilla* not ideal for biotechnology. So far, none of these approaches has thus delivered a commercial cell or organ-tissue culture system for vanillin production. Moreover, the genetic engineering of *Vanilla* plants is problematic mainly due to the incomplete understanding of the vanillin biosynthetic route and of the enzymes involved (Walton *et al.*, 2003; Havkin-Frenkel & Belanger, 2008).

1.7.3 Use of microorganisms

Microorganisms, on account of their rapid growth rates and amenability to molecular genetics, are much ideal targets for biotechnology and can be selected for their ability to grow on a putative precursor of vanillin as a sole source of carbon and energy. A large number of microbes such as bacteria, fungi and yeast have been used for the laboratory-scale production of vanillin from various substrates, such as lignin, eugenol, isoeugenol and ferulic acid as well as vanillic acid, phenolic stilbenes, aromatic amino acid and also glucose.

A major problem of microbial production of vanillin is the over-oxidation and/or the reduction of end product to vanillic acid and vanillyl alcohol, respectively. Both reactions lead to a decrease in the vanillin concentration. To prevent these side reactions and hence to improve the vanillin yield, process optimization (addition of adsorbent resin or anti-oxidation agent) or metabolic engineering (inactivation of relevant enzymes, such as vanillin dehydrogenase) was applied.

Table 3 enlists several native as well as engineered microorganisms, which were used to produce vanillin from various substrates.

Substrate	Microorganism	Yield (g/L)	Reference
Eugenol	*Pseudomonas* sp. TK2102	0.28	Washisu *et al.*(1993)*
	Pseudomonas sp. HR199	0.44	Overhage *et al.*(1999c)
	P. resinovorans SPR1	0.24	Ashengroph *et al.*(2011)
	Two-step process: *E. coli* XL1-Blue (pSK*vao*mP*cal*Am*calB*) and *E. coli*(pSKechE/Hfcs)	0.3	Overhage *et al.*(2003)
Isoeugenol	*Senatia marcescens* DSM 30126	3.8	Rabenhorst&Hopp(1991)*
	Bacillus subtilis B2	0.9	Shimoni *et al.*(2000)
	B. subtilis HS8	8.1	Zhang *et al.*(2006)
	B. fusiformis SW-B9	32.5	Zhao *et al.*(2005)
	Pseudomonas putida IE27	16.1	Yamada *et al.*(2007)
	Pseudomonas chlororaphis CDAE5	1.2	Kasana *et al.*(2007)
	Bacillus pumilus S-1	3.75	Hua *et al.*(2007)
	Candida galli PG06	0.58	Ashengroph *et al.*(2010)
	Psychrobacter sp. CSW4	1.28	Ashengroph *et al.*(2012)
	E.coli BL21(DE3)	28.3	Yamada *et al.*(2008)
Ferulic acid	*Amycolatopsis* sp. (DSM9991 or DSM9992)	11.5	Rabenhorst&Hopp(1997)*
	Streptomyces setonii ATCC 39116	13.9	Müller *et al.*(1998)*
	S. setonii	6.4	Muheim&Lerch(1999)
	Streptomyces sp. V-1	19.2	Hua *et al.*(2007)
	Pycnoporus cinnabarinus	0.126	Tilay *et al.*(2010)
	Pseudomonas sp.	0.0085	Agrowal *et al.*(2003)
	Pseudomonas fluorescens AN103	- **	Martinez-Cuesta *et al.*(2005)
	P. fluorescens BF13	- **	Calisti *et al.*(2008)
	P. putida KT2440	> 10	Plaggenborg *et al.*(2003)
	mutant *P. putida*	2.247	Cheetham *et al.*(2000)*
	Two-step process: *A. niger* I-1472 and *P. cinnabarinus* MUCL39533	0.584	Lesage-Meessen *et al.*(2002)
	E. coli strain JM109/pBB1	0.851 mol/L	Torre *et al.*(2004)
	E. coli strain JM109/pBB1	2.52	Barghini *et al.*(2007)

Table 3 (continued)

Substrate	Microorganism	Yield (g/L)	Reference
	E. coli XL1-Blue (pSk*ech*E/H*fcs*)	trace amounts	Overhage *et al.*(2003)
	E. coli (pDAHEF)	0.58	Yoon *et al.*(2005a)
	Recombinant *E. coli*	1.1	Yoon *et al.*(2005b)
	E. coli DH5α (pTAHEF)	1.0	Yoon *et al.*(2007)
	E. coli (pTBE-FP)	2.1	Song *et al.*(2009)
	E. coli NTG-VR1	2.9	Yoon *et al.*(2007)
	E. coli DH5α (pTAHEF-gltA)	1.98	Lee *et al.*(2009)
	E. coli BW25113 (pTAHEF)	5.14	Lee *et al.*(2009)
Glucose	*E. coli* KL7	trace amounts	Li&Frost(1998)
	Engineered *Schizosaccharomyces pombe*	0.065	Hansen *et al.*(2009)
	Engineered *S. cerevisiae*	0.045	Hansen *et al.*(2009)
	Engineered *S. cerevisiae*	25***	Brochado *et al.*(2010)

***Patented work**
**** not reported by authors**
*****vanillin β-$_D$-glucoside**

Table 3. Bioconversion of various substrates to vanillin by several microorganisms.

1.7.3.1 Bioconversion of lignin

Lignin, a complex aromatic polymer, is a cell wall constituent in plants and represents one of the most abundant natural source of flavoring compounds. Lignin harbors vanillin subunits in its polymeric structure and it is formed by the dehydrogenative polymerization of three cinnamyl alcohols (monolignols), i.e. *p*-coumaryl, coniferyl, and sinapyl alcohol. Lignin is an abundant by-product of the paper industry and is also the precursor for vanillin production by chemical oxidation. Despite of this, only few

reports have been published on microbial vanillin production from lignin (Priefert *et al.*, 2001).

The degradation and depolymerization of lignin were investigated in several white-rot fungi, including *Phanerochaete chrysosporium* and *Pleurotus eryngii* (Tien & Kirk, 1983; Martinez *et al.*, 2001). The enzyme lignin peroxidase, manganese peroxidase, and laccase are responsible for lignin depolymerisation. However, vanillin is released only in trace amounts as well as other metabolites, i.e. dehydrodivanillin, vanillic acid, coniferyl aldehyde, ferulic acid, *p*-hydroxycinnamyl aldehyde, *p*-hydroxycinnamic acid, guaiacylglycerol-b-coniferyl ether, and guaiacylglycerol beside lignin fragments (Ishikawa *et al.*, 1963a; Kirk & Farrell, 1987).

Based upon the scientific literature, six independent lignin degradation pathways were identified, of those only two, i.e. β-aryl ether cleavage and ferulate catabolic pathways, grabbed much interest of the microbiologists as vanillin was found as an intermediate metabolite. β-aryl ether cleavage pathway was characterized in *Sphingomonas paucimobilis* (Figure 5), *Delftia acidovorance* and *Rhodococcus* sp. (Masai *et al.*, 2002), but currently these studies have only scientific interest.

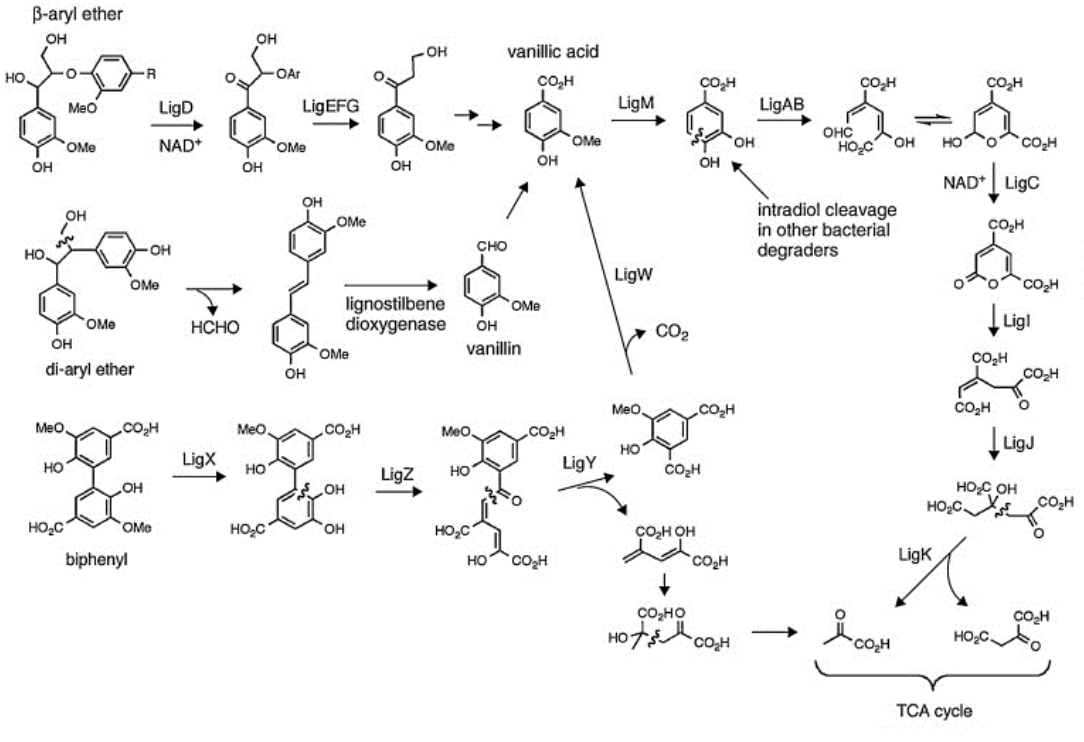

Figure 5. Pathways and gene products identified in *Sphingobium* sp. SYK-6 for degradation of lignin components (Bugg *et al*., 2011).

Within the commercial context, companies utilize enzyme-catalyzed oxidative degradation of lignin to obtain vanillin. The process yields around 1% vanillin as well as a vast array of other by-products. However, the starting substrate, degradable lignin fragments obtained by harsh chemical treatments, may not be regarded as a natural material and so the resulting vanillin is considered synthetic (Havkin-Frenkel and Belanger, 2008).

1.7.3.2 Bioconversion of propenylbenzenes

Propenylbenzenes are aromatic compounds with various substitutions, physically extracted from plant essential oils and widely used as starting materials for synthesizing various products with applications as food preservatives and flavors (Xu *et al.*, 2007). Although propenylbenzenes are usually toxic for most microbes (Koeduka *et al.*, 2006), they can be transformed into high-valued flavors by certain microorganisms. In particular, eugenol (2-propenylbenzene) and isoeugenol (1-propenylbenzene) extracted from the essential oil of the clove tree *Syzygium aromaticum* can be transformed into vanillin. Despite of the great potential of both eugenol or isoeugenol as cheap precursor for vanillin production, most of the so far described biotransformation processes showed rather low vanillin yields, due to the toxicity either of the substrates or of the end product. Studies on the bioconversion of eugenol and isoeugenol by using several microbial and fungal strains are summarized below.

Eugenol

The metabolic pathway of eugenol was investigated in many bacteria and fungi, such as *Corynebacterium*, *Pseudomonas*, *Byssochlamys*, *Penicillium* and *Rhodococcus*. Two oxidation reactions take part to the degradation of eugenol, where first step involves oxidation of double bond of side chain to coniferyl alcohol, and in the second oxidation step, it is converted into ferulic acid via coniferyl aldehyde. The oxidative hydrolysis of eugenol involves many intermediates, such as ferulic acid, an important precursor which can yield vanillin through different metabolic pathways (Priefert *et al.*, 2001) (Figure 6).

In most of the eugenol-converting bacterial species, only trace amounts of vanillin were accumulated.

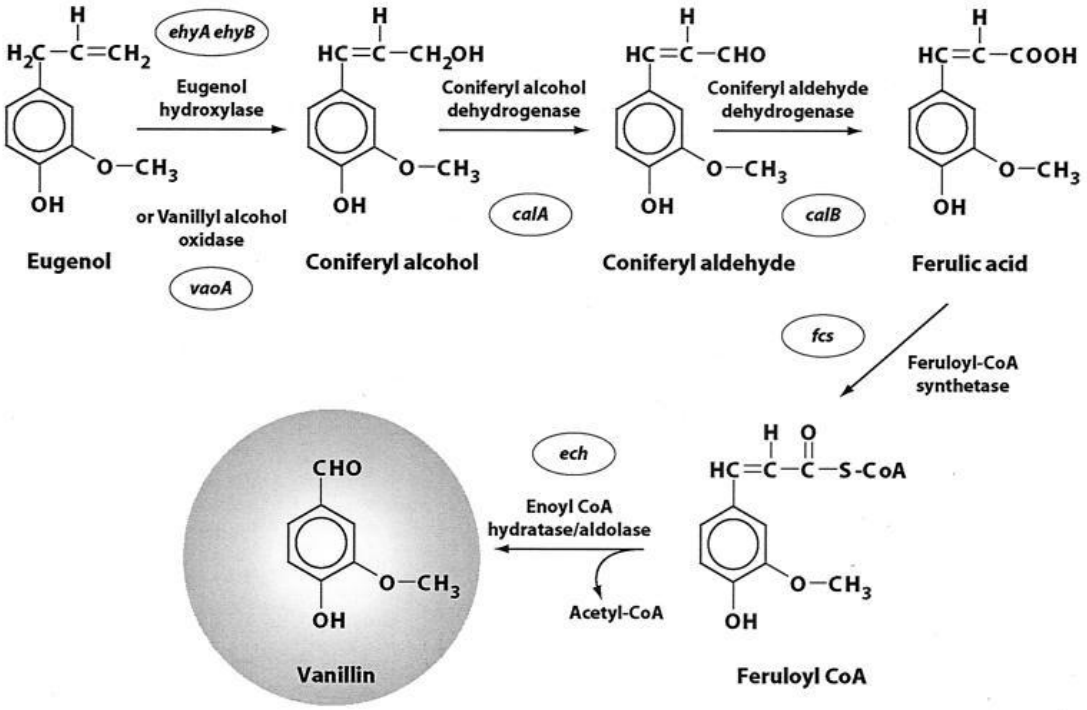

Figure 6. Eugenol conversion to vanillin (Overhage *et al.*, 2003).

In 1993, Washisu *et al.* patented the production of vanillin from eugenol, using a strain of *Pseudomonas* spp TK2102, which accumulated vanillin up to 280 mg/L, and other metabolites such as coniferyl alcohol, ferulic acid and vanillic acid. The same metabolites were also found as the main product in the biotransformation of eugenol catalysed by *Pseudomonas* sp. and *Rhodococcus opacus* PD630. To improve the yield of vanillin, metabolic engineering was introduced. A mutant strain of *Pseudomonas* sp. strain HR199 was constructed by insertion of an omega element into the vanillin dehydrogenase gene, and an accumulation of 2.9 mM (0.44 g/L) vanillin was achieved with resting cells from 6.5 mM eugenol within 17 h without further optimization. However, the accumulated vanillin was further oxidized due to the unspecificity of coniferyl aldehyde dehydrogenase, which also exhibited vanillin dehydrogenase activity (Overhage *et al.*, 1999b).

Penicillium simplicissimum carries out two exceptional reactions, as outlined by Ramachandra Rao and Ravishankar (2000a). Firstly, it has an enzyme that converts eugenol into coniferyl aldehyde. Secondly, it has an aromatic alcohol oxidase that converts vanillyl alcohol into vanillin. The vanillyl alcohol oxidase gene (vaoA) from *P. simplicissimum* CBS170.90 was thus expressed in *R. opacus* PD630 and *Amycolatopsis sp.* HR167, together with the coniferyl alcohol dehydrogenase (calA) and coniferyl aldehyde dehydrogenase (calB) genes from *Pseudomonas sp*. HR199. The recombinant strains converted eugenol to ferulic acid, which could be transformed to vanillin (Shimoni *et al.*, 2000).

The concept of metabolic engineering for vanillin production was further exploited in recombinant strains of *Ralstonia eutropha* H16 (Overhage *et al.*, 2002), *Rhodococcus strains* PD630 (Plaggenborg *et al.*, 2006), *E. coli* XL1-Blue (pSK*vao*mP*cal*A*calB*) (Overhage *et al.*, 2003) and *Amycolatopsis* sp. HR167 (Overhage *et al.*, 2006). Although vanillin was produced in small amounts or detected not at all, these mutant strains may be suitable candidates for vanillin production from eugenol.

Recently, Srivastava *et al.* (2010) for the first time established the eugenol bioconversion pathway in fungal systems that lead to hypothesize the metabolic fate of eugenol in eukaryotic systems. Using the 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*.

In the same year, 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. However, 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).

Isoeugenol

Isoeugenol is metabolized into vanillin through an epoxide-diol pathway involving oxidation of side chains of propenylbenzenes. The biotransformation products of isoeugenol, and related 1-propenylbenzenes, differ from those obtained from eugenol, and related 2-propenylbenzenes, as these are decarboxylated to corresponding substituted benzoic acid (Priefert *et al.*, 2001). To date the yields of vanillin produced from isoeugenol are higher than those obtained from eugenol.

In 1988, Abraham *et al.* reported the first biotransformation of isoeugenol 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 vanillyl alcohol and vanillic acid.

The production of vanillin from isoeugenol was further investigated in *Pseudomonas paucimobilis* TMY 1009 (Yoshimoto *et al.*, 1990), as well as in strains of the genera *Klebsiella, Enterobacter* and *Serratia* (Rabenhorst & Hopp, 1991). As reported by Rabenhorst and Hopp (1991), the strain *Serratia marcescens* DSM 30126 was capable of converting 20.5% of isoeugenol to vanillin under optimized conditions, and 3.8 g/L vanillin was obtained after 9 days. Eugenol was also transformed by this organism, but the yield of vanillin was much lower (0.018 g/L).

A vanillin molar yield of 58% was achieved when 3-day-old cultures of *Rhodococcus rhodochrous* were fed with isoeugenol at a concentration of 15% (w/v) (Chatterjee *et al.*, 1999).

Shimoni *et al.* (2000) isolated the soil strain *Bacillus subtilis* B2, which converted isoeugenol with a molar yield of 14% to vanillin (0.9 g/L) by using cell free extracts. The low yield was mainly due to the toxicity of end product.

Zhang *et al.* (2006) found that another strain of *Bacillus subtilis*, i.e. strain HS8, is able to convert isoeugenol into vanillin via isoeugenol-diol. Vanillin was produced after 96 h, with molar yield of 14.7% and product concentration of 1.36 g/L. Vanillin toxicity was overcome with the addition of HD-8 adsorbent resin, which allowed to converted 50 g/L isoeugenol and to accumulate 8.1 g/L vanillin.

Using 60% (v/v) isoeugenol as substrate and solvent at pH 4.0, vanillin was produced at 32.5 g/L over 72 h of bioconversion by the strain *B. fusiformis* SW-B9 (Zhao *et al.*

2005). This is the highest vanillin yield from isoeugenol by microbial biotransformation to date.

Similarly, a very high vanillin yield from isoeugenol using *Pseudomonas putida* IE27 cells was reported (Yamada *et al.*, 2007a). Under optimized reaction conditions, strain 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.

Later, Yamada and coworkers (2008) proposed the production of vanillin using metabolically engineered *Escherichia coli* cells, which over-expressed isoeugenol monooxygenase of *Pseudomonas putida* IE27. The recombinant *E. coli* BL21(DE3) cells produced 28.3 g/L vanillin from 230 mM isoeugenol, with a molar conversion yield of 81% at 20°C after 6 h. In the reaction system, no accumulation of undesired by-products, such as vanillic acid or acetaldehyde, was observed.

Few more studies on the production of moderate amounts of vanillin by using biotransformation capabilities of various bacterial and fungal strains have been summarized below. *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). A strain of *Bacillus pumilus* S-1 capable of transforming isoeugenol to vanillin through isoeugenol epoxide and isoeugenol-diol as intermediates was isolated and characterized. With the growing culture of *B. pumilus* S-1, 10 g/L isoeugenol was converted to 3.75 g/L vanillin in 150 h, with a molar yield of 40.5 % (Hua *et al.*, 2007). Strains of *Arthrobacter* sp. TA13 (Shimoni *et al.*, 2003) and *Pseudomonas nitroreducens* Jin 1 (Unno *et al.*, 2007) were also found to produce vanillin, but at very low concentrations. Isolated *Candida galli* PGO6 can produce vanillin and vanillic acid in concentrations of 583.2±5.7 mg/L (molar yield 48 %) and 177.3±1.7 mg/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 isolated, 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 isoeugenol at 10 g/L concentration after a 48-h reaction.

1.7.3.3 Bioconversion of ferulic acid

The conversion of ferulic acid to vanillin via microbial routes is one of the most intensely studied biotransformation and also the most promising process for commercial production of biovanillin.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a ubiquitous plant constituent that is produced from phenylpropanoid metabolism and, together with dihydroferulic acid, is a component of lignocelluloses, conferring cell wall rigidity by cross linking lignin and polysaccharides (Ou & Kwok, 2004). Ferulic acid (FA) is the main phenolic component found in cell walls of monocotyledons, therefore it can be obtained from agro industrial by-products such as 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). FA can be released by treatment with strong alkali or by enzymatic hydrolysis. The latter is the best choice for the production of vanillin with "natural" label (Hasyierah *et al.*, 2008; Mathew & Abraham, 2006). Moreover ferulic acid may be the major suitable candidate for biovanillin production, being least toxic of all the investigated precursors.

The biotransformation of FA to vanillin was widely investigated in several microorganisms, including gram-negative bacteria of the *Pseudomonas* genus (Barghini *et al.*, 1998; Plaggenborg *et al.*, 2003), actinomycetes of the genera *Amycolatopsis* and *Streptomyces* (Sutherland *et al.*, 1983; Oddou *et al.*, 1999; Muheim and Lerch, 1999; Achterholt *et al.*, 2000; Brunati *et al.*, 2004), gram-positive bacteria, such as *Bacillus subtilis* (Plaggenborg *et al.*, 2001) and *Rhodococcus* sp. (Plaggenborg *et al.*, 2006), and the basidiomycete fungi, such as *Pycnoporus cinnabarinus* (Lesage-Meessen *et al.*, 1996; Tilay *et al.*, 2010), *Polyporous versicolor* and *Fomes fomentarius* (Ishikawa *et al.*, 1963b) were proposed for the bioconversion of ferulic acid to vanillin. The production of vanillin from ferulic acid was also studied in microalgae *Spirulina platensis* (Ramachandra Rao *et al.*, 1996) and *Haematococcus pluvialis* (Tripathi *et al.*, 2002). In addition, more recent and preliminary studies investigated the capability of producing vanillin from ferulic acid by the bacteria *Staphylococcus aureus* (Sarangi & Sahoo, 2010) and *Enterobacter* sp. (Li *et al.*, 2008), as well as by *Lactobacillus* sp. (Bloem *et al.*, 2007; Szwajgier & Jakubczyk, 2010).

In almost all of the microorganisms studied so far, vanillin was produced as transient metabolite in trace amounts and was either rapidly converted to other products or utilized by the microorganism as carbon source due to its toxic effect and product inhibition. Optimization of the process, such addition of resin, and/or techniques of metabolic engineering were therefore investigated.

Bacteria belonging to genera *Pseudomonas sp*. were found to be interesting candidates for their capability of converting ferulic acid as sole carbon source to vanillin. However, FA was quickly oxidized or reduced to vanillic acid and vanillyl alcohol, respectively. An attempt to improve vanillin yields in *P. fluorescens* AN103 was based on the disruption of the *vdh* gene. Nonetheless, inactivation of the *vdh* gene did not result in the expected accumulation of vanillin in the medium despite the absence of other enzymes exhibiting vanillin dehydrogenase activity (Martinez-Cuesta *et al.*, 2005).

The highest vanillin concentrations from FA in the literature were reported by using two actinomycetes belonging to genera *Amycolatopsis* and *Streptomyces.*

In 1997 Rabenhorst and Hopp described the isolation of a new *Amycolatopsis sp.* strain HR167 (DSM9991 or DSM9992) that was capable of converting 19.92 g/L of ferulic acid to 11.5 g/L vanillin within 32 h at a 10-l scale, corresponding to a molar yield of 77.8%. One year later, Müller and coworkers (1998) disclosed a process for the bioconversion of FA to vanillin by *S. setonii* strain ATCC39116. At a 10-l scale, 13.9 g/L vanillin (molar yield 75%) was obtained from 22.5 g/L ferulic acid after 17 h of incubation; 0.4 g/L guaiacol was detected as by-product. At a 340-l scale, 9 g/L (molar yield of 51%) vanillin was obtained from 20.75 g/L ferulic acid within 25.5 h of incubation. Both of these actinomycete strains exhibited an exceptional tolerance towards vanillin and the works were also patented. However, the biochemical basis for these very high accumulations is not well understood (Walton *et al.*, 2000).

Muheim and Lerch (1999) reported a reasonable high yield of vanillin (concentration up to 6.4 g/L with molar yield 68%) from conversion of ferulic acid by *Streptomyces setonii*.

Recently, Hua *et al.* (2007) reported that high vanillin production was achieved in batch biotransformation of FA by *Streptomyces* sp. V-1. When 8% resin DM11 (wet w/v) was added to the biotransformation system, 45 g/L ferulic acid could be added continually and 19.2 g/L vanillin with molar yield of 55% was obtained within 55 h of culture.

Lesage-Meessen *et al.* (1996) described a two-step process for production of biovanillin from sugar beet pulp-derived ferulic acid, by using two filamentous fungi. In the first bioconversion, *Aspergilllus niger* converted FA to vanillic acid. In the second step, the produced vanillic acid was reduced to vanillin by 3-days-old *Pycnoporus cinnabarinus* cultures. The authors observed that low level of vanillin (237 mg/L and 22% molar yield) was obtained mainly due to the laccase activity, which was associated with the formation of ferulic acid polymers and the loss of phenolic monomers from the culture medium (Lomascolo *et al.*, 1999). The process was further optimized by using the laccase deficient *P. cinnabarinus* MUCL 39533 strain, which produced 767 mg/L of vanillin in the presence of cellobiose and XAD-2 resin (Lesage-Meessen *et al.*, 2002). Addition of cellobiose channeled the flow of vanillic acid to its reduction to vanillin, instead of its decarboxylation to the by-product methoxyhydroquinone (Bonnin *et al.*, 1999). Within this work, the researchers reported that *P. cinnabarinus* MUCL 39533 could be fed with the autoclaved fraction of maize bran as a ferulic acid source and *A. niger* I-1472 culture filtrate as an extracellular enzyme source. Under these conditions, 584 mg/L of vanillin were obtained.

Recently, Tilay and coworkers (2010) proposed a statistically optimized one-step biotransformation process for vanillin production from FA using *P. cinnabarinus.* Under statistically optimum conditions, 126 mg/L of vanillin were produced (molar yield 54 %) in the presence of glucose as carbon source, and corn steep liquor and ammonium chloride as organic and inorganic nitrogen source, respectively.

Although much higher vanillin yields were obtained with actinomycetes, the filamentous growth, resulting in highly viscous broths, unfavorable pellet formation and uncontrolled fragmentation and lysis of the mycelium, might complicate the rheology of the production processes, reduce their productivity and determine an increase in the downstream processing costs (Barghini *et al.*, 2007).

Catabolic pathways of ferulic acid

According to the scientific literature, four main pathways have been proposed for the initial reaction of ferulic acid degradation, namely non-oxidative decarboxylation, side-chain reduction, coenzyme-A-independent and coenzyme-A-dependent deacetylation (Priefert *et al.*, 2001). Mathew and Abraham (2006) mentioned two additional catabolic

routes, namely demethylation and oxidative coupling. The microbial demethylation of FA was observed in anaerobic *Clostridium methoxybenzovorans* (Mechichi *et al.*, 1999) and in facultative aerobic *Enterobacter cloacae* (Grbic-Galic and La Pat-Polasko, 1985), which are capable of converting ferulic acid to caffeic acid via O-demethylation pathway. The oxidative coupling mechanism was observed using a laccase enzyme purified from the basidiomycete *Marasmius quercophilus* (Farnet *et al.*, 2004). Ferulic acid in the presence of this oxidase enzyme was degraded leading to the formation of various polymerized compounds by radical mediated reactions, as also observed in *Pycnoporus cinnabarinus* (Lesage-Meessen *et al.*, 2002).

The proposed pathway for *non-oxidative decarboxylation* catalyzed by ferulic acid decarboxylase involves the initial enzymatic isomerization of FA to the quinoid intermediate 4-vinylguaiacol, which further decarboxylates spontaneously (Huang *et al.*, 1993). This mechanism was observed in many fungi and yeasts and also in some bacteria. Beside 4-vinylguaiacol, the occurrence of additional metabolites, such as dihydroferulic acid, vanillin, vanillyl alcohol, vanillic and protocatechuic acid, were identified in some bacterial and fungal strains. In contrast to ferulic acid decarboxylation, the reactions leading from 4-vinylguaiacol to the other metabolites have been not well established (Priefert *et al.*, 2001). Recently, Li and coworkers (2008) isolated the *Enterobacter* sp. Px6-4 from *Vanilla* roots with the ability to utilize FA as the sole carbon source to produce vanillin. However, this novel pathway from 4-vinylguaiacol to vanillin needs to be confirmed by isotope analysis, as suggested by the authors.

Another mechanism of initial degradation involves the *side-chain reduction* of ferulic acid. This reaction leads to the formation of dihydroferulic acid and it is typical for the anaerobic degradation of FA, but it was observed also under aerobic conditions. Rosazza *et al.* (1995) proposed the mechanism of side-chain reduction via hydride attack of a quinoid intermediate, which is initiated by an isomerization analogous to the decarboxylation reaction. As observed in *Nocardia* sp., the reaction also includes the activation of carboxyl groups with ATP to yield highly reactive carbonyl-AMP intermediates readily reduced to aldehydes which are then reduced to their corresponding alcohol products (Li & Rosazza, 2000).

The *coenzyme-A-independent deacetylation* involves the initial elimination of an acetate moiety from the unsaturated ferulic acid side-chain, that directly yields vanillin, as reported in *Streptomyces setonii* (Sutherland *et al.*, 1983), *Fusarium solani* (Nazareth & Mavinkurve, 1986), *Pseudomonas mira* (Jurková & Wurst, 1993). The pathway proposed by Rosazza *et al.* (1995) for the acetate cleavage from FA involves the hydration of the double bond to give a transient hydroxy-intermediate, followed by aldolase cleavage to vanillin and acetate (Figure 7A).

A *coenzyme-A-dependent* mechanism analogous to the β-oxidation pathway of fatty acid catabolism was proposed for the degradation of substituted cinnamic acids in *Pseudomonas putida* (Zenk *et al.* 1980), and of ferulic acid in *Rhodotorula rubra* (Huang *et al.* 1994). This pathway includes the thioclastic cleavage of 4-hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA to yield acetyl-CoA and vanillyl-CoA, catalyzed by a β- ketothiolase (Figure 7B).

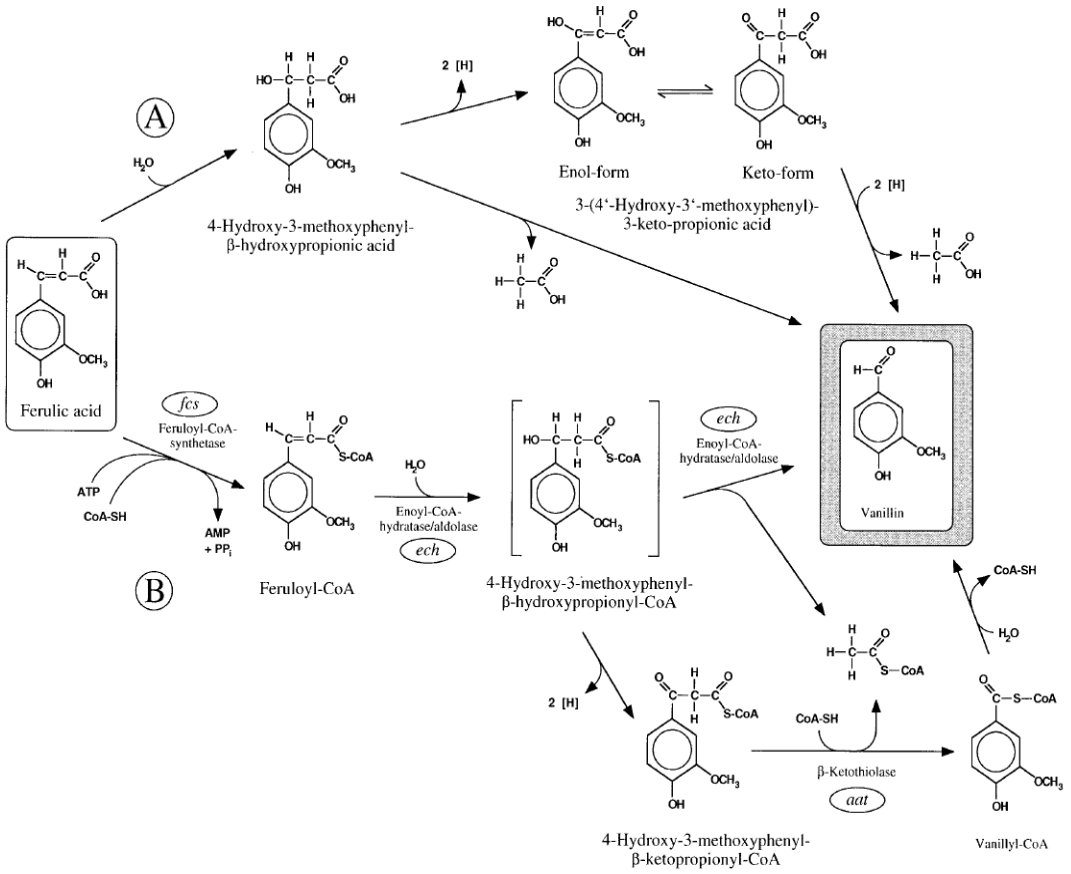

Figure 7. Coenzyme-A-independent (A) and coenzyme-A-dependent deacetylation (B) of ferulic acid to yield vanillin (Priefert *et* al., 2001).

Later, a novel *coenzyme-A-dependent, non-β-oxidative pathway*, was identified and then reported as the most common mechanism of FA degradation in bacteria. In the proposed mechanism, ferulic acid is initially activated to the CoA thioester by feruloyl-CoA synthetase. Feruloyl-CoA is subsequently hydrated and non-oxidatively cleaved to vanillin and acetyl-CoA. Both reactions are catalyzed by one distinct enzyme, designated as enoyl-CoA hydratase/aldolase.

The genes and enzymes involved in this pathway have been characterized in *Pseudomonas fluorescens* AN103 (Gasson *et al.*, 1998; Narbad & Gasson, 1998), *Pseudomonas* sp. strain HR199 (Overhage *et al.*, 1999a), *Pseudomonas putida* KT2440 (Plaggenborg *et al.*, 2003), *Amycolatopsis* sp. strain HR167 (Achterholt *et al.*, 2000), *Streptomyces setonii* (Muheim & Lerch, 1999) and *Delftia acidovorans* (Plaggenborg *et al.* 2001). These genes are organized in a catabolic cluster which includes the genes *ech*, *vdh* and *fcs* (encoding feruloyl-CoA hydratase/aldolase, vanillin dehydrogenase and feruloyl-CoA synthetase, respectively), or at least *ech* and *fcs* (Plaggenborg *et al.*, 2006).

1.7.3.4 Biotransformation of vanillic acid

Vanillic acid is a main intermediate in lignin and ferulic acid degradation, and in it is often accumulated in remarkable amounts, unlike vanillin (Andreoni *et al.*, 1995; Eggeling & Sahm, 1980). The vanillin production from vanillic acid was investigated in the white-rot fungus *P*ycnoporus *cinnabarinus*, where vanillic acid is either oxidatively decarboxylated to methoxyhydroquinone or reduced to vanillin and vanillyl alcohol (Falconnier *et al.*, 1994).

To avoid the predominant decarboxylation reaction, resulting in low yields of vanillin (Lesage-Meessen *et al.* 1996), a different carbon source, i.e. cellobiose, was added prior to vanillic acid supplementation. This expedient allowed to limiting the formation of methoxyhydroquinone and thus to favoring vanillin production, leading to a molar yield of 51.7%. (Bonnin *et al.*, 1999; Lesage-Meessen *et al.*, 1997). The vanillin yield was further improved by use of high-density cultures (Oddou *et al.*, 1999) and different types of bioreactors (Stentelaire *et al.*, 2000), and by the additional application of

selective XAD-2 resin to reduce the vanillin concentration in the medium. The ability of converting ferulic acid, obtained from sugar beet pulp, into vanillic acid by the fungal strains *Aspergillus niger* was combined with the biotransformation of recovered vanillic acid by *P. cinnabarinus* (Lesage-Meessen *et al.*, 1994, 1996, 1999). More recently, this two-steps process was explored by Zhang *et al.* (2007), using ferulic acid prepared from waste residue of rice bran oil.

The conversion of vanillic acid to vanillin was also examined in *Nocardia* sp. strain NRRL 5646, and the carboxylic acid reductase, responsible of reducing vanillic acid to vanillin, was expressed in recombinant *E. coli* cultures for direct use in whole-cell biocatalytic conversions of natural or synthetic carboxylic acids, such as vanillic acid. (Li & Rosazza, 2000; He *et al.*, 2004).

1.7.3.5 Biotransformation of phenolic stilbenes

As reviewed by Priefert *et al.* (2001), phenolic stilbenes are commonly found in spruce bark and can be oxidatively cleaved to the corresponding aromatic aldehydes catalyzed by ligno stilbene-α,β-dioxygenases from *Pseudomonas paucimobilis* strain TMY 1009. Corresponding genes have been cloned and expressed in *Escherichia coli*. With cell-free extracts of *P. paucimobilis* strain TMY 1009, naturally occurring isorhapotin was oxidized to gain vanillin with a yield of up to 70%. This process has also been patented (Yoshimoto *et al.* 1990a, b).

1.7.3.6 Biotransformation of aromatic amino acids

The essential amino acid phenylalanine is deaminated to *trans*-cinnamic acid by phenylalanine ammonia lyase, 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. The metabolism of L-phenylalanine was studied in several white-rot fungi as well as in bacterium *Proteus vulgaris*, which also possess phenylalanine ammonia lyase activity (Jensen *et al.*, 1994; Krings *et al.*, 1996; Casey & Dobb, 1992). *P. vulgaris* (CMCC2840)

deaminates methoxytyrosine to the corresponding phenylpyruvic acid, which is then converted to vanillin by mild caustic treatment (Casey & Dobb, 1992).

1.7.3.7 De novo synthesis of vanillin from glucose

De novo biosynthesis of vanillin, that is outside the *Vanilla planifolia* seed pod or other plants, from a primary metabolite like glucose may be a very attractive approach, since glucose costs less than $0.30 per kilogram (U.S. Census Bureau, 2004).

Li and Frost (1998) combined *de novo* conversion of glucose via shikimic acid pathway to vanillic acid by recombinant *Escherichia coli* KL7 strain, with in vitro enzymatic reduction of vanillic acid to vanillin by aryl aldehyde dehydrogenase isolated from *Neurospora crassa.* Despite of its attractive novelty, the process showed serious drawbacks such as the lack of an *in vivo* step for the enzymatic reduction of vanillic acid, demanding the addition of isolated carboxylic acid reductase and costly cofactors such as ATP, NADPH, and Mg^{2+}, and the generation of isovanillin as a contaminating side product (Frost, 2000).

Later, Hansen and coworkers (2009) proposed a true *de novo* biosynthetic pathway for vanillin production from glucose using two metabolically engineered yeast, i.e. *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Vanillin was produced at concentrations of 65 mg/L and 45 mg/L in *S. pombe* and *S. cerevisiae*, respectively, freely from contaminating isomers and without any specific optimization of media and growth conditions. The heterologous pathway for vanillin biosynthesis was engineered in both organisms by the expression of three genes, i.e. one gene encoding dehydroshikimate dehydratase from the dung mold *Podospora pauciseta*, one gene encoding aromatic carboxylic acid reductase (ACAR) from *Nocardia* sp., and one gene encoding an O-methyltransferase from *Homo sapiens*. Reduction of vanillin to vanillyl alcohol in *S. cerevisiae* was prevented by knockout of the host alcohol dehydrogenase ADH6. Despite of these expedients, the major drawback of this study was the glycosylation step which implies reduction in the maximum theoretical yield.

Nevertheless, this process leads to increase in toxicity and decrease in solubility of vanillin.

To reduce vanillin toxicity towards *S. cerevisiae* and to improve the product yields, an *in silico* metabolic engineering strategy of this vanillin β-$_D$-glucoside-producing yeast was designed and a glycosyltransferase from *Arabidopsis thaliana* was expressed in the host strain, respectively. This mutant strain exhibit to be able of growing in the presence of vanillin β-$_D$-glucoside at extracellular concentration up to 25 g/L. Moreover it showed a 5-fold improvement of free vanillin production compared to the previous study of Hansen and coworkers on *de novo* vanillin biosynthesis in baker's yeast (Brochado *et al.*, 2010). As outlined by the authors of this work, an elegant solution to overcome the toxicity of vanillin is the glycosylation to its conjugated form of β-$_D$-glucoside, which is observed in the natural producer *Vanilla planifolia*.

1.7.4 Metabolic engineering of *E. coli* for vanillin production

Beside the genetic manipulation of single enzymes, a different approach of metabolic engineering is to express the genes, which are involved in ferulate-catabolic pathways, in a host organism. For this purpose, ferulic-catabolic genes isolated principally from native ferulate-degrading strains of *Pseudomonas fluorescens* BF13 and *Amycolatopsis* sp. HR104, but also from *Penicillium simplicissimum* and *Delftia acidovorans*, were cloned in recombinant cells of *E. coli*.

Converti and coworkers (2003) proposed the recombinant *E. coli* strain JM109, which harbored the plasmid pBB1 including the ferulic catabolic genes from *P. fluorescens* BF13, as a suitable candidate for ferulic acid conversion to vanillin.

The recombinant plasmid pBB1 was generated by cloning a 5098-bp fragment, which contained the *ech* and *fcs* genes (encoding enzymes hydratase/aldolase and feruloyl-CoA synthetase, respectively) from a *vdh* negative mutant strain of *P. fluorescens* BF13 under the control of native P_{fer} promoter, into the low-copy vector pJB3Tc19 (Figure 8).

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). The use of integrative or low-copy number plasmids overcame the rapid decrease of end product by recombinant strains of *E. coli* due to the genetic instability of the vanillin-producing mutants, ss suggested by the researches of the studies following described (Barghini *et al.*, 2007; Ruzzi *et al.*, 2008).

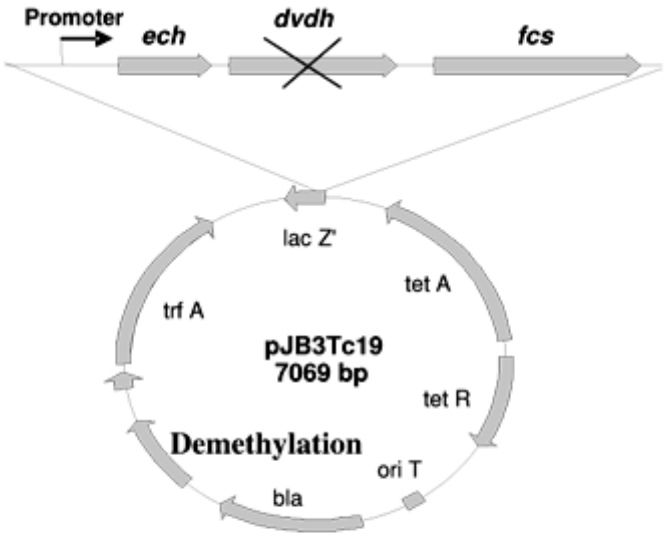

Figure 8. Construction of plasmid pBB1 containing genes from P. fluorescens BF13 to produce vanillin from FA using the vector pJB3Tc19. The plasmid was subsequently transformed into E. coli JM109 (Converti *et al.*, 2010).

Barghini *et al.* (2007) reported that resting cells of *E. coli* JM109/pBB1 with a low-copy number plasmid led to the final concentration of 3.5 mM vanillin after 6 h incubation by sequential induction with 1.1 mM ferulic acid. The authors proposed also the successful reuse of resting cells in four subsequent bioconversion cycles, which allowed to increase the end product concentration up to 2.52 g of vanillin per liter of culture. The biomass recycling may be a suitable strategy either to improve the vanillin productivity by using a continuous system or to reduce the costs of vanillin recovery by concentrating end product in the medium.

To develop a more stable recombinant strain, E. coli JM109 was engineered by cloning *ech* and *fcs* genes of *P. fluorescens* BF13 into a vector (pFR12) with a temperature-sensitive replicon, designed for chromosomal integration into the *lacZ* gene of *E. coli*. The resulting strain, namely FR13, was found to be more stable and efficient in vanillin

production than strains expressing the same genes from a low copy plasmid vector (Ruzzi *et al.*, 2008).

A recombinant strain of *E. coli* XL1-Blue(pSK*ech*E/H*fcs*), which harbored a hybrid plasmid with *fcs* and *ech* genes of *Pseudomonas* sp. HR199 under the control of *lacZ* promoter, could convert ferulic acid to vanillin at millimolar levels, under resting cells condition (Overhage *et al.*, 2003).

Similarly, the gene loci *fcs* and *ech* isolated from bacterium *Amycolatopsis* sp. strain HR167 were expressed in recombinant strains of *E. coli*, which were capable of transforming ferulic acid to vanillin (Achterholt *et al.*, 2000).

Two recombinant plasmids, namely pDAHEF and pDDAEF, carrying *fcs* and *ech* genes from *Amycolatopsis* sp. strain HR104 and *Delftia acidovorans*, respectively, were inserted into *E. coli* strains. As reported by the authors, 160 mg/L of vanillin was obtained by conversion of ferulic acid using *E. coli* with pDAHEF plasmid, whereas 10 mg/L of vanillin was observed with pDDAEF. Further optimization of the biotransformation process with *E. coli* harboring pDAHEF was performed by the addition of 13.3 mM arabinose as metabolic inducer and supplementation of 0.2% ferulic acid at 18 h of culture, which led to a vanillin production of 580 mg/L (Yoon *et al.*, 2005a).

In a separate research, Yoon and coworkers (2005b) reported that higher yield of vanillin was achieved with recombinant *E. coli* engineered by cloning *fcs* and *ech* genes from *Amycolatopsis* sp., under the isopropylthiogalactoside-inducible (IPTG) *trc* promoter. Vanillin concentration of 1.1 g/L was thus obtained with cultivation for 48 h in 2YT medium with 0.2% (w/v) ferulate, without IPTG and no supplementation of carbon sources.

Later, the production of vanillin from ferulic acid in *E. coli* DH5α cells transformed with plasmid pTAHEF containing *fcs* and *ech* genes cloned from *Amycolatopsis* sp. strain HR104 was tested. Vanillin was recovered at concentration of 1.0 g/L from 2.0 g/L ferulic acid within 48 h of culture (Yoon *et al.*, 2007).

Song *et al.* (2009) proposed a substrate channeling approach by dimer formation between leucine-zippers of *fcs* and *ech* genes, in order to channelize feruloyl-CoA from fcs to ech and thus increase vanillin production using recombinant *E. coli*. Mutant *E. coli* harboring a plasmid pTBE-FP forming an efficient dimer of Bait-Ech and Fcs-Prey

produced 2.1 g/L of vanillin at initial concentration of 3 g/L ferulic acid within 30 hours of culture, which was improved by 2.3-fold from vanillin production of 0.9 g/L of control strain harboring pTAHEF with no leucine-zipper.

To improve the vanillin production by reducing its toxicity, two strategies were proposed, i.e. the creation of a vanillin-resistant mutant, namely NTG-VR1, *via* nitrosoguanidine mutagenesis and removal of vanillin from medium by XAD-2 resin absorption. Using 5 g/L of ferulic acid, the production of vanillin with NTG-VR1 increased to three times when compared with its wild-type strain. Adding 50% (w/v) of XAD-2 resin to the culture, NTG-VR1 converted 10 g/L of ferulic acid to 2.9 g/L of vanillin. The concentration of end product was 2-fold higher than that obtained without resin addition (Yoon *et al.*, 2007).

E. coli DH5α (pTAHEF-gltA) harboring the amplification of *gltA* gene (encoding citrate synthase gene required for conversion of acetyl-CoA) converted 3 g/L ferulic acid to 1.98 g/L vanillin in 48 h of culturing. In the same study, the authors showed that the deletion of *icdA* gene encoding isocitrate dehydrogenase of TCA cycle enhanced the conversion of acetyl-CoA to CoA in comparison with TCA cycle. The vanillin production by the new mutant of *E. coli* BW25113 carrying plasmid pTAHEF together with deletion of *icdA* gene was 2.6-fold enhanced. The real synergistic effect of *gltA* amplification and *icdA* deletion was observed with addition of XAD-2 resin reducing the vanillin toxicity. Vanillin at 5.14 g/L concentration and molar yield 86.6% was recovered in 24 h of the culture. So far, this is the highest vanillin production from ferulic acid using recombinant *E. coli* (Lee *et al.*, 2009).

Within the metabolic engineering of *E.coli* for vanillin production, genes responsible for degrading either eugenol or isoeugenol were also isolated and cloned in the host recombinant strains.

In an earlier study, Overhage *et al.* (2003) proposed a two-step process for eugenol bioconversion to vanillin by resting cells of two metabolically engineered *E. coli* strains. In the first step, eugenol was converted to 8.6 g/L ferulic acid (molar yield 91%) within 15 h by *E. coli* XL1-Blue(pSK*vao*mP*calA*m*calB*). This strain carried a hybrid plasmid (pSK*vao*mP*calA*m*calB*), which was constructed by cloning the *vaoA* gene from *Penicillium simplicissimum* CBS 170.90 under the control of the *lac* promoter, together

with the genes *calA* and *calB* from *Pseudomonas* sp. strain HR199. In the second step, the produced ferulic acid was transformed to vanillin by the strain *E. coli* XL1-Blue (pSK*ech*E/H*fcs*) described above. This process led to 0.3 g/L of vanillin, besides 0.1 g/L of vanillyl alcohol and 4.6 g/L of ferulic acid.

The recombinant strain of *E. coli* BL21(DE3) without vanillin-degrading activity was engineered by introduction of a plasmid harboring the isoeugenol monooxygenase gene of *Pseudomonas putida* IE27 under the control of T7 promoter. Transformed cells were able to convert 230 mM isoeugenol to 28.3 g/L vanillin with a molar conversion yield of 81% after 6 h of culturing at 20°C (Yamada *et al.*, 2008). The wild type *P. putida* strain IE27 was previously investigated for its capability of efficiently converting isoeugenol to vanillin (Yamada *et al.*, 2007).

1.7.5 *Pseudomonas fluorescens* BF13 as potential candidate for vanillin production from ferulic acid

Recently, Di Gioia *et al.* (2011) proposed the use of a new metabolically engineered strain of *Pseudomonas fluorescens* for ferulic acid conversion to vanillin. The developed strain produced up to 8.41 mM vanillin, which is the highest final titer of vanillin produced by a *Pseudomonas* strain to date.

Biochemical and molecular data indicated that in *P. fluorescens* BF13, as in other members of the genus *Pseudomonas*, ferulic acid is degraded through a CoA-dependent non-oxidative route (Calisti *et al.*, 2008), leading to a transient formation of vanillin (Figure 9). In *P. fluorescens* BF13, the catabolic genes involved in the ferulic acid degradation are located in the *ech–vdh–fcs* operon, under the control of FerR regulator, with both activation and repression functions and which is induced by ferulic acid (Calisti *et al.*, 2008). In order to obtain a mutant *P. fluorescens* BF13 which retained the ability to bioconvert ferulic acid into vanillin but lost the ability to further oxidize the aldehyde to vanillic acid, the vanillin dehydrogenase (*vdh*)-encoding gene was inactivated *via* insertional mutation by using a kanamycin resistance gene cassette.

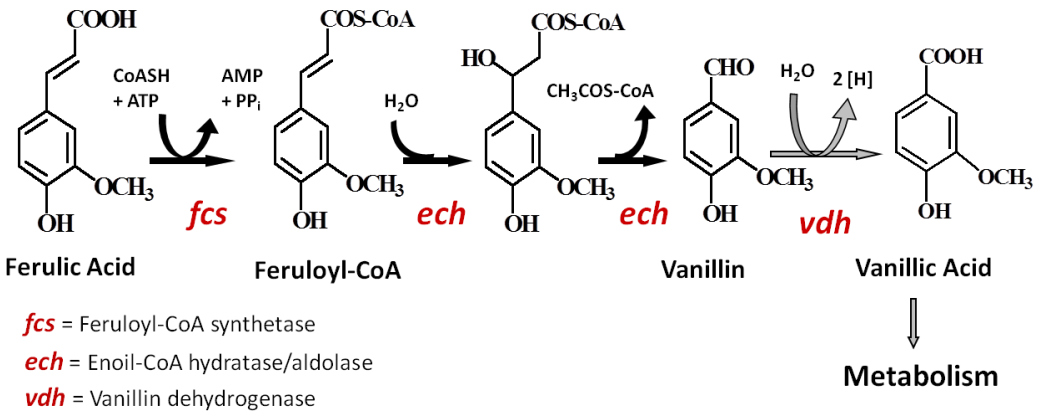

Figure 9. Schematic pathway for the degradation of ferulic acid in *P. fluorescens* BF13.

Since the insertional inactivation of *vdh* gene had strong polar effects on the expression of downstream functional genes in the operon and blocked the transcription of feruloyl-CoA synthetase (*fcs*)-encoding gene, a low-copy plasmid containing the *fcs* gene under its native promoter was introduced (Figure 10). The developed *P. fluorescens* BF13-1p4(pBB1) is thus able to bioconvert FA into vanillin and to accumulate the aldehyde by preventing its oxidation to vanillic acid, under resting cells conditions (Di Gioia *et al.*, 2011).

This transformed strain was also the object of study for biovanillin production from ferulic acid in the present PhD thesis.

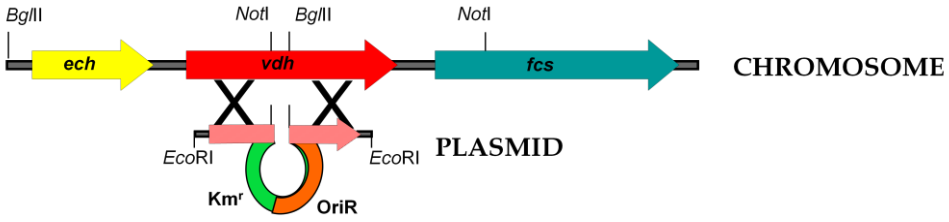

Figure 10. Metabolic engineering in *P. fluorescens* BF13-1p4(pBB1).

1.7.6 Valorization of agro-industrial wastes for biovanillin production

Agro-industrial wastes (or by-products) are "several kinds of biomass materials produced chiefly in food and fibre processing industries", as defined by Bioenergy and Food Security (BEFS).

They are the most abundant source of organic components in the world and hence important natural renewable resources, cheap and readily available. Straw, cereal bran, citrus peelings, cobs, stalks, bagasse are examples of agricultural residues..The use of agro-industrial residues as substrates in biotechnological processes could be a valuable alternative to overcome the high manufacturing costs of industrial fermentations (Bicas *et al.*, 2010).

Torres and coworkers (2009) investigated the ability of *E. coli* JM109/pBB1 to produce vanillin from alkaline hydrolyzate of corn cob. At initial biomass concentration of 0.5 g (dry weight)/L, maximum values of vanillin concentration (239±15 mg/L), vanillin yield on consumed ferulic acid (0.66±0.03 mol/mol) and vanillin volumetric productivity (10.9±0.7 mg $L^{-1}h^{-1}$) were observed after 22 h.

Shin *et al.* (2006) reported that an actinomycete strain of *Streptomyces setonii* (ATCC391161) was capable of producing vanillin from ferulic acid extracted from corn bran by using the enzyme pool of a filamentous fungus (*Neosartorya spinosa* NRRL185). 98% ferulic acid was converted to 0.43 mmol vanillin with molar yield 43% after 12 h of culturing.

Vaithanomsat and Apiwatanapiwat (2009) obtained by steam explosion a Jatropha curcas stem hydrolysate containing 1.55 g/L of ferulic acid, which was successfully used as substrate for one-step vanillin production by *Aspergillus niger* and *Pycnoporus cinnabarinus*.

Ferulic acid derived from sugar beet pulp was used as a precursor in a two step process to produce vanillin by employing two fungal strains. In the first step, the micromycete *A. niger* bioconverted ferulic acid to vanillic acid via propenoic chain degradation, to give rise to 920 mg/L of vanillic acid (molar yield 88%) and was subsequently decarboxylated to methoxyhydoquinone. The latter, was reported as the limiting pathway in the process. In the second step, vanillic acid was reduced to 237 mg/L of

vanillin (molar yield 22%) by a laccase deficient strain of basidiomycete *Pycnoporus cinnabarinus* (Lesage-Meessen *et al.*, 1999).

In a further study, Lesage-Meessen *et al.*, (2002) reported that *P. cinnabarinus* MUCL39533 could be fed with the autoclaved fraction of maize bran as a ferulic acid source and *A. niger* I-1472 culture filtrate as an extracellular enzyme source. Under these conditions, 584 mg/L of vanillin were obtained.

Rice bran was also investigated as a potential source of ferulic acid. Co-culture of ferulic-hydrolyzing *A. niger* CGMCC0774 and *P. cinnabarinus* CGMCC1115 were used for the production of vanillin on waste residue of rice bran oil involving vanillyl alcohol as an important intermediate. The yield of vanillin reached up to 2.8 g/L when 5 g/L of glucose and 25 g of HZ802 resin were supplemented in the bioconversion medium (Zhang *et al.*, 2007).

Recently, Barbosa *et al.* (2008) evaluated the possibility of vanillin production by solid-state fermentation on green coconut residue using the basidiomycete *Phanerochaete chrysosporium*. The sun-dried green coconut husk was selected as the better solid support for vanillin production by using the Plackett-Burman experimental design.

1.8 Wheat bran: a natural source for ferulic acid recovery

Wheat, together with maize and rice, is one of the most-produced cereals in the world. In 2010, world production of wheat was approximately 653 million tons. The European Union (EU-27) is the main producer of wheat (~140 million tons) (FAOSTAT, 2010) and in consequence is a major producer of wheat bran.

The most widely cultivated specie of wheat is *Triticum aestivum* and, to a lesser extent, *T. durum*. Wheat can be used as an animal feed (whole, crushed grain) as well as for flour production (Jerkovic *et al.*, 2010).

Bran is a major by-product generated during the milling of wheat grain for flour production. The composition of wheat bran depends greatly on wheat variety and cultivation conditions as well as wheat milling procedure. Nevertheless, it is particularly rich in carbohydrates (mostly dieter fibers) and essential fatty acid and contains

significant quantities of starch, protein, vitamins and phytonutrients (also called phytochemicals), such as ferulic acid. Due to its nutritional properties and cheap market availability, wheat bran (also known as a "brown gold") is one of the most attractive agro-industrial source for production of value-added compounds by biotransformation (Javed *et al.*, 2011).

The bran fraction comprises approximately 14-19% of the kernels weight and consists of the outer layer of the grain and the germ (Figure 11) (Liu, 2007).

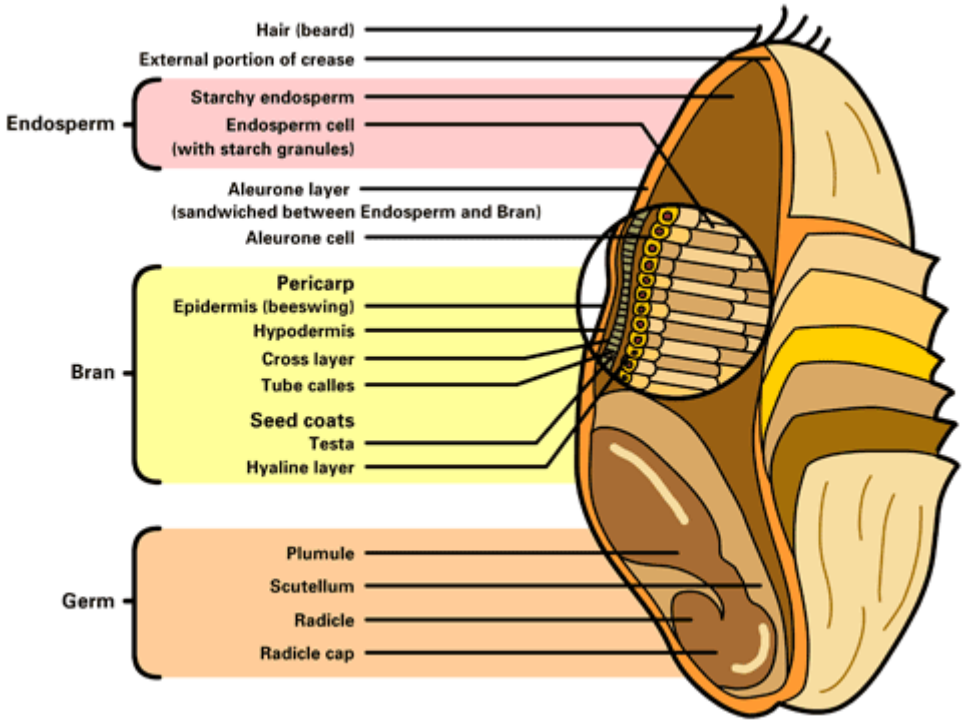

Figure 11. Wheat kernel dissection.

Bran, as a technical fraction from the milling industry, generally comprises the kernel wall and contains small amounts of the starchy endosperm and germ.

Wheat bran is composed mainly of polysaccharides, including arabinoxylans, xyloglucans and cellulose, but also contains significant amounts of phenolic acids, lignin and some proteins.

Phenolic acids are found mainly in wheat bran layer as well as in aleurone layer. The latter is actually the protective outer layer of cells in the endosperm, but it adheres to the bran and is removed with the bran during milling. Phenolic acids such as ferulic acid

play in the cell wall an important part in the cross-linking of polysaccharides with other cell-wall components, including lignin through ester and ether bonds, and also in the cross-linking of polysaccharide chains (Parker *et al.*, 2005). Among the phenolic acids, ferulic acid is the major component, accounting for 0,66% dry weight of the cell wall (Wong, 2006), and occurs principally in insoluble-bound form.

Wheat contains free, soluble-conjugated and insoluble-bound phenolic acids. Free and water conjugated phenolics represent approximately 5% and 20% of the total phenolic acid complement in wheat, respectively (Adom *et al*., 2003). Whilst the presence of individual bound phenolic acids is correlated with the bran there is no clear distinction for phenolics in their free and conjugated form; so it is presumed that these occur in all wheat tissues. The profile of free phenolic acids may vary with wheat type and cultivars conditions as well as the wheat processing for flour production. Nonetheless, ferulic acid tends to be the major source (Lempereur *et al.*, 1997; Gelinas & McKinnon, 2006; Mpofu *et al.*, 2006).

Particularly in monocots such as wheat, ferulic acid is attached to cell wall polymers by either ester bonds through its carboxylic acid group with the C5-hydroxyl of a-L-arabinosyl side chains of xylans (Figure 12) or *via* ether bonds to lignin, with its hydroxyl group covalently linked to lignin monomers (Shin *et al.*, 2006; Buonafina, 2009).

Ferulic acid endows structural rigidity and strengthens cell wall architecture by cross-linking pentosan chains, arabinoxylans and hemicelluloses, rendering these components less susceptible to hydrolytic enzymes during germination (Graf, 1992). Hydroxycinnamic acid is also involved in protein protection against pathogen invasion and control of extensibility of cell walls and growth.

Since its discovery, FA has also been reported to exhibit a wide range of important biological and therapeutic properties, which can be attributed to its antioxidant capacity, making it potentially useful as food additives and medicines (Ou & Kwok, 2004). In addition, ferulic acid probably represents the best candidate as a precursor for vanillin production by biotechnological routes, as extensively described in previous sections.

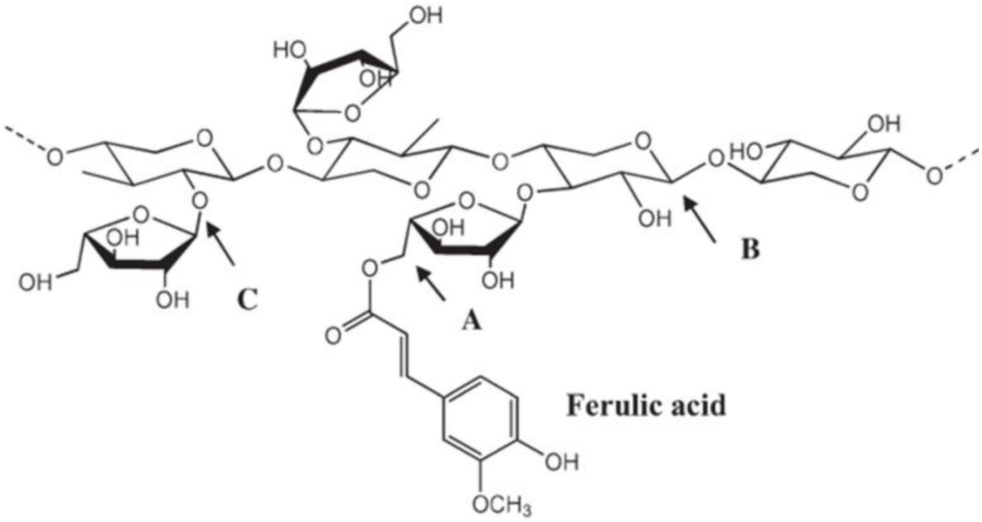

Figure 12. Structure of ferulic acid esterified to arabinoxylan in monocots (Buonafina, 2009)
(A) FA linked to O-5 of arabinose chain of arabinoxylan.
(B) b-1,4-linked xylan backbone.
(C) a-1,2-linked L-arabinose.

1.8.1 Release of ferulic acid from wheat bran

Ferulic acid can be released from wheat bran mainly by chemical and enzymatic hydrolysis. In addition, several wheat bran processing, such as mechanical (milling), physical, thermo-physical (such as steam explosion and DIC technology) and/or enzymatic pre-treatments, can be combined with enzymatic hydrolysis to improve and thus to facilitate the availability of the complex biomass matrix for enzyme activity. Chemical hydrolysis with strong alkali allows the almost complete release of ferulic acid from wheat bran (Kim *et al.*, 2006). However, beside the several disadvantages affecting chemical processes, the ferulic acid gained from chemical hydrolysis cannot be considered as a natural source for vanillin production. In fact, according to current EU and US regulations, vanillin through microbial conversion can be labeled as natural, when ferulic acid is derived from a natural source, such as wheat bran, and the recovery method is mild (such as biotechnological method), including the use of enzymes with GRAS (Generally regarded as safe) status (Mathew & Abraham, 2006). In order to

produce vanillin that can be labeled as a natural product, enzymatic treatment is thus considered as a growing interesting option.

1.8.2 Enzymatic hydrolysis

The degradation of lignocellulosic materials including wheat bran requires the combined and synergistic action of several enzymes with different activities, which essentially can be grouped into three categories, i.e. cellulase, hemicellulase and lignin-degrading enzymes. The latter group includes "accessory enzymes", such as α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterases, and feruloyl esterase, which help xylanases and pectinases to break down plant cell wall hemicelluloses. Among the accessory enzymes, feruloyl esterases play a key role in hydrolyzing ferulate ester groups involved in the cross-linking between hemicelluloses and between xylans and lignin. Feruloyl esterases (EC 3.1.1.73), also known as ferulic acid esterases (FAEs), belong to a subclass of carboxylic esterases (EC 3.1.1) (Fazary & Ju, 2008; Wong, 2006).

Despite its importance, FAE alone is not sufficient to release ferulic acid from the biomass matrix. Synergistic use of FAE and hemicellulases (especially xylanase and arabinofuranosidase) as well as cellulase and also protease, is necessary (Shin *et al.*, 2006; Sørensen *et al.*, 2003).

Many microorganisms have been reported to produce FAE. Among these, *Aspergillus* species, such as *Aspergillus flavipes*, *Aspergillus awamori*, *Aspergillus niger*, and *Aspergillus oryzae*, are the most active producers of feruloyl esterases. In spite of its extensive research, purified FAE is still not commercially available. This may explain the lack of industrial technology based on the recovery of ferulic acid from wheat bran (Fazary & Ju, 2008).

Nevertheless, several commercial enzyme cocktails are reported to have feruloyl esterase activity, such as Depol 740 L and Pectinase PE (Biocatalysts Ltd, Wales, UK), Pentopan Mono BG® and Novo® 188 (Novozymes, Bagsvaerd, Denmark), and Grindamyl™ S100 (Danisco, Brabrand, Denmark). FAE activity is usually a side activity, the major being hemicellulase or cellulase activity, except for Depol 740 L, in

which FAE activity is standardized to 36 Ug/L (Giet *et al.*, 2010). Some research works on enzymatic recovery of ferulic acid from wheat bran are summarized in Table 4.

1.8.3 Physicochemical pretreatments

Combined chemical and physical treatment systems are of importance in dissolving hemicellulose and lignin structure, providing an enhanced accessibility of the bran matrix for hydrolytic enzymes (Hendriks & Zeeman, 2009). The steam explosion is considered to be one of the most promising methods to make the industrial biomass more accessible to enzymes digestion. The process consists to expose the plant-derived materials up to high pressure (15 to 50 bar) and temperature (180 to 250 °C) in presence of steam during a determined time up to 90 minutes (Sassner *et al.*, 2008), followed by a rapid reduction in pressure, in order to breakdown the lignocellulosic structure. This technology was implemented at industrial scale with batch processes (Stake and Iotech) (Ballerini & Alalzadrd-Toux, 2006). The treatment leads to a partial self-hydrolysis of hemicelluloses, depolymerisation of lignin and a destructuration of cellulose, largely dependent on treatment temperature. The instant controlled pressure drop technology (D.I.C, in french: Détente Instantanée Contrôlée) process is close to steam explosion technology (Zhang *et al.*, 2008; Viola *et al.*, 2008) with the difference that the D.I.C treatment comprises two additional steps. The first step involves the instauration of initial vacuum before steam injection and allows to reduce the resistance of air and thus to facilitate the diffusion of steam into the product. Consequently, the time necessary to reach the steam equilibrium temperature is reduced. The second step consists of an abrupt decompression which carries out towards the vacuum (50 mbar) instead of atmospheric pressure as it happens with the steam explosion process (Maache-Rezzoug *et al.*, 2009). Due to the instantaneous character of this transformation as well as the adiabatic nature of the transition of steam inside the product, the water vaporisation induces a fast cooling. The temperature is quickly stabilized at a balance temperature of the considered final pressure, limiting the reactions of degradation.

Substrate	Ferulic acid (%w/w)		Release of ferulic acid (%)	Reference
Wheat bran	-	1%(w/v), AFAE III & xylanase of *Trichoderma viride*	95	Faulds & Williamson (1995)
	-	1%(w/v), PFAEB & xylanase of *T. viride*	98	Kroon *et al.* (2000)
	-	5%(w/v), FAE-II & xylanase from *Sporotrichum thermophile* (1.5 U FAE & 300 U xylanase)*	23	Topakas *et al.* (2003b)
	-	5%(w/v), FAE-I & xylanase from *S. thermophile* (4 U FAE & 100 U xylanase)*	92	Topakas *et al.* (2003a)
	-	2.5–3.0%(w/v), TsFaeC & xylanase of *T. viride*	66	Garcia-Conesa *et al.* (2004)
	0.288	1%(w/v), Ultraflo L (1U FAE & 2.43 xylanase)*	90	Faulds *et al.* (2004)
	-	2%(w/v), NcFaeD & xylanase of *T. viride* (0.1 U FAE & 200 U xylanase)*	36	Crepin *et al.* (2004)
Wheat-WIP	0.49	0.5%(w/v), 4 hemicellulases, 8 hemicellulases	96.9; 97.5	de Vries *et al.* (2000)
Wheat-WAIR	1.03	2%(w/v), FaeA/Xln11	29.2	Faulds *et al.* (2005)

Table 4. Release of ferulic acid from wheat bran by enzymatic hydrolysis (Adapted from Shin *et al.*, 2006).

*The enzyme load was calculated based on the enzyme units per gram of substrate.
WIP, water-insoluble pentosan from wheat; **WAIR**, wheat bran-alcohol-insoluble residues; **FaeA**, feruloyl esterase A from *A. niger*; Xln11, family 11 xylanase from *T. viride*; Ultraflo L, multifunctional b-glucanase from *H. insolens* (Novozymes, Denmark); **TsFaeC**, feruloyl esterase C from *Talaromyces stipitatus* CBS 375.48; **AFAE III**, ferulic acid esterase (FAE-III) from Aspergillus niger; **PFAEB**, feruloyl esterase B from *Penicillium funiculosum*; **FAE-II**, feruloyl-esterase-II from *Fusarium oxysporum*; **FAE-I**, feruloylesterase-I from *F. oxysporum*; **NcFaeD**, feruloyl esterase D from *Neurospora crassa*;

2 AIM OF THE THESIS

The present research activity was performed in the frame of the European project NAMASTE (New Advances in the integrated Management of food processing wAste in India and Europe: use of Sustainable Technologies for the Exploitation of by-products into new foods and feeds), which aims at the valorization of food by-products into new ingredients and compounds for food and feed industry, using innovative biotech processes.

In particular this PhD work focused on the production of vanillin, which is one of the most important commercial flavoring compound, from ferulic acid, a natural substrate abundant in the cells wall of cereals and available from bran processing by-products.

About 12.000 tons of vanillin is annually consumed in the world and only less than 1% of total production is extracted from vanilla pods, with a market price between $1.200 and $4.000 per kilogram. The main amount of vanillin is instead obtained from guaiacol and lignin by chemically synthesis and costs about $11-15/kg (Berger, 2007). Despite of the low price of synthetic vanillin, the world flavours and fragrances market prefers natural vanillin due to the increasing trend of consumers toward natural food ingredients. However the naturally vanillin production cannot meet the worldwide demand, because of several reasons, such as the limited vanilla plants growing areas and agro-climatic conditions and the labour-intensive cultivation and product recovery.

According to the European regulation on flavours (EC N° 1334/2008), vanillin obtained from materials of natural origin (vegetable, animal or microbiological sources) using physical, enzymatic and microbiological processes can be labelled as "natural vanillin". Therefore, biotechnological routes and in particular biotransformations represent and alternative and feasible way of producing biovanillin. This has attracted the interest of flavour producing companies, such as Solvay-Rhodia, whose commercial product Rhovanil® Natural is natural vanillin obtained by fermentation from ferulic acid (Maureen Rouhi, 2003).

A number of bacteria, including *Pseudomonas* spp., actinomycetes of the genera *Amycolatopsis* and *Streptomyces*, and the gram-positive *Bacillus subtilis*, have been proposed for the production of vanillin from ferulic acid, even though the produced vanillin is used as carbon source or further transformed by most of these microorganisms (Priefert *et al*., 2001).

In this work, the possibility of producing vanillin from ferulic acid by employing the *Pseudomonas fluorescens* strain BF13-1p4 (Barghini *et al*., 1998; Civolani *et al*., 2000) was investigated. The wild type strain is able to use ferulic acid as the sole carbon source by a CoA-dependent non-β-oxidative route with transient formation of vanillin, so it was modified by inactivation of the vanillin dehydrogenase (*vdh*) gene and amplification, on a low copy number plasmid, of BF13 ferulic catabolic genes required for conversion of ferulic acid to vanillin (*ech* and *fcs*) under the control of the native P_{fer} promoter, which is induced by ferulic acid (Ruzzi *et al*., 1997). The mutated strain is not able to use ferulic acid as carbon source but has the capability of converting ferulic acid into vanillin under resting cell conditions.

The experimental activities focused on the development and the optimization of protocols for the growth of the mutant strain *P. fluorescens* BF13-1p4 and for its use, under resting cells conditions, in the bioconversion of ferulic acid into vanillin. The goals were, for the growth phase optimization, to obtain biomass with the highest ferulic acid to vanillin bioconversion capability and, for the bioconversion phase, to obtain the highest vanillin molar yield, selectivity and productivity. The research work allowed the optimization either of the biomass growth step or of the bioconversion phase by the classical method of changing one variable while fixing the others at a certain level. A large array of parameters and conditions were investigated using this approach, i.e. time of metabolic induction with ferulic acid, inducer concentration, medium pH and duration of induction for the biomass production phase. On the other hand, the effect of buffer pH, cell concentration and ferulic acid concentration was studied in order to optimize the bioconversion phase.

An alternative approach based on statistical methods was further investigated. The response surface methodology (RSM) (Box and Wilson, 1951) is a commonly used method to assess the optimal fermentation conditions and also an efficient statistical technique for optimization of multiple variables and for interpretation of the

interactions among variables (Chaari *et al*., 2012; De Faveri *et al*., 2007). A three-variables response surface model for the biomass growth optimization and a five-variables model for the bioconversion experiments were thus designed.

In order to improve the sustainability of the vanillin production process, the suitability of the developed bioconversion protocol using ferulic acid obtained from wheat bran hydrolysates was investigated.

Wheat bran constitutes a major agricultural processing byproducts and there is great interest toward innovative strategies for valorizing this residue through its transformation into added value biomolecules. In fact the cell walls of wheat contain high amounts of ferulic acid, i.e., the most important precursor for the microbial production of vanillin. Ferulic acid acts an important role for the structure and biology of cell wall as cross-linking agent among carbohydrates (Mathew and Abraham, 2004). It is covalently linked to arabinoxylans through ester bonds and can be released in free form, available for its use in vanillin production, by either chemical digestion or through the action of specific enzymes, i.e. feruloyl esterases. The latter is the most interesting choice for a more environmentally friendly process as well as for obtaining a product that can be labeled as natural. A number of studies have therefore focused on the development of enzymatic methods to recover ferulic acid form cereal bran. Feruloyl esterases, capable of hydrolyzing the ester links between ferulic acid and polysaccharides, have been isolated mainly from fungal strains, belonging to the genera *Fusarium* (Shin and Chen, 2006; Topakas *et al*., 2003), *Neurospora* (Crepin *et al*., 2004), *Aspergillus* (Mathew and Abraham, 2004; de Vries *et al*., 2000; Bonnin *et al*., 2002), *Penicillium* (Kroon *et al*., 2000), *Talaromyces* (Garcia-Conesa and Kroon, 2004), *Humicola* (Faulds *et al*., 2004) and *Sporotrichum* (Topakas *et al*., 2005) and *Neosartorya* (Shin *et al*., 2006). Several of the isolated enzymes, used in combination with other extracellular enzyme activities such as cellulases and hemicellulases, which degrade the polysaccharide fraction of the plant tissue, thus helping the access of feruloyl esterases to their substrate, allowed a nearly complete recovery of ferulic acid from wheat bran (Faulds and Williamson, 1995; Faulds *et al*., 2004). However, the enzymes used are not commercially available and their application in full-scale processes is still far from being sustainable. Conversely, much lower recovery yields were obtained using

commercial, technical-grade enzyme preparations containing ferulic acid esterase activity combined with xylanase. In addition, only a few studies have investigated the use of ferulic acid-containing wheat bran hydrolysates as substrate for the biotechnological production of vanillin and evidenced that purification of ferulic acid from the carbohydrates occurring in the hydrolysate might be necessary to avoid increase in oxido-reductive enzyme activity that converts vanillin into side-products, thus reducing the vanillin yield.

The aim was thus to develop environmentally friendly bioconversion protocols based on the combination of (thermal) pre-treatments and enzymatic digestion of wheat bran with different commercially available enzyme preparations able to act on different cell wall constituents, in order to obtain a high recovery of ferulic acid, followed by ferulate purification using solid phase extraction.

3 MATERIALS AND METHODS

3.1 Chemicals

Reagents of analytical grade used for the preparation of culture medium were purchased from Biolife Italia (Milan, Italy), whereas antibiotics, salts for solutions, culture medium and buffer were from Sigma-Aldrich (Milan, Italy). Industrial grade Luria-Bertani (LB) components for biomass growth in bioconversion steps were kindly provided by Gnosis S.P.A. (Desio, MB, Italy). Food-grade ferulic acid (purity grade 97%) was obtained from Wuhan Yuancheng Co. Ltd (China). Untreated wheat bran for enzymatic hydrolysis experiments was supplied by G.R. Wright & Sons Ltd (Enfield, Essex, UK), whereas enzyme preparations were purchased from Sigma-Aldrich (Milan, Italy), Biocatalysts Ltd (Wales, UK), AB Enzymes (Darmstadt, Germany) and Megazyme (Wicklow, Ireland). High purity solvents for the preparation of samples and solutions used in high performance liquid chromatography (HPLC) were purchased from Carlo Erba (Milan, Italy) and Sigma-Aldrich (Milan, Italy). Columns ISOLUTE ENV$^+$ (20 mL volume, 1 g of packing) for bran hydrolizates purification were purchased from Biotage (Uppsala, Sweden). Solutions and media were prepared using distilled water, unless otherwise stated.

3.2 Bacterial strain

The microorganism used in this study for the production of vanillin from ferulic acid is a derivative of *Pseudomonas fluorescens* BF13 strain, named BF13-1p4. The mutated bacterial strain carries an insertional inactivation of *vdh* gene and *ech* and *fcs* BF13 genes under the control of native P_{fer} promoter on a low copy number plasmid. *P. fluorescens* BF13-1p4 is not able to use ferulic acid as carbon source but has the capability of converting ferulic acid into vanillin under resting cell conditions.

3.3 Culture medium, buffer and solutions

The bacterial strain was grown at 30°C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) having the following composition (Table 5):

LB medium composition	
Tryptone	10 g/L
Yeast Extract	5 g/L
Sodium Chloride (NaCl)	5 g/L
pH	7.0

Table 5. Composition of LB medium.

LB is a nutritive rich medium designed for growth of pure cultures of recombinant strains. The tryptone (an enzymatic digest of casein) and yeast extract supply essential growth factors such as nitrogen, carbon, sulfurs, minerals and vitamins, particularly B-group and other metabolites that the microorganism would otherwise have to synthesize. Sodium chloride supplies essential electrolytes for transport and osmotic balance. The LB formulations generally differ in the amount of sodium chloride, thus providing selection of the appropriate osmotic conditions for the particular bacterial strain and desired culture conditions. In this LB formulation, sodium chloride level was half than in the original composition, allowing a quicker growth of *P. fluorescens* strain.

LB medium was used in liquid form for inocula and cultivation broths, in solid form with addition of 15 g/L agar for plates preparation.

Medium prepared with industrial grade components was employed for the biomass growth, both in flask or fermentor, in the bioconversion experiments. LB of analytical grade plus 50 μg/mL kanamycin and 35 μg/mL tetracycline was used only for inocula preparation.

Saline phosphate buffer (Barghini *et al.*, 1998) used in the bioconversion experiments and for cells washing had the follow composition (Table 6):

Saline phosphate buffer composition	
Disodium Hydrogen Phosphate (Na_2HPO_4)	6 g/L
Potassium Dihydrogen Phosphate (KH_2PO_4)	3 g/L
Ammonium Chloride (NH_4Cl)	1 g/L
Sodium Chloride (NaCl)	0.5 g/L
pH	7.0

Table 6. Composition of saline phosphate buffer.

Different pHs of the buffer were obtained by changing the phosphate salts amount. Stock solution of food-grade ferulic acid at 20 g/L was prepared in saline buffer as indicated in Table 7. Sodium hydroxide in pellet form was added to the saline solution to completely dissolve ferulic acid.

Ferulic acid stock solution composition	
Disodium Hydrogen Phosphate (Na_2HPO_4)	8.66 g/L
Sodium Dihydrogen Phosphate (NaH_2PO_4)	6.24 g/L
Ferulic Acid	20 g/L
pH	7.0

Table 7. Composition of stock solution of food-grade ferulic acid.

Solutions, buffers and media used were sterilized in autoclave at 121°C for 20 min before use. Heat labile solutions, such as antibiotics and ferulic acid solutions, were filter-sterilized.

3.4 Bioreactor

The 2L stirred tank reactor (STR) Esedra Plus 2.0 (Solaris Biotechnology S.r.l., Porto Mantovano, Italy) was used in experiments of biomass growth optimization.

This bench top bioreactor consists of a cylindrical vessel made in borosilicate glass with a motor-driven central shaft that supports two Rushton 6-blade impellers. Sterile air is sparged into the fermenter liquid below the bottom impeller through of a perforated ring (1mm diameter) sparger. A stainless steel head plate is located on top of the vessel. Threaded ports in the head plate are provided for: acid-base addition, thermowells for temperature resistance and sensor, an internal stainless steel cooling coil, a sampling tube, dissolved oxygen (DO) and pH probes.

The measurement and control system has the same hardware control configuration of pilot and industrial bioreactors and is based on the supervisory Solaris SBC-12. This applications program is designed to provide a high level of automated management of the fermentation processes.

The process controller uses a touch screen interface for setting up, calibrating and monitoring of fermentation conditions, i.e. pH, DO, temperature and agitation speed. Three variable-speed peristaltic pumps are connected to the instrument and can be assigned to acid, base and antifoam or feed addition parts.

For these experiments of biomass growth with the *Pseudomonas* strain, the propylene glycol antifoam (provided by Solaris Biotechnology S.r.l.) was added at 0.2 g/L to the culture medium before autoclaving.

The pH is controlled in the range of 2.0-12.0 by automatically adding 1M H_2SO_4 and 2M NaOH sterile solutions. The pH probe is calibrated prior to the autoclave cycle outside the vessel using a two-point calibration method with standard pH 4.0 and 7.0 buffers.

The removable motor is connected to the agitation shaft with a mechanical seal and ranges from 0-1500 rpm. It is possible to cascade the dissolved oxygen to agitation so the agitation speed will vary between the user-specified minimum and maximum set points in order to maintain a set percentage of DO. The DO probe is calibrated using a standard two-point calibration method: 0 % (often referred to as the zero point) and 100 % (often referred to as span). The zero can be achieved by

disconnecting the DO cable (the electronic zero), by submersing the probe in a saturated solution of sodium sulfite Na_2SO_3 or by sparging nitrogen gas N_2 into the media to achieve a level stable near zero (used in this process). The 100 % calibration point is achieved by bringing the vessel filled with medium to all of the operational set points, i.e. agitation, temp, etc. DO is calibrated pre-autoclave and needs a six hour polarization period before any readings. The airflow rate is manually selected in the range 0-4 NL/min via a rotameter located on the instrument.

3.5 Process of biovanillin production from ferulic acid with *P. fluorescens* BF13-1p4

3.5.1 Overview of the process and methodology

The process of biovanillin production from ferulic acid using the bacterial strain *P. fluorescens* BF13-1p4 consists of two main phases, i.e., i) the biomass production phase and ii) the bioconversion phase. During the biomass production phase, cells metabolic induction with ferulic acid was performed in order to induce expression of functional genes coding for enzymes responsible for ferulic acid bioconversion into vanillin. Resting cell conditions were used in the bioconversion phase to avoid/reduce vanillin consumption due to the production of nonspecific oxidoreductase enzymes, typically occurring under growing conditions. A biomass washing step was thus necessary between the biomass production phase and the bioconversion phase, in order to remove residual carbon sources.

The main steps of the protocol are following outlined.

- A frozen stock culture of the bacterial strain *P. fluorescens* BF13-1p4 was firstly used to prepare a pre-culture on analytical-grade LB medium.

- The overnight grown pre-culture was used to inoculate fresh LB medium prepared with industrial grade components, either in flasks or in the 2L stirred tank reactor (STR), for biomass production.
- During biomass growth, ferulic acid was added as metabolic inducer.
- At the end of the biomass production phase, biomass was collected by centrifugation, washed to remove residual growth medium components and metabolites and suspended in saline phosphate buffer at the desired pH and final cell concentration.
- At this stage, biomass suspension was either immediately used for ferulic acid bioconversion to vanillin or stored overnight at 4°C.
- Bioconversion was performed either in flasks or in STR by adding ferulic acid to the biomass suspension in saline phosphate buffer.
- When using wheat bran hydrolysates as the source of ferulic acid, the collected and washed biomass was suspended at the desired concentration in bran hydrolysate to start bioconversion.

The general scheme of the process is outlined in Figure 13.

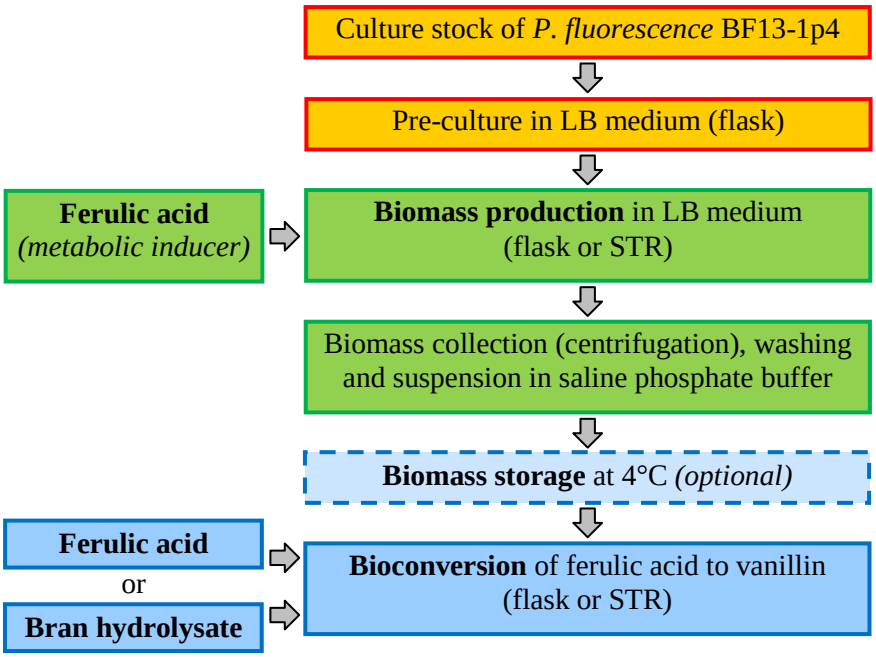

Figure 13. General scheme of vanillin production from ferulic acid/wheat bran hydrolysates with *P. fluorescens* BF13-1p4.

The above described process was optimized with respect to the biomass production phase, the biomass storage step and the bioconversion phase, in order to achieve the highest vanillin production in terms of vanillin molar yield, final vanillin concentration in the reaction medium and productivity. The three evaluation parameters were calculated as follows:

Vanillin molar yield (%): $[\text{Vanillin}]_{\text{produced}} / [\text{Ferulic acid}]_{\text{initial}} \times 100$
Selectivity (%):$[\text{Vanillin}]_{\text{produced}} / [\text{Ferulic acid}]_{\text{consumed}} \times 100$
Ferulic acid conversion (%):$[\text{Ferulic acid}]_{\text{consumed}} / [\text{Ferulic acid}]_{\text{initial}} \times 100$

All optimization experiments were performed using food-grade ferulic acid as bioconversion substrate. The suitability of the optimized protocol for the bioconversion of ferulic acid in wheat bran hydrolysates was tested on crude and purified bran hydrolysates obtained in paragraph 4.3.2. Details on protocol optimization of each phase are described in the following sections.

3.5.2 Protocol of the biomass producing phase optimization

Procedure in steps

- Defrost in ice a cryovial containing a stock culture of *P. fluorescens* BF13-1p4 frozen at -20°C in LB medium plus glycerol 20% v/v.
- Use the defrosted stock culture to inoculate (1% v/v) a 500 mL flask containing 50 mL of analytical-grade LB medium plus 50 µg/mL kanamycin and 35 µg/mL tetracycline solutions.
- Grow the pre-culture overnight at 30°C and 150 rpm on a rotary shaker.
- For biomass production in flask, inoculate (2% v/v) a 1 L flask containing 100 mL of industrial-grade LB medium with the overnight pre-culture. Grow biomass at 30°C on a rotary shaker (150 rpm).
- For biomass production in 2L STR, inoculate (2% v/v) 1 L of fresh industrial-grade LB medium plus 0.2 g/L antifoam with the overnight pre-culture. Cultivation was performed at 30°C, 500 rpm stirring, 4 NL/min aeration. The

desired pH value was maintained constant by automated addition of 2M NaOH and 1M H_2SO_4.

- Read hourly absorbance (600 nm) of cultivation broth to follow biomass growth. At the desired time, induce the culture by adding food-grade ferulic acid 2.5 mM to the desired final concentration.
- At the end of biomass growth, collect biomass by centrifugation at 4°C and 6000xg for 10 min.
- Discard the supernatant and wash the pellet twice with saline phosphate buffer.
- Suspend the pellet in saline phosphate buffer pH 7.0 at 6 g(wet weight)/L for bioconversion.

3.5.3 Protocol of the bioconversion phase optimization

Procedure in steps

- Suspend cells into sterile saline phosphate buffer at desired pH and cell final concentration and transfer 19 mL aliquots of cells suspension into sterile 100 mL flasks.
- Add 1 mL of ferulic acid stock solution in phosphate buffer at desired pH to obtain the desired ferulic acid final concentration.
- Incubate on orbital shaker at 30°C, 150 rpm.
- At time zero and then hourly, transfer 0.2 mL aliquots from the bioconversion flask into a 1.5 mL Eppendorf tube containing 0.8 mL HPLC-grade water plus 0.05 mL 2M trichloroacetic acid.
- Centrifuge at 12000xg for 10 min and recover supernatant for HPLC analysis of ferulic acid, vanillin and by-products vanillic acid and vanillyl alcohol according to paragraph 3.8 “Analytical assays”.

3.6 Two stage optimization procedure for enhancing vanillin production by Surface Response Methodology (RSM)

The optimization of vanillin production process with the response surface methodology (RSM) was performed in batch experiments using the following two stage procedure:

Step (1): Optimization of biomass producing phase by keeping the bioconversion conditions constant.

Step (2): Optimization of bioconversion conditions using the optimized growth conditions in step 1.

The simultaneous effect of three independent variables [ferulic acid concentration (mM) for induction, cell concentration (OD_{600}) at the time of induction, duration of induction (h)] for biomass growth optimization, and five independent variables [ferulic acid concentration (mM), cell concentration (g/L), pH of bioconversion buffer, reaction temperature (°C) and reaction time (h)] for bioconversion experiments were tested.

3.6.1 Experimental design

A central-composite statistical design, with the three variables for the biomass producing phase and the five variables for the bioconversion step, was used to study the response pattern and to determinate the optimum combination of variables for each steps of the process. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors. The independent variables were coded at five levels, i.e. −1.681, −1, 0, +1, +1.681, where 0 corresponded to central point. The coded values were calculated according to the following equation:

$$x_i = (X_i - X_0)/\Delta X_i, \quad i = 1,2, 3, \ldots \qquad (1)$$

Where x_i in Eq. (1) is the dimensionless value of an independent variable; X_i is the real value of an independent variable; X_0 is the real value of the independent variable at the central point; ΔX_i is the step change value.

The production of vanillin is the response variable. A second order polynomial model was fitted for the production of vanillin (Y), giving an equation of the following form:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \qquad (2)$$

where x_1, x_2, x_3 represent the coded levels of the independent variables and b_0, b_i, b_{ij} ($_{i,\ j}$ = 1, 2, 3) the coefficient estimates, where b_0 is the interception, b_1 the linear terms, b_2 the quadric terms and b_3 is the interaction terms.

The statistical analysis of the data were performed using MINITAB Statistical Software (Minitab, 2009) and the level of significance was 95%. The proportion of variance explained by the polynomial models obtained was given by the multiple coefficient of determination, R^2, which indicates the goodness of fit of the model based on RSM. The closer the R^2 value is to 1, the stronger the model is and the better it predicts. The significance of each coefficient was determined using the Student's *t*-test and *P*-value, which also indicated the interaction strength between each independent variable. The larger the magnitude of the t-value and smaller the *P*-value, the more significant is the corresponding coefficient. The regression Eq. (2) obtained using central composite design was maximized employing constrain search procedure using MATLAB software (The Mathworks, 2001). In general, the Fisher's test for analysis of variance (ANOVA) was employed to evaluate the statistical significance of the quadratic polynomial. The *F*-value is calculated as ratio of mean square regression and mean square residual. Generally, the calculated *F* value should be several times greater than the tabulated *F*-value if the model is a good prediction of the experimental results and the estimated factor effects are real.

3.7 Enzymatic hydrolysis of wheat bran for ferulic acid release

3.7.1 Characteristics of wheat bran used in hydrolysis experiments

Wheat bran used in the following experiments of enzymatic hydrolysis was supplied by G.R. Wright & Sons Ltd (Enfield, Essex, UK) as untreated wheat bran derived from their mill B fraction, as named within the miller.

The bran was stored at IFR (Institute of Food Research, Norwich, UK) at 10-12°C, moisture content ~ 10% and relative humidity < 70% to avoid microbial spoilage. Chemical analysis for bran characterization were performed at IFR by routine laboratory methods.

As shown in Table 8, the composition of wheat bran is mainly polysaccharide (~ 40% non-starch polysaccharide and ~ 15% starch), but is also rich in protein (~ 18%) and lignin (~ 5%).

Wheat bran composition (% w/w)	
Non-starch polysaccharide	39.0
Starch	15.7
Protein	17.6
Lipid	4.3
Bound phenolics	0.07
Lignin	5.1
Ash	5.2
Moisture	10.6
Total	(98)

Table 8. Composition of wheat bran used in this study (% w/w).

Cell wall bound phenolics are a minor (0.07%) but important component of wheat bran. The phenolics profile suggests that ferulic acid and its dimer –DiFA (diferulic acid) are the major phenolic acids and of interest for exploitation (Table 9).

Wheat bran phenolics profile (mg/g)	
Coumaric acid	0.09±0.01
8,8'-DiFA (AT)	0.00
Ferulic acid	5.23±0.20
8,8'-DiFA	0.07±0.01
8,5'-DiFA	0.41±0.04
5,5'-DiFA	0.32±0.03
8-0-4'-DiFA	0.67±0.03
8,5'-DiFA (BF)	0.28±0.01
Sinapic acid	0.12±0.01
Total	7.19

Table 9. Phenolics profile of wheat bran used in this study (mg/g).

3.7.2 Overview of the process and methodology

The process of the ferulic acid recovery from wheat bran treatment is outlined in Figure 14 and consisted of three main phases, i.e. i) bran pre-treatment, ii) enzymatic release of ferulic acid, and iii) purification of ferulic acid from carbohydrates.

Two protocols for bran pre-treatment (*Method 1* and *Method 2*) were tested. *Method 1* consisted in thermal treatment of wheat bran suspension in autoclave at 121°C for 20 minutes, while *Method 2* consisted in enzymatic destarching and deproteinating pre-treatment of wheat bran with the amylase Termamyl and the protease Alcalase respectively.

Enzymatic release of ferulic acid from pre-treated bran was performed with different enzyme combinations. In particular, thermally pretreated bran (from *Method 1*) was digested with enzymes acting on different components of the cell wall, namely with a combination of Fungamyl® 800L, a commercial amylase formulation, and Celluclast® 1.5L, a cellulase commercial formulation, and with a combination of Pentopan Mono BG®, a xylanase commercial formulation, and Celluclast® 1.5L.

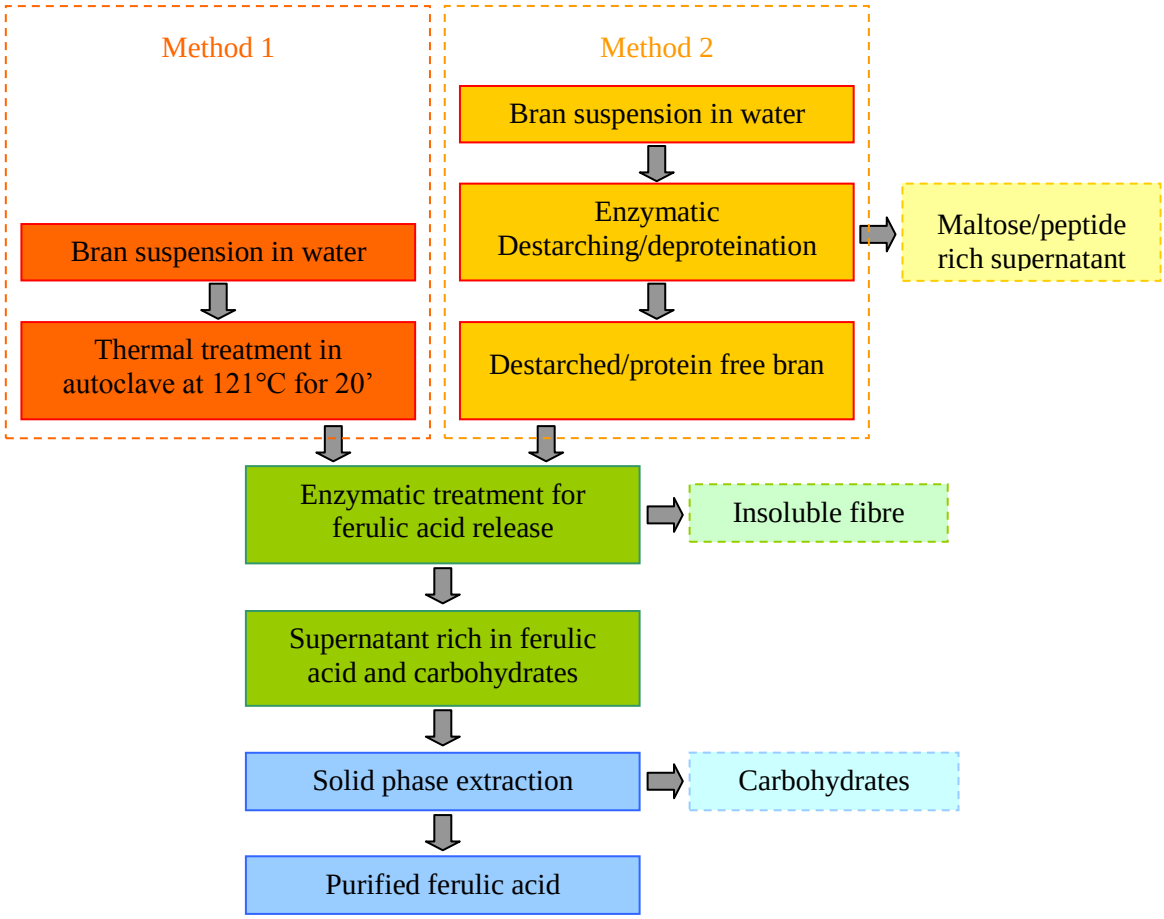

Figure 14. General scheme of the wheat bran treatment protocol for the release and purification of ferulic acid.

Destarched/deproteinised pre-treated wheat bran (from *Method 2*), instead, was digested with xylanase commercial preparations, namely Depol 740L, Econase, Pentopan Mono BG®, and with a combination of the xylanases Depol 740L or Pentopan Mono BG® with a commercial feruloyl esterase (rumen microorganism) since the other major components of the cell wall were assumed to be removed during the pre-treatment phase. The features of the enzymes tested are reported in Table 10.

Enzyme	Activity	Provider
Fungamyl® 800L	α-amylase	Sigma (A8220)
Celluclast® 1.5L	cellulase	Sigma (C2730)
Pentopan Mono BG®	Xylanase	Sigma (X2753)
Termamyl	α-amylase	Sigma (A3403)
Alcalase	protease	Sigma (P4860)
Depol 740L	xylanase	Biocatalyst
Econase® CE	xylanase	AB Enzymes
Feruloyl esterase (rumen microorganism)	feruloyl esterase	Megazyme

Table 10. Enzymes used in the protocols.

The purification of ferulic acid from carbohydrates was tested only on the hydrolysate obtained from *Method 2* destarching/deproteinating pre-treatment and further digestion with Pentopan Mono BG® + feruloyl esterase. The selected purification method consisted of solid phase extraction of ferulic acid with ISOLUTE ENV+ columns, followed by elution with absolute ethanol and solvent evaporation. Protocol details for each phase are described in the following sections.

3.7.3 Protocol of enzymatic hydrolysis of wheat bran for ferulic acid release: *Method 1*

Procedure in steps

- Suspend 40 g of wheat bran in 280 mL of distilled water (ratio 1:7) into a 0.5 l Pyrex bottle.
- Incubate the suspension in autoclave at 121°C for 20 minutes.
- Cool down to 30°C.
- Add enzyme cocktail (Fungamyl + Celluclast , 1% w/w each, or Pentopan + Celluclast , 1% w/w each) and incubate at 30°C on a rotary shaker (60 rpm) for 20 h.

- Filter the suspension on a nylon membrane to remove lignin compound.
- Centrifuge the filtrate at 5000xg for 20 min to remove starch and arabinoxylans. Recover the supernatant.
- Filter the supernatant on a filter paper (2.5 μm) and then on a cellulose acetate filter (0.2 μm).
- The filter-sterilised hydrolysate could be stored at -20°C. Ferulic acid concentration was analysed by HPLC-UV (for details, see paragraph 3.8 "Analytical assays").

3.7.4 Protocol of enzymatic hydrolysis of wheat bran for ferulic acid release: *Method 2*

Procedure in steps

- Suspend 400 g wheat bran (10% moisture, i.e., 360 g dry weight) in 4 liters of deionised water (pH 5.5-7.0).
- Heat to 90-100°C for 20 min in a water bath to gelatinise starch, with occasional stirring.
- Cool to 60°C and add Termamyl (200U/g bran).
- Incubate 30 min with occasional stirring.
- Add ammonium carbonate (30 mM final conc.) to give required pH for Alcalase digestion (pH ~8.5-8.7) and add Alcalase (20 μl/g bran).
- Incubate at 60°C for 3-4 h with stirring.
- Remove supernatant (filter using cloth) and wash pellet with ~4L water (x3). (The destarched/deproteinated wheat bran could be stored at -20°C or freeze-dried and stored at room temperature).
- Suspend 500 mg dry weight of whole wheat bran in 5 mL deionised water or 500 mg destarched/deproteinated wheat bran in 10 mL deionised water into 10 mL Corning Tubes.
- Incubate tubes in a water bath at 50°C and allow sample to hydrate for 4-5min.
- Add enzyme cocktail and incubate for set times (e.g. 0, 2, 4, 6, 16, 24h).

- At pre-set time, centrifuge at 3000 rpm for 3 min to pellet undigested sample.
- Remove supernatant to a clean tube and refrigerate until required for assay, or heat the pellet at 100°C for 10 min to inactivate enzymes.
- Assay ferulic acid released via HPLC-UV analysis (for details, see paragraph 3.8 “Analytical assays”).

3.7.5 Protocol of ferulic acid purification from carbohydrates in wheat bran hydrolysates

Procedure in steps

- Equilibrate the non-polar ISOLUTE ENV^{+} column (hydroxylated polystyrene-divinylbenzene co-polymer) with 9 mL of methanol and then with 9 mL of distilled water.
- Load 20 mL of crude hydrolyzate on the column.
- Discard the water phase effluent from the column.
- Wash the column with 30 mL of distilled water.
- Elute the adsorbed ferulic acid and phenolics with 2x 12 mL absolute ethanol.
- Remove solvent by evaporation under a gentle nitrogen flux.
- Re-suspend ferulic acid in the desired volume of phosphate buffer pH 7.0 (purified hydrolysate).
- Assay ferulic acid and reducing sugars occurring in the purified hydrolysate by HPLC-UV and spectrometric measurement respectively (for details, see paragraph 3.8 “Analytical assays”).

3.8 Analytical assays

Optical density (OD) of the culture broths was measured at 600 nm using a double beam UV-Vis spectrophotometer (mod. Varian Cary® 100, USA).

Qualitative and quantitative analysis of ferulic acid, vanillin, vanillic acid and vanillyl alcohol in the bioconversion buffer was done via a HPLC-UV system (Beckman Coulter, USA) equipped with a Beckman Ultrasphere 4.6 mm × 250 mm ODS column (5 μm particle diameter). Column temperature was 35 ◦C; injection volume was 20 μl.

The eluents employed were: (A) water additioned with 1% (v/v) acetic acid and (B) methanol additioned with 1% (v/v) acetic acid. The isocratic method used was 70% (A) and 30% (B), with flow rate of 1 mL/min and analysis duration of 16 min. Compounds were identified comparing their retention time with those of authentic sample. They were eluted at the following retention time: vanillyl alcohol, 3.5 min; vanillic acid, 5.1 min; vanillin, 6.5 min; ferulic acid, 9.5 min. For quantification, all intermediates were calibrated with external standards.

The amount of ferulic acid in wheat bran hydrolysates was quantified using the eluents gradient method described in Table 11.

Gradient	% (A)	% (B)	Duration (min)
none	90	10	1
linear, to	62	38	30
none	62	38	10
linear, to	0	100	1
none	0	100	8
linear, to	90	10	5

Table 11. Eluents gradient method for HPLC analysis of bran hydrolysates.

Spectrophotometric analysis of reducing sugars in wheat bran hydrolysates was performed as follows (Miller, 1959):

- dissolve 12 g of potassium sodium tartrate in 8 mL of 2M NaOH solution.
- Prepare a 96 mM solution of 3,5-dinitrosalicylic acid in 20 mL of distilled water.
- Coloring solution (DNSA reagent): mix (freshly prepared) the potassium sodium tartrate solution with the 96 mM 3,5-dinitrosalicylic acid solution and bring volume to 40 mL with distilled water (Store the coloring solution at 4°C and protected from light)
- STANDARD CURVE: prepare a stock solution of D(+)-Maltose monohydrate at 0.2 % w/v in distilled water and dilute to provide a serial dilution 0 – 2 mg/mL to construct a calibration curve.
- Add in 2 mL eppendorf:
    - 200 µL maltose standard or sample
    - 200 µL distilled water
    - 200 µL coloring solution
- Rapidly mix and incubate for 15 min in boiling water for color to develop.
- 15 min at boiling water for color to develop.
- Cool in ice to room temperature.
- Transfer all the content in 4 mL cuvette and add:
    - 1.800 µL distilled water
- Mix well and read the adsorbance at 540 nm, using distilled water plus the coloring solution as blank.
- Use the calibration curve to determinate the reducing sugars amount in the wheat bran hydrolizates.

4 RESULTS

4.1 Production of biovanillin from ferulic acid with *P. fluorescens* BF13-1p4

4.1.1 Optimization of the biomass producing phase

Following the biomass growth procedure described in paragraph 3.5.2, optimal cultivation parameters to obtain cells capable of efficiently performing the bioconversion were investigated (Table 12).

Parameters	Tested values
Time of metabolic induction with ferulic acid	0 and 4 h after culture inoculation
Inducer concentration	1 and 2.5 mM
Medium pH	6.8; 7.2; 7.6
Cells recovery time	5.5 and 6.5 h after culture inoculation

Table 12. List of investigated cultivation parameters.

The bioconversion efficiency of cells grown under different conditions was evaluated as vanillin molar yield and vanillin molar concentration obtained in bioconversion experiments performed in phosphate buffer pH 7.0 with 6 g(wet weight)/L cells and 5 mM ferulic acid, after 3 hours of incubation.

The detailed protocol of the bioconversion process is reported in paragraph 3.5.3.

As shown in Figure 15, when induction with 2.5 mM ferulic acid was performed at the beginning of the incubation and followed by cells recovering after 5.5 h of growth in the absence of pH control, vanillin molar yield obtained was 37.2%. Vanillin molar yield increased to 41.8% when induction with ferulic acid 2.5 mM was performed in the middle of the exponential growth phase (after 4.5 hours of growth). On the other hand, cells induced in the middle of the exponential growth

phase with lower ferulic acid concentration (1 mM) exhibited a decrease in bioconversion efficiency (31.6% vanillin molar yield) under the same culture conditions (Figure 15).

The pH of the growth medium significantly affected the cell bioconversion efficiency. Remarkable higher vanillin molar yields (63.4% and 62.8%) were obtained at pH values close to neutrality (pH 6.8 and 7.2, respectively), whereas slightly alkaline pH values (pH 7.6) significantly reduce the bioconversion efficiency to 43.3% vanillin molar yield (Figure 15). Finally, increasing the contact time of cells with the inducer (cells induced after 4.5 hours of growth and harvested after 6.5 hours of growth) remarkably reduced vanillin molar yield to 54.4% (Figure 15).

The most active cells for the bioconversion of ferulic acid into vanillin were thus obtained at pH 6.8 by inducing cells after 4.5 hours of growth with 2.5 mM ferulic acid and harvesting them 1h after inducer addition.

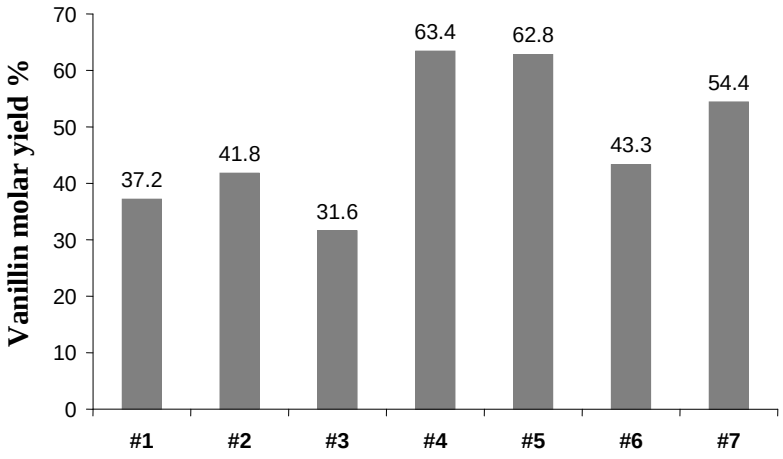

Figure 15. Vanillin molar yields obtained after 3 hours of bioconversion (cells concentration: 6 g/L; 5 mM ferulic acid; pH 7.0) using *P. fluorescens* cells grown under different conditions (experiments #1 to #7). Experiment #1: induction with 2.5 mM ferulic acid at the beginning of cultivation, cells recovered after 5.5 h of growth. Experiment #2: same as experiment #1 except for induction time (after 4.5 hours of growth). Experiment #3: same as experiment #2 except for inducer concentration (1 mM ferulic acid). Experiments #4, #5 and #6: same as experiment #2 with pH control at 6.8, 7.2 and 7.6, respectively. Experiment #7: same as experiment #4 except for cells recovery time (after 6.5 h of growth). Cells were grown in shaken flasks in experiments #1 to #3 and in stirred tank reactor in experiments #4 to #7.

4.1.2 Optimization of the bioconversion phase

Cells produced under the optimized growth conditions were used in a set of experiments aiming at the optimization of the bioconversion conditions. In order to monitor hourly the bioconversion process until almost complete consumption of ferulic acid, cells harvested at the end of the biomass production step were stored overnight at 4°C in ferulic acid-free bioconversion buffer at the concentration of 12 g/L and used the following day.

The bioconversion parameters investigated are reported in Table 13.

The bioconversion efficiency was evaluated after 8 hours in terms of vanillin molar yield, selectivity and ferulic acid conversion percentages.

Parameters	Tested values
buffer pH*	6.5, 7.0, 7.5
cell concentration	3.0, 6.0, 12.0, 24.0 g/L
ferulic acid concentration	2.5, 5.0, 7.5, 10 mM

*Different buffer pHs were obtained by changing the phosphate salts amount.

Table 13. List of parameters investigated during the bioconversion phase optimization.

4.1.2.1 Optimization of pH

pH had a modest effect on the bioconversion; the highest vanillin molar yield (82.8±0.9 %) and bioconversion selectivity (87.5±1.2 %) were obtained at pH 7.0 (Figure 16). Under these conditions, 94.6±0.8 % of the initially available 5.14±0.01 mM ferulic acid was consumed after 8 hours of bioconversion and converted into 4.26±0.03 mM vanillin, 0.54±0.002 mM vanillic acid and 0.12±0.002 mM vanillyl alcohol.

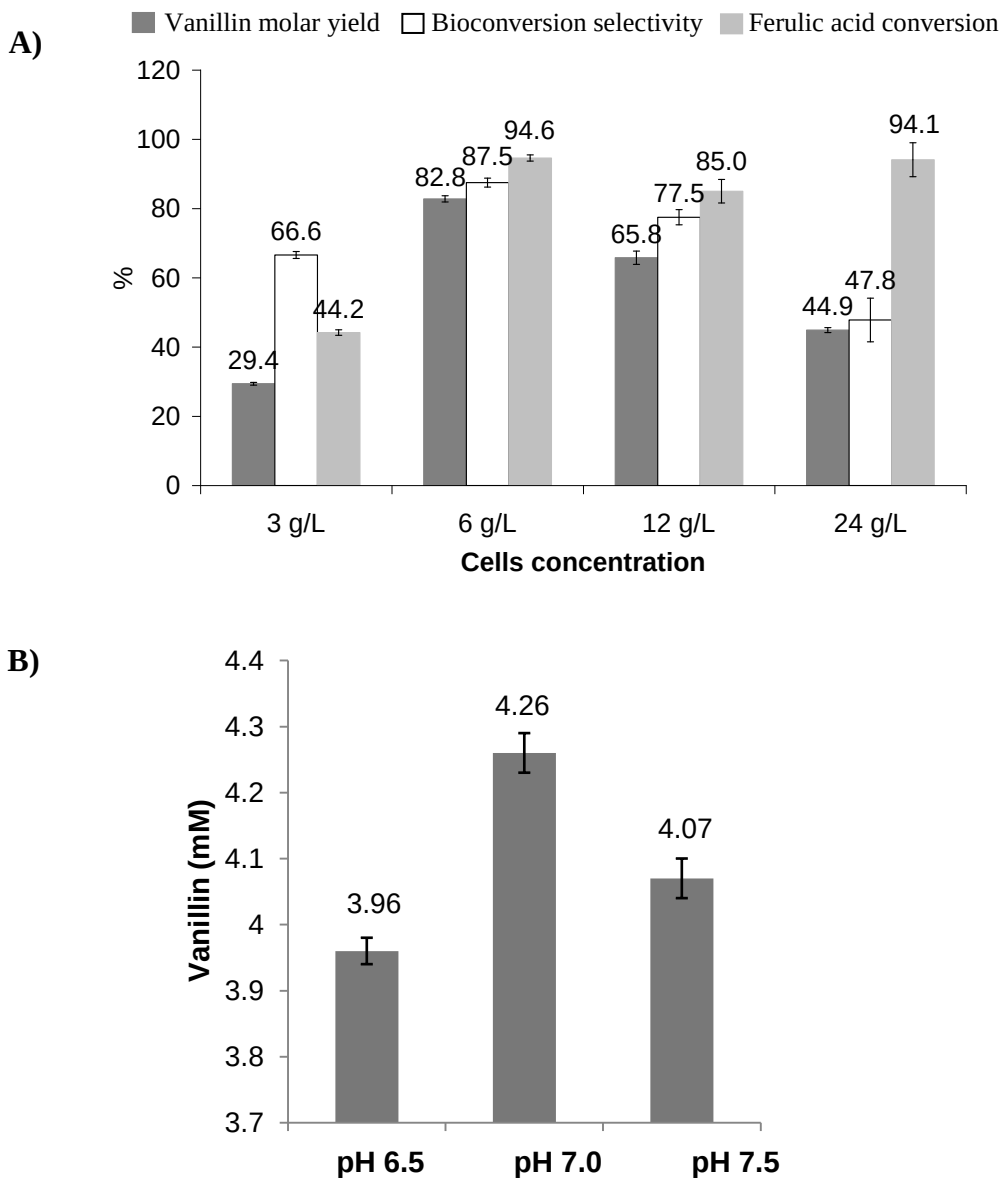

Figure 16. Effect of pH on the ferulic acid bioconversion into vanillin, in terms of A) vanillin molar yield (%), bioconversion selectivity (%) and ferulic acid conversion (%) and B) mM of vanillin produced after 8 hours of bioconversion performed with 6 g/L cells and 5 mM initial ferulic acid.

4.1.2.2 Optimization of cell concentration

The use of cells at 3 g/L gave remarkably low ferulic acid conversion, thus leading to very low vanillin molar yields. On the other hand, the use of higher cell concentrations (12 and 24 g/L) mainly resulted in lower bioconversion selectivity,

which in turn reduced vanillin molar yield. Using cells at 6 and 12 g/L resulted in the same final concentration of vanillin. However, it may be better to use 6 g/L, since this allows to produce less biomass for the following bioconversion step. Moreover less by-products were formed, that is higher selectivity, and therefore less problems and costs of purification. The highest ferulic acid conversion (94.6%), bioconversion selectivity (87.5%) and thus vanillin molar yield (82.8%) were obtained using cells at 6 g/L (Figure 17).

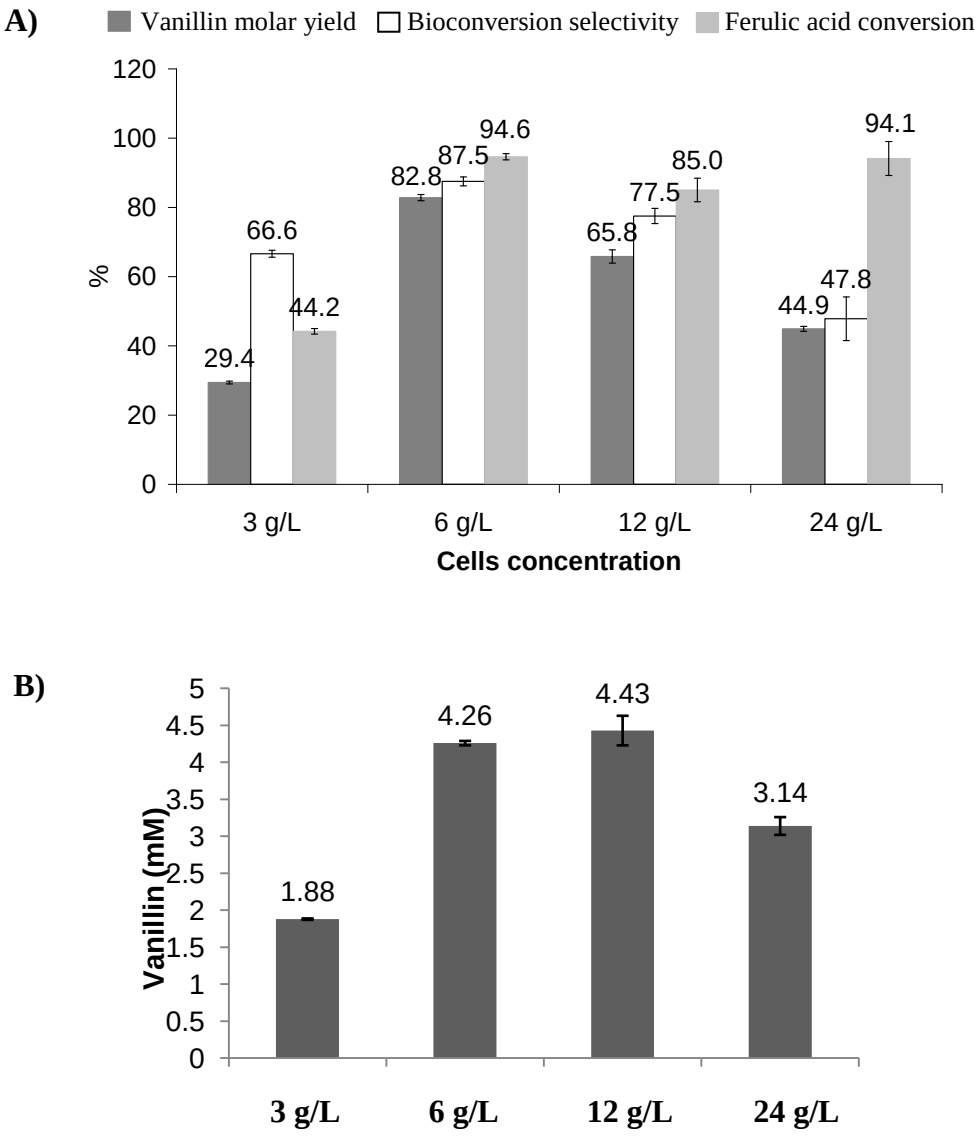

Figure 17. Effect of cell concentration (wet weight) on the ferulic acid bioconversion into vanillin, in terms of A) vanillin molar yield (%), bioconversion selectivity (%) and ferulic acid conversion (%) and B) mM of vanillin produced after 8 h of bioconversion performed in phosphate buffer pH 7.0 with 5 mM initial ferulic acid.

4.1.2.3 Optimization of ferulic acid concentration

Increasing ferulic acid initial concentrations from 5 mM to 10 mM resulted in increasing final concentrations of vanillin, the highest being 8.4 mM after 24 hours of incubation with 10 mM initial ferulic acid (Figure 18). Increasing ferulic acid initial concentrations up to 5 mM also increased vanillin productivity, whereas increasing initial concentrations of ferulic acid above 5 mM decreased vanillin productivity (Figure 18). The highest vanillin productivity (0.61 mmoles L^{-1} h^{-1}) was thus obtained with 5 mM ferulic acid. This behaviour was probably related to substrate inhibition phenomena.

Similar molar yields (82.9% and 80.2%) and selectivity (87.5% and 91.6%) were obtained with ferulic acid at 5 mM and 10 mM concentration. The use of 5 mM ferulic acid resulted in the highest productivity, whereas the use of 10 mM ferulic acid produced the highest final vanillin concentration in the bioconversion buffer. The best option depends on the costs related to the downstream processing for product recovery and purification from low-concentrated and high-concentrated vanillin solutions.

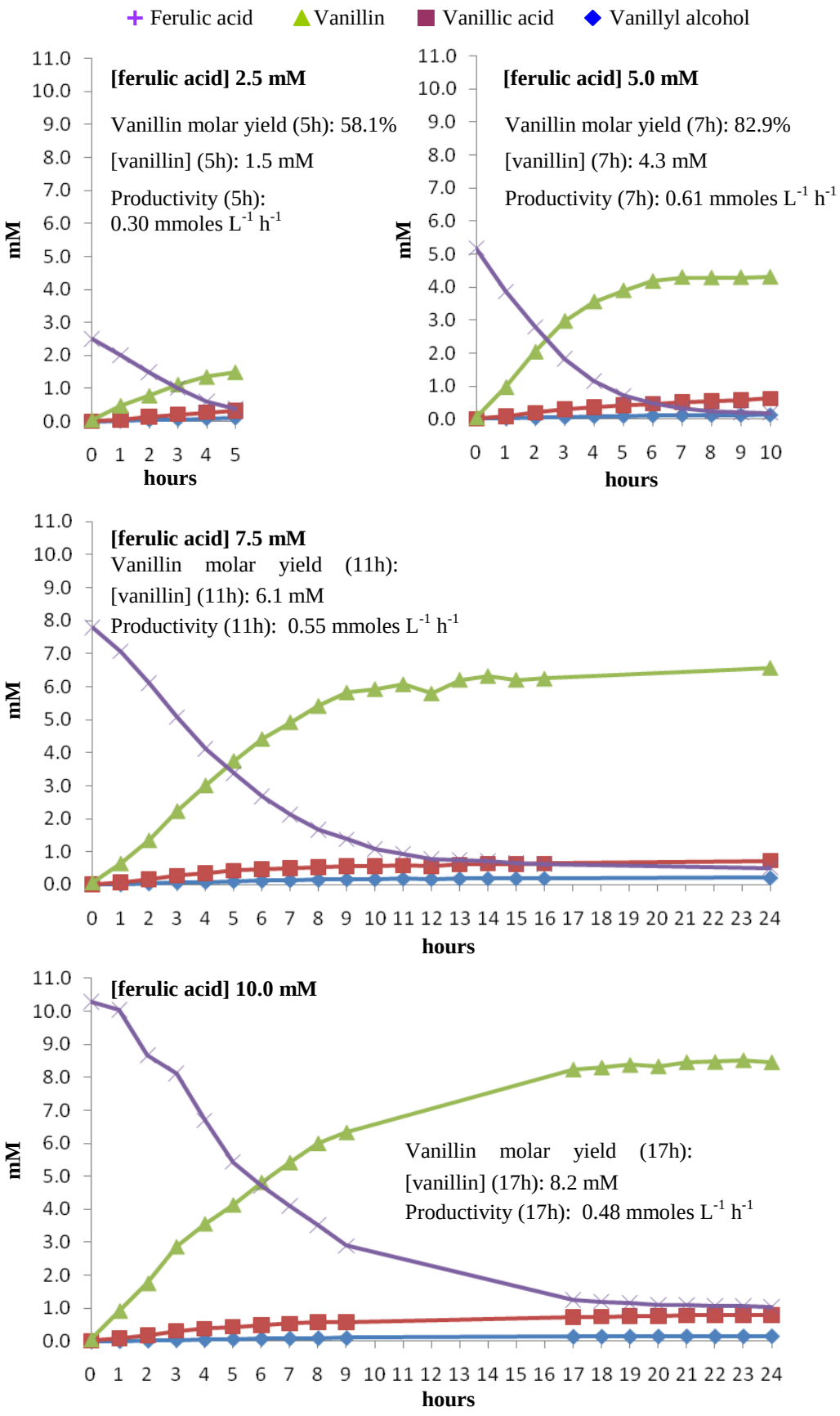

Figure 18. Effect of ferulic acid concentration on bioconversion (pH 7.0; 6 g/L cell).

4.1.3 Optimization of bioconversion in fed-batch process

Fed-batch addition of ferulic acid was investigated in order to increase vanillin concentration and molar yields. Vanillin 3.5 mM was obtained after two sequential additions of ferulic acid 2.5 mM, corresponding to 68% vanillin molar yield. Thus vanillin concentration and molar yield lower than those obtained after a single spike of 5 mM ferulic acid (4.3 mM vanillin and 83% vanillin molar yield) were obtained. Similarly, 5.3 mM vanillin was achieved after three sequential additions of ferulic acid 2.5 mM, corresponding to 64% vanillin molar yield, which were lower than vanillin concentration (6.6 mM) and molar yield (84%) obtained after a single spike of 7.5 mM ferulic acid. Finally, 6.4 mM vanillin resulted after two sequential additions of 5 mM ferulic acid, corresponding to 64% vanillin molar yield. Vanillin concentration and molar yields were thus lower than those obtained after a single spike of 10 mM ferulic acid (8.2 mM vanillin and 80% vanillin molar yield). Thus, the sequential addition of ferulic acid (fed-bath bioconversion) reduced the overall concentration of vanillin produced, i.e., the vanillin bioconversion yield, regardless of the overall amount of substrate provided and the number of substrate spikes. The process should be hence performed under batch conditions.

4.1.4 Optimization of biomass storage

Additional experiments were performed in order to evaluate the effect of biomass storage conditions on the bioconversion efficiency (Figure 19). Very similar bioconversion results were obtained with cells immediately used after harvesting and cells stored at 6 and 12 g/L in bioconversion buffer, whereas significant decreases in vanillin molar yield and ferulic acid conversion were observed with cells stored at higher concentrations (24 g/L) or as pellet (Figure 19). This indicated that biomass can be stored overnight at 4°C in bioconversion buffer at concentrations lower than 12 g/L without affecting their capability to efficiently bioconvert ferulic acid into vanillin.

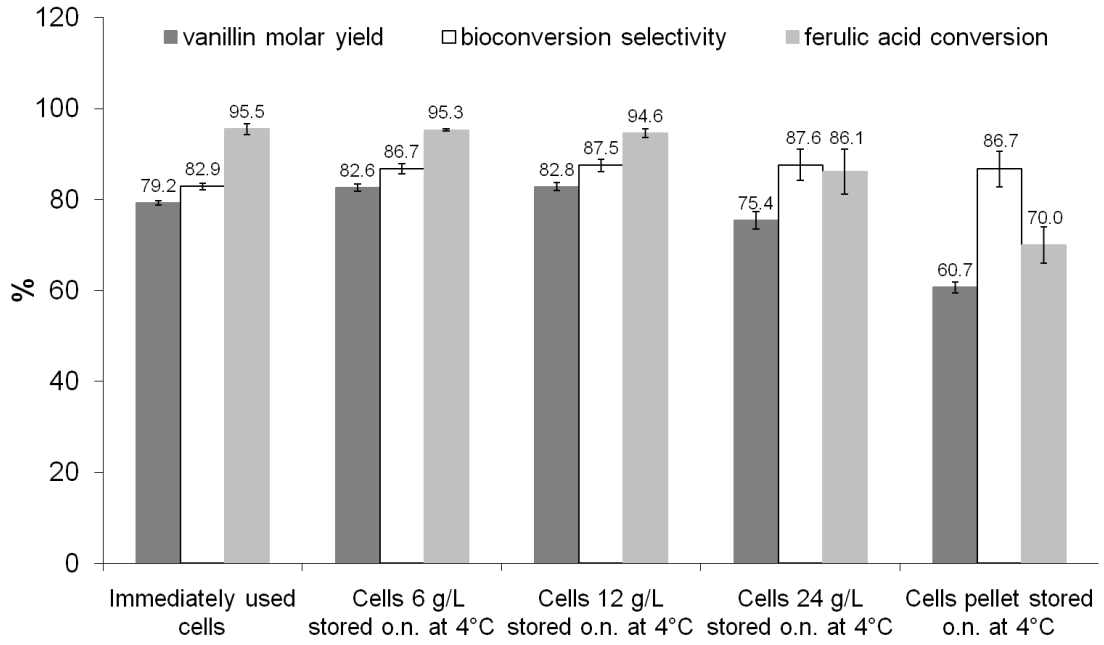

Figure 19. Effect of biomass storage at different cell concentrations on the bioconversion process.

4.1.5 Effect of biomass reuse in successive bioconversion steps

In order to lower the cost of the process, biomass reuse was investigated since this could allow to reduce the amount of biomass required for the bioconversion of a given amount of ferulic acid, thus reducing the volume and/or the number of the biomass production vessels required. Biomass produced under the optimized conditions was washed and stored overnight at 12 g(wet weight)/L in ferulic acid free bioconversion buffer at 4°C between each bioconversion, since the bioconversion capability of the biomass was not affected by this storage procedure (Figure 19); however, biomass could be reused immediately after the end of the bioconversion step. Very similar ferulic acid consumption and vanillin accumulation were obtained at the first and second use of the biomass, being vanillin molar yields obtained at the second use of the biomass lower by only 8.9% than those obtained at its first use (Figure 20). Conversely, at the third use of biomass vanillin was rapidly consumed after 4 hours of incubation and converted mainly into vanillic acid, which was

probably further oxidised and used as carbon and energy source. Thus, biomass use is possible for two consecutive bioconversion without any remarkable effect on the bioconversion efficiency.

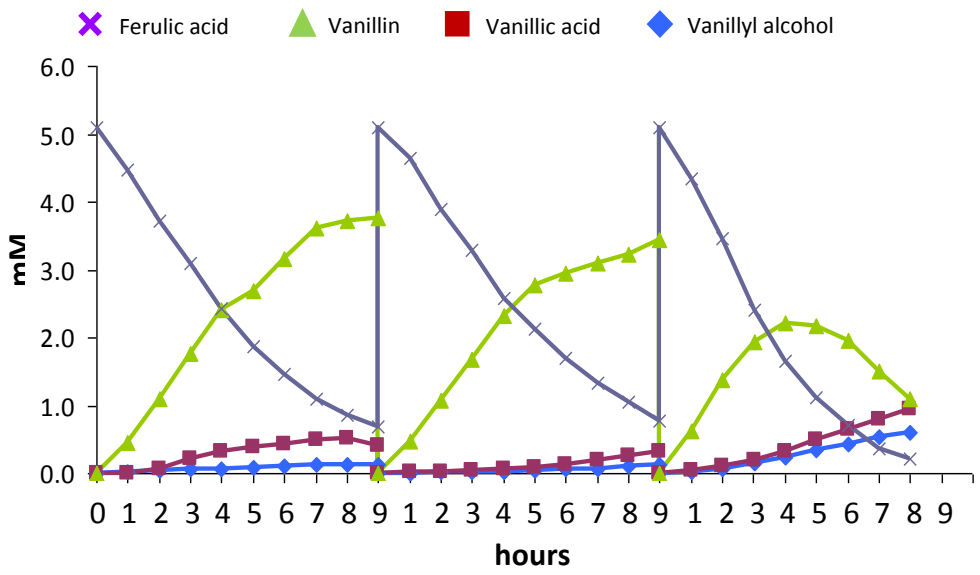

Figure 20. Biomass reuse in sequential bioconversions of food grade ferulic acid 5 mM (pH 7.0, cells 6g/L).

4.1.6 Final protocol for the production of natural vanillin from ferulic acid

The optimized protocol to obtain natural vanillin from ferulic acid bioconversion is reported in Figure 21.

The bacterial strain *P. fluorescens* BF13-1p4 was firstly grown in a shaken flask containing analytical-grade LB medium at 30°C and 150 rpm of stirring. The overnight grown pre-culture was used to inoculate at 2% v/v fresh LB medium prepared with industrial grade components, either in flasks (30°C, stirring at 150rpm) or in a 2L stirred tank reactor (30°C, 500rpm, aeration at 4NL/min and pH 6.8). After 4.5 hours of growth cells were induced with ferulic acid 2.5 mM for 1 hour before

harvesting. At the end of the growth phase, biomass was collected by centrifugation, washed to remove residual growth medium components and metabolites, and suspended in saline phosphate buffer at pH 7.0 and final cell concentration 6 g(wet weight)/L . At this stage, biomass suspension was either immediately used for ferulic acid bioconversion to vanillin or stored overnight at cell concentration 12 g(wet weight)/L and 4°C . Bioconversion was performed at 30°C in saline phosphate buffer pH 7.0 with 5 or 10 mM ferulic acid as substrate and 6 g(wet weight)/L cells. Under these conditions, similar vanillin molar yields (82.9% and 80.2%) were obtained with initial ferulic acid concentrations of 5 and 10 mM. However, the highest productivity (0.61 mmoles L-1 h-1) was obtained with ferulic acid 5 mM, whereas the highest vanillin concentration (8.4 mM) with ferulic acid 10 mM.

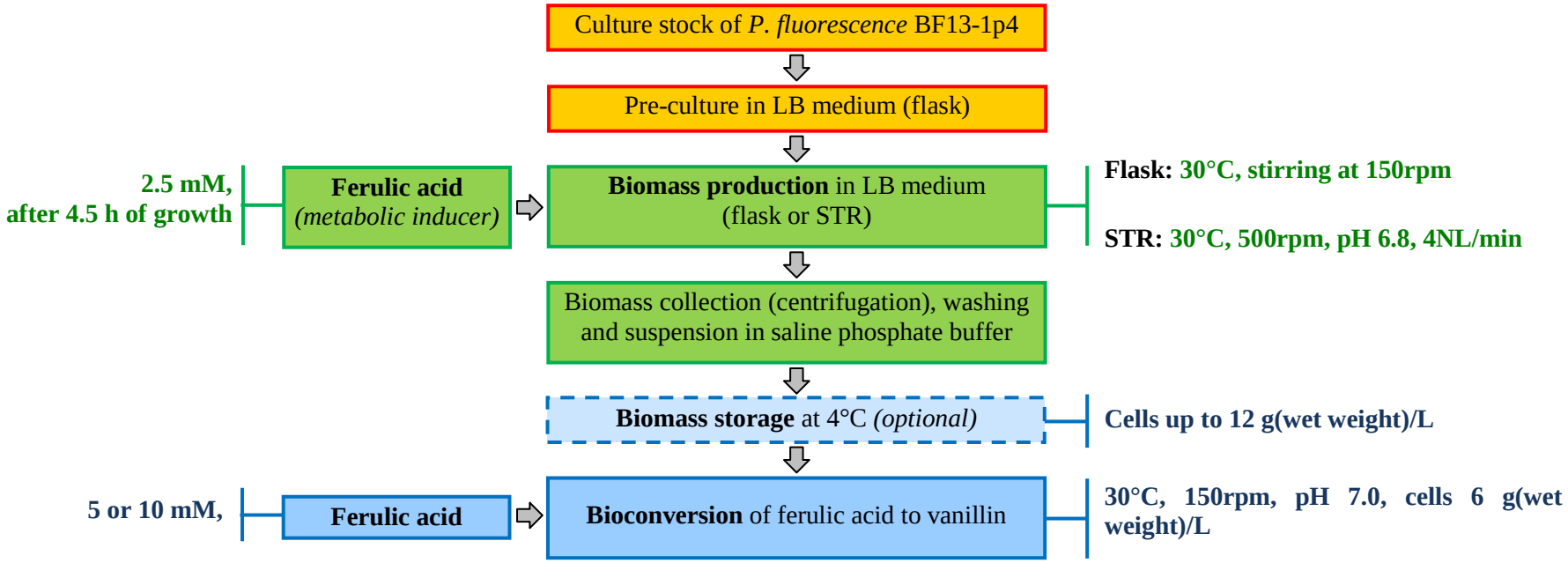

Figure 21. Final protocol for food grade ferulic acid bioconversion to vanillin by *Pseudomonas fluorescens* BF13-1p4.

4.2 Optimization of the vanillin production process by Surface Response Methodology (RSM)

The process of vanillin production from ferulic acid by metabolically engineered *Pseudomonas fluorescens* BF13-1p4 was further optimized in flask experiments by using the response surface methodology (for details on the methodology, see paragraph 3.6).

4.2.1 Optimization of biomass producing phase by RSM: *Step 1*

The individual and interactive effects of the ferulic acid concentration for induction (x_1) (values range: 0 – 7 mM), cell concentration at the time of induction OD_{600} (x_2) (values range: 0.5-3.5), duration of induction (x_3) (values range: 0.5 – 4 h) on the vanillin production were studied by using RSM. Central composite statistical design for the study of the three independent variables each at five levels (Table 14) was carried out as shown in Table 15.

Indipendent variables	**Coded symbol**	**Coded levels**				
		-1.681	-1	0	1	1.681
Ferulic acid concentration (mM)	x_1	0	1.42	3.5	5.58	7
$(OD)_{600}$ at the time of induction	x_2	0.5	1.11	2	2.89	3.5
Duration of induction (h)	x_3	0.5	1.21	2.25	3.29	4

Table 14. Experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors for biomass growth phase.

Run N°	x_1 (mM)	x_2 $(OD)_{600}$	x_3 (h)	Vanillin Production (mM)
1	3.5	2	2.25	2.06
2	3.5	2	2.25	1.95
3	3.5	0.5	2.25	2.04
4	1.42	2.89	3.29	1.73
5	3.5	2	2.25	1.91
6	5.58	1.11	1.21	1.64
7	3.5	2	4	1.98
8	7	2	2.25	1.65
9	1.42	1.11	1.21	1.37
10	0	2	2.25	1.27
11	3.5	3.5	2.25	2.25
12	3.5	2	2.25	2.11
13	5.58	2.89	1.21	2.05
14	5.58	2.89	3.29	1.34
15	3.5	2	2.25	2.37
16	3.5	2	2.25	2.39
17	1.42	2.89	1.21	2.38
18	3.5	2	0.5	2.37
19	5.58	1.11	3.29	2.38
20	1.42	1.11	3.29	2.47

x_1 – Ferulic acid concentration (mM); x_2 – $(OD)_{600}$ during induction; x_3 – Duration of induction period (h)
Other variables that are kept constant: Temperature – 30°C; stirring speed – 150 rpm
Constant Bioconversion conditions: Ferulic acid concentration- 5 mM; Cell mass – 6 g/L; pH of reaction buffer – 7.0; Temperature - 30°C; Reaction period – 3 h

Table 15. The central composite design (CCD) matrix of independent variables used in RSM studies with corresponding experimental values of vanillin production [Stage I – Biomass growth phase].

By applying multiple regression analysis on the Table 15 data, the experimental results of the full factorial central composite design were fitted to the polynomial Eq. (2) (see paragraph 3.6.1). The model obtained for vanillin production is shown in the following polynomial equation:

$$Y= 2.38– 0.087\ x_1 – 0.0289\ x_2 – 0.1313\ x_3 – 0.3418\ x_1^2 – 0.0377\ x_2^2 – 0.209\ x_3^2 – 0.0325\ x_1\ x_2 - 0.022\ x_1\ x_3 +0.04\ x_2\ x_3 \quad (3)$$

where Y is the amount of vanillin produced (mM), x_1, x_2 and x_3 are the coded values of variables ferulic acid concentration (mM) for induction, cell concentration $(OD)_{600}$ at the time of induction and duration of induction (h) respectively.

The regression analysis from the data of central composite design (CCD) experiments were shown in Table 16. Analysis of variance for vanillin production for cell growth optimization is shown in Table 17.

Codified Variables	**Regression Coefficient**	**Standard Error**	**Computed *t* value**	**Significance level, *p* value**
Constant	2.38757	0.05855	40.777	0.000
x_1	-0.08700	0.03885	-2.240	0.049
x_2	-0.02896	0.03885	-0.745	0.473
x_3	-0.13137	0.03885	-3.382	0.007
$x_1\ x_1$	-0.34182	0.03782	-9.039	0.000
$x_2\ x_2$	-0.03776	0.03782	-0.999	0.342
$x_3\ x_3$	-0.20924	0.03782	-5.533	0.000
$x_1\ x_2$	-0.03250	0.05076	-0.640	0.536
$x_1\ x_3$	-0.02250	0.05076	-0.443	0.667
$x_2\ x_3$	0.04000	0.05076	0.788	0.449
S = 0.143562	PRESS = 1.51931			
R-Sq = 92.41%	R-Sq(pred) = 44.04%	R-Sq(adj) = 85.58%		

x_1 – Ferulic acid concentration (mM); x_2 – $(OD)_{600}$ during induction; x_3 – Duration of induction period (h)

Table 16. Estimated coefficients of multiple determinations (R^2) for vanillin production for cell growth optimization.

Sources of Variation	Degrees of freedom	Sum of Squares	Mean Squares	*F* value	*p* value
Regression	9	2.50900	0.278777	13.53	0.000
Linear	3	0.35051	0.116836	5.67	0.016
Square	3	2.13319	0.711062	34.50	0.000
Interaction	3	0.02530	0.008433	0.41	0.750
Residual Error	10	0.20610	0.020610		
Lack of Fit	5	0.19877	0.039753	27.10	0.001
Pure Error	5	0.00733	0.001467		
Total	19	2.71509			

Table 17. Analysis of variance (ANOVA) for vanillin production for cell growth optimization.

The fit of the model was checked with the coefficient of determination R^2, which was calculated to be 0.924 (Table 16), indicating that only 7.6% of the variability cannot be explained by the model. This value of R2 suggested thus a close agreement between experimental and predicted values of vanillin production.

The significance of each coefficient was determinate using the Student's *t* test and *p* value in Table 16. The corresponding variables will be more significant if the absolute t value becomes larger and the p value becomes smaller and anyway less than 0.05. Table 16 shows that the variable with the largest effect was the quadratic term of ferulic acid concentration for induction (x_1 x_1), followed by the quadratic and the linear term of duration of induction (x_3 x_3 and x_3 respectively).

The goodness of the regression model was also demonstrated by the analysis of the variance using the Fischer *F* test at 95% confidence level (Table 17). The high tabulated *F* value of 13.53 and the probability *p* value of 0.000 indicated that the second order polynomial equation is highly significant.

The response surface plots and their corresponding contour plots for the vanillin production are shown in Figure 22.

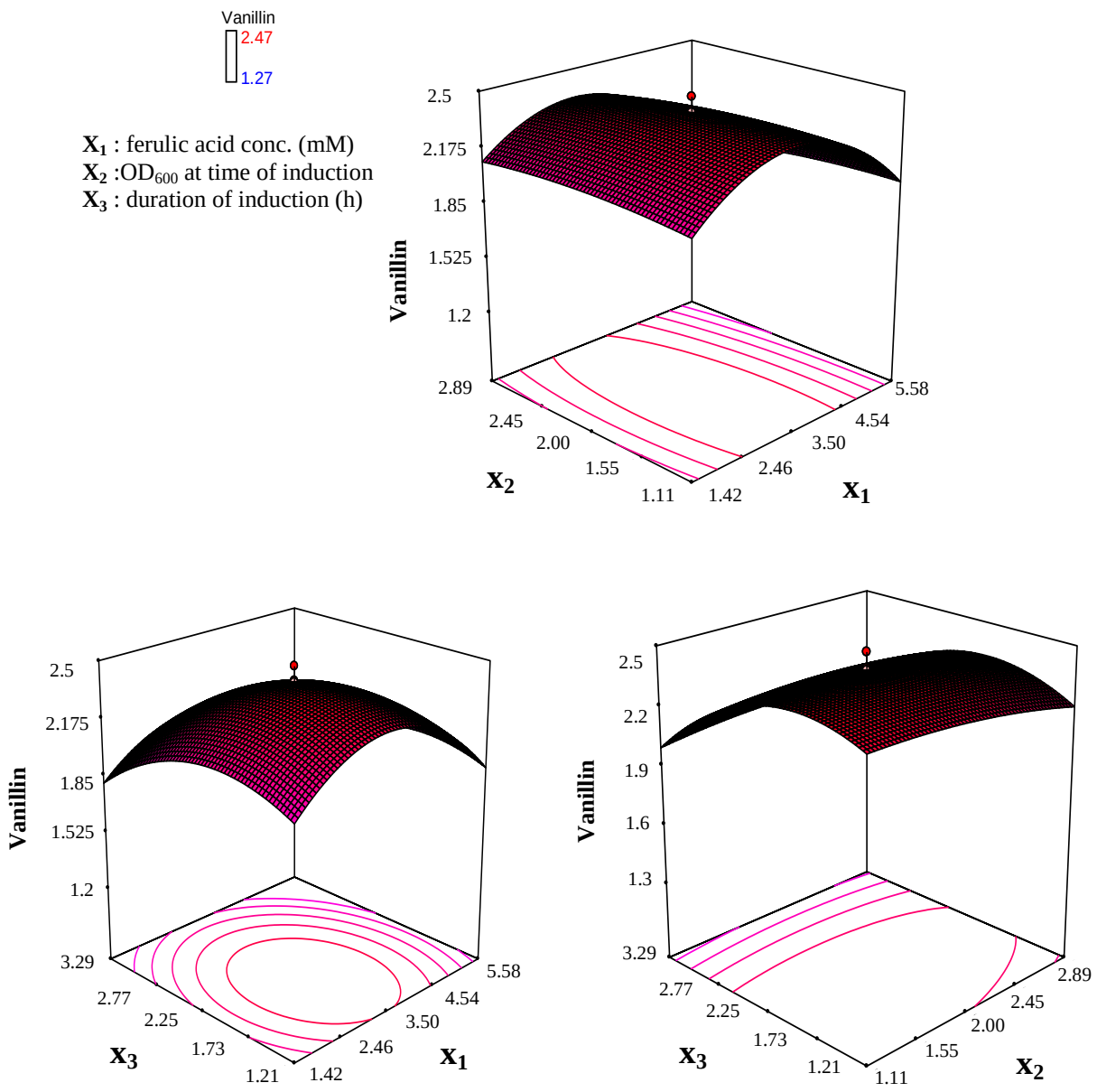

Figure 22. Response surface and contour plots for vanillin production [Step (1): Optimization of biomass growth by keeping the bioconversion conditions constant].

The optimum values of the tested variables were found to be: 3.25 mM ferulic acid for induction, 1.62 $(OD)_{600}$ cell concentration at the time of induction and duration of induction of 1.91 h. At these optimizes conditions, the highest predicted vanillin molar concentration after 3 hours of bioconversion was 2.42 mM.

4.2.2 Optimization of bioconversion phase by RSM: *Step 2*

Central composite statistical design for the study of the five independent variables each at five levels was carried out as shown in Table 18. Experiments in Table 19 were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors. The coded levels were −2, −1, 0, +1, +2.

Variables with designate	Coded levels				
	-2	-1	0	1	2
Ferulic acid concentration (mM)	2	6.5	11	15.5	20
Cell concentration (g/L)	2	7.5	13	18.5	24
pH of bioconversion buffer	6	6.5	7	7.5	8
Reaction temperature (°C)	25	28.75	32.5	36.25	40
Reaction time (h)	4	9	14	19	24

Table 18. Experimental range and levels of the five independent variables used in RSM in terms of actual and coded factors for bioconversion step.

Multiple regression analysis of the central composite experimental design gave the following quadratic polynomial equation for vanillin production.

$$Y = 3.37409 + 0.45917\,x_1 - 0.37417\,x_2 + 0.4325\,x_3 - 0.4241\,x_4 - 0.11\,x_5 - 0.444\,x_1^2 - 0.362\,x_2^2 - 0.464\,x_3^2 - 0.714\,x_4^2 - 0.295\,x_5^2 + 0.111\,x_1x_2 + 0.247\,x_1x_3 - 0.302\,x_1x_4 - 0.0325\,x_1x_5 + 0.0162\,x_2x_3 - 0.026\,x_2x_4 - 0.1787\,x_2x_5 - 0.4\,x_3x_4 - 0.242\,x_3x_5 + 0.057\,0.0475\,x_4x_5 \quad (4)$$

Where Y is the amount of vanillin produced (mM), x_1, x_2, x_3, x_4 and x_5 are the coded value of variables ferulic acid concentration (mM), cell concentration (g/L), pH of bioconversion buffer, reaction temperature (°C) and reaction time (h) respectively. The regression analysis from the data of central composite design experiments are shown in Table 21. Analysis of variance for vanillin production for bioconversion optimization is shown in Table 20.

Experiment N°.	Ferulic acid concentration (mM)	Cell concentration (g/L)	pH of bioconversion buffer	Reaction temperature (°C)	Reaction time (h)	Vanillin (mM)
	A	**B**	**C**	**D**	**E**	
1	6.5	7.5	6.5	28.75	19	0.71
2	15.5	7.5	6.5	28.75	9	1.32
3	6.5	18.5	6.5	28.75	9	0.02
4	15.5	18.5	6.5	28.75	19	1.18
5	6.5	7.5	7.5	28.75	9	1.66
6	15.5	7.5	7.5	28.75	19	5.11
7	6.5	18.5	7.5	28.75	19	0
8	15.5	18.5	7.5	28.75	9	7.03
9	6.5	7.5	6.5	36.25	9	0.56
10	15.5	7.5	6.5	36.25	19	1.01
11	6.5	18.5	6.5	36.25	19	0.23
12	15.5	18.5	6.5	36.25	9	0.56
13	6.5	7.5	7.5	36.25	19	0.12
14	15.5	7.5	7.5	36.25	9	1.25
15	6.5	18.5	7.5	36.25	9	0.13
16	15.5	18.5	7.5	36.25	19	0.33
17	2	13	7	32.5	14	1.23
18	20	13	7	32.5	14	3.21
19	11	2	7	32.5	14	3.55
20	11	24	7	32.5	14	0.54
21	11	13	6	32.5	14	0.38
22	11	13	8	32.5	14	3.9
23	11	13	7	25	14	1.15
24	11	13	7	40	14	0.13
25	11	13	7	32.5	4	2.34
26	11	13	7	32.5	24	2.29
27	11	13	7	32.5	14	8.4
28	11	13	7	32.5	14	8.9
29	11	13	7	32.5	14	8.3
30	11	13	7	32.5	14	8.35
31	11	13	7	32.5	14	8.6
32	11	13	7	32.5	14	8.8

Other variables that are kept constant: stirring speed – 150 rpm
Constant cell growth conditions:
Ferulic acid concentration (mM) for induction = 3.25
Cell concentration $(OD)_{600}$ at the time of induction = 1.62
Duration of induction (h) = 1.91

Table 19. The CCD matrix of independent variables used in RSM studies with corresponding experimental values of vanillin production [Stage II – Bioconversion optimization step].

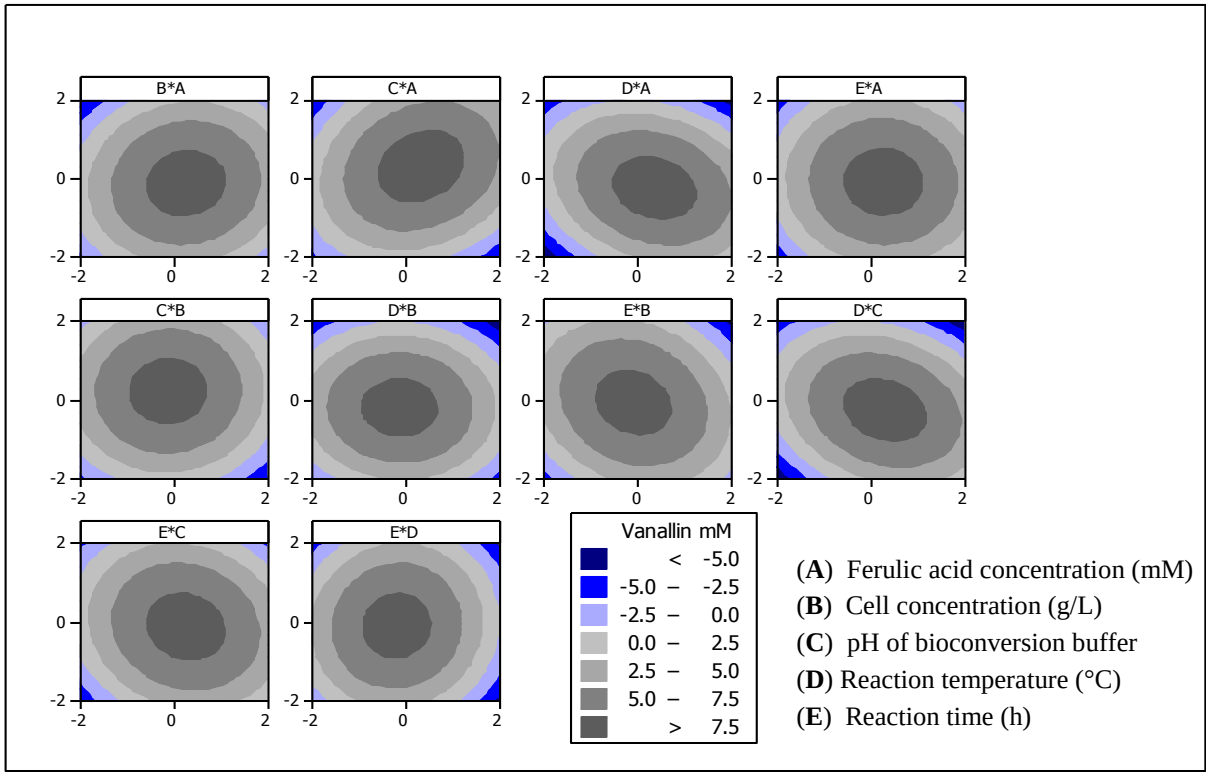

Figure 23. Response surface and contour plots for vanillin production [Step (2): Optimization of bioconversion conditions].

Sources of Variation	Degrees of freedom	Sum of Squares	Mean Squares	*F* value	*p* value
Regression	20	305.890	15.2945	15.41	0.000
Linear	5	38.869	7.7737	7.83	0.002
Square	5	240.567	48.1134	48.49	0.000
Interaction	10	26.455	2.6455	2.67	0.062
Residual Error	11	10.914	0.9922		
Lack of Fit	6	10.602	1.7671	28.31	0.001
Pure Error	5	0.312	0.0624		
Total	31	316.805			

Tale 20. Analysis of Variance for vanillin production (mM).

Codified Variables	Regression Coefficient	Standard Error	Computed *t* value	Significance level, *p* value
Constant	8.35364	0.3973	21.026	0.000
A	0.76333	0.2033	3.754	0.003
B	-0.34500	0.2033	-1.697	0.118
C	0.71167	0.2033	3.500	0.005
D	-0.62000	0.2033	-3.049	0.011
E	-0.16417	0.2033	-0.807	0.437
A*A	-1.37989	0.1839	-7.503	0.000
B*B	-1.42364	0.1839	-7.741	0.000
C*C	-1.39989	0.1839	-7.611	0.000
D*D	-1.77489	0.1839	-9.650	0.000
E*E	-1.35614	0.1839	-7.374	0.000
A*B	0.19250	0.2490	0.773	0.456
A*C	0.57875	0.2490	2.324	0.040
A*D	-0.63375	0.2490	-2.545	0.027
A*E	-0.07625	0.2490	-0.304	0.765
B*C	0.06000	0.2490	0.241	0.814
B*D	-0.07000	0.2490	-0.281	0.784
B*E	-0.51000	0.2490	-2.048	0.065
C*D	-0.69375	0.2490	-2.786	0.018
C*E	-0.32375	0.2490	-1.300	0.220
D*E	0.13875	0.2490	0.557	0.589

S = 0.996105 PRESS = 262.887

R-Sq = 96.55% R-Sq(pred) = 17.02% R-Sq(adj) = 85.58%

(**A**) **Ferulic acid concentration (mM)**
(**B**) **Cell concentration (g/L)**
(**C**) **pH of bioconversion buffer**
(**D**) **Reaction temperature (°C)**
(**E**) **Reaction time (h)**

Table 21. Estimated Regression Coefficients for vanillin production (mM).

As shown in Table 21, the Student's t test and p value indicated that the more significant variables were the linear and squared effect of (*A*) ferulic acid concentration (mM), (*C*) pH of bioconversion buffer and (*D*) reaction temperature (°C), the squared effect of (B) cell concentration (g/L), and (E) reaction time (h). Also the interactive effect of (A*C), (A*D) and (C*D) were found to be significant as the *P*-value is less than 0.05 (Table 21).

From the variance analysis (Table 20), the determination coefficient R^2 of 0.965 indicated that the mathematical model based on RSM was adequate and significant to represent the experimental results. The model could be considered statistically significant also according to the F test with 95% of confidence, as the high tabulated F value of 15.41 and the very low p value indicate that the second order polynomial equation (Eq 4) is significant at 95% level confidence.

The response surface plots and their corresponding contour plots for the vanillin production are shown in Figure 23.

The highest predicted value of vanillin concentration was 8.81 mM and was achieved after 13.2 hours of bioconversion using 12.9 mM ferulic acid, 12.67 g(wet weight)/L of cells, buffer pH 7.21 and reaction temperature 31.21°C.

4.3 Enzymatic hydrolysis of wheat bran for ferulic acid release

4.3.1 Enzymatic hydrolysis of wheat bran for ferulic acid release: *Method 1*

The protocol of bran hydrolysis described in paragraph 3.7.3 was tested with two different batches of enzymes, which both allowed the release of very limited amounts of ferulic acid (Table 22).

In general, higher amount of released ferulic acid was obtained with the xylanase Fungamyl® 800L plus the cellulase Celluclast® cocktail.

Doubling the enzymes concentration resulted in doubling the amount of released ferulic acid. However, the amount of released ferulic acid obtained (0.29 g/kg) was still very low, corresponding approximately to only 5% of the total ferulic acid occurring in wheat bran (~ 5.5 g/kg) and did not justify the cost increase due to the use of higher amounts of enzymes (Table 22).

	Amount of ferulic acid released (g/kg of wheat bran)	
	Fungamyl® 800L + Celluclast® 1.5L	Pentopan Mono BG® + Celluclast® 1.5L
Enzymes batch #1	0.17 ±0.00	0.04 ±0.00
Enzymes batch #2	0.13 ±0.00	0.08 ±0.01
Enzyme concentration doubled (Enzyme batch #1)	0.29 ±0.02	0.16 ±0.01
Additional wheat bran mechanical pre-treatment via chopping with a mixer (Enzyme batch #1)	0.28 ±0.04	*nd*
Wheat bran suspension in higher amount of water (bran:water ratio 1:10) (Enzyme batch #1)	0.23 ±0.03	*nd*

***nd* : not determined**

Table 22. Amount of ferulic acid released from wheat bran with *Method 1* by using different batches of enzymes, additional mechanical pre-treatment and suspension of wheat bran in higher amount of water.

Additional mechanical pre-treatment (i.e., chopping), in order to increase the surface available to enzymatic attack, and suspension of wheat bran in a higher amount of water (bran:water ratio of 1:10) in order to improve the mixing of the bran suspension, allowed to obtain a higher release of ferulic acid, which however still corresponded approximately to only 5% of ferulic acid in bran (Table 22).

This protocol was thus considered ineffective in the release of ferulic acid from wheat bran.

4.3.2 Enzymatic hydrolysis of wheat bran for ferulic acid release: *Method 2*

Enzymatic digestion with *Method 2* (for details on the protocol, see paragraph 3.7.4 "Protocol of enzymatic hydrolysis of wheat bran for ferulic acid release: *Method 2*") was carried out on whole and destarched/deproteinated wheat bran with the two xylanases Depol 740L (36 µl/g bran) and Econase (60 µl/g bran) at 50°C for 24 hours (Table 23).

The destarching/deproteination pre-treatment of wheat bran remarkably increased the specific release of ferulic acid. However, the amount of ferulic acid released was less than 5% of that measured in whole wheat bran following chemical release using 4M alkali (~ 5.5 g/kg).

Bran pre-treatment, enzyme	**Ferulic acid released (g/kg whole bran)**
Whole bran, Depol 740L	0.04±0.01
Destarched/deproteinized bran, Depol 740L	0.26±0.02
Whole bran, Econase	0.09±0.00
Destarched/deproteinized bran, Econase	0.15±0.00

Table 23. Effect of destarching/deproteination pre-treatment on the release of ferulic acid from wheat bran after 24h digestion with Depol 740L (36 µl/g bran) and Econase (60 µl/g bran).

Destarched/deproteinated wheat bran was then digested with Depol 740L at higher concentration (140 µl/g bran). In addition, the xylanase Pentopan Mono BG® (1% w/w) was tested. Finally, the two xylanases were tested in combination with the Feruloyl esterase (0.25 U) (Table 24).

Enzyme	Ferulic acid released (g/kg whole bran)
Depol 740L	0.35±0.04
Depol 740L + Feruloyl esterase	0.80±0.07
Pentopan Mono BG ®	0.06±0.01
Pentopan Mono BG ® + Feruloyl esterase	0.92±0.15

Table 24. Release of ferulic acid from destarched/deproteinated wheat bran after 24 h digestion with Depol 740L (140 µl/g bran), Pentopan Mono BG® (1% w/w), Depol 740L + Feruloyl esterase (0.25 U) and Pentopan Mono BG® + Feruloyl esterase.

A four-fold increase of Depol 740L concentration resulted in increase of about 30% of the released ferulic acid, while the use of Pentopan Mono GB® permitted a release of only about 1% of the ferulic acid occurring in wheat bran (Table 24). However, the combination of Depol 740L or Pentopan Mono GB® with the feruloyl esterase remarkably increased the release of ferulic acid to 0.8-0.9 g/kg whole bran, corresponding approximately to 15% of the ferulic acid occurring in whole bran (~ 5.5 g/kg). This suggested that feruloyl esterase activity was a limiting factor in the release of ferulic acid.

Feruloyl esterase (U)	Set A. Incubation time (h)	Set B. Incubation time (h)
0.5	2	24
0.25	3.3	24
0.063	16	24
0.032	24	-

Table 25. Incubations of destarched/deproteinated wheat bran with Pentopan Mono BG® (1% w/w) and different amounts of feruloyl esterase.

The release of ferulic acid over time as a function of feruloyl esterase activity was then investigated, in order to find out if there was an upper limit in the release of ferulic acid or/and the release of the latter was dependent on the feruloyl esterase activity present (Tables 25; 26). To this purpose, reaction with Pentopan Mono BG® (1% w/w) was carried out on destarched/deproteinated wheat bran with feruloyl esterase at decreasing concentrations and incubated for increasing times (Table 25).

Feruloyl esterase [U]	Time (h)	Ferulic acid released (g/kg)
0.5	2	0.73
0.5	24	0.93
0.25	3.3	0.87
0.25	24	0.80
0.063	16	0.54
0.063	24	0.71
0.032	24	0.88

Table 26. Ferulic acid released over time from destarched/deproteinated wheat bran by Pentopan Mono BG® (1% w/w) and different amounts of feruloyl esterase.

Similar amounts of ferulic acid were released from destarched/deproteinated wheat bran after 24 h of incubation with increasing amounts of added feruloyl esterase. This indicated that there was a plateau limit in the ferulic acid release from wheat bran. This limit could be achieved with lower amounts of feruloyl esterase and longer incubation times, which allows to choose the optimal enzyme concentration and the corresponding digestion duration according to the cost balance and productivity evaluation of whole process.

4.3.3 Enzymatic digestion of bran samples pre-treated with two different thermal processes

The enzymatic digestion of wheat bran milling fractions pre-treated with the steam explosion and the thermomechanical D.I.C. process (in french: Détente Instantanée Contrôlée), was tested in order to investigate the suitability of these thermo-phisical treatments for improving the enzyme accessibility of the bran and thus to increase the release of ferulic acid. Bran pre-treated samples were provided by IFR (Institute of Food Research, Norwich, UK) and AZTI-Tecnalia (Unidad de Investigación Alimentaria, Spain). The investigation for optimal conditions of steam explosion and DIC treatment by IFR and AZTI-Tecnalia are still in progress.

Table 27 shows the characteristics of bran samples, the specific conditions of thermal pretreatment and the release of ferulic acid (g of ferulic acid/ kg of wheat bran) after enzymatic digestion with Pentopan Mono GB® (1% w/w) and feruloyl esterase (0.25 U/g). Mill A" and "Mill B" refer to bran samples obtained from different flour milling fractions within the same company and correspond to the untreated bran as supplied by the miller (G.R. Wright & Sons Ltd, UK). Differences in bran composition among the milling fractions are not expected but some physical milling distinctions may arise through variation in engineering between the mills. The "Mill B Pentopan" samples were the residues recovered from destarching/deproteination of Mill B bran with Pentopan Mono BG®. In theory all available ferulic acid should be released in the oligosaccharides produced during digestion. An estimated ~ 75% of the original ferulic acid content remains associated with the undigested residue, therefore any further and significant release of ferulic acid should indicate an effect of the DIC treatment. The bran samples were dried and rehumidified to 10% or 30% humidity before DIC treatment, as specified in Table 27. Two different sets of pressure and temperature for DIC treatment were tested: PT*1* (135 °C for 20s, 5 bar) and PT*2* (100 °C for 40s, 2 bar), followed by sudden decompression. On the contrary, the decompression step was not performed in the DIC-control treatment.

The "WM" samples refer to steam exploded samples of bran which was not destarched/deproteinated prior to steam explosion. The main difference in the "WM" samples was the pressure conditions as shown in Table 27.

Sample N°	Bran Type	Treatment	Ferulic acid released (g/kg)	St. dev.
1	**MILL A** 10%H pre-DIC	Control	0.15	0.01
2		DIC-PT*1*	0.58	0.03
3		DIC-PT*1*-control	0.58	0.04
4		DIC-PT*2*	0.36	0.02
5		DIC-PT*2*-control	0.11	0.00
6	**MILL A** 30%H pre-DIC	Control	0.11	0.01
7		DIC-PT*1*	0.36	0.03
8		DIC-PT*1*-control	0.44	0.06
9		DIC-PT*2*	0.12	0.01
10		DIC-PT*2*-control	0.12	0.01
11	**MILL B** 10%H pre-DIC	Control	0.10	0.01
12		DIC-PT*1*	0.31	0.03
13		DIC-PT*1*-control	0.56	0.26
14		DIC-PT*2*	0.09	0.01
15		DIC-PT*2*-control	0.27	0.03
16	**MILL B** 30%H pre-DIC	Control	0.06	0.00
17		DIC-PT*1*	0.57	0.00
18		DIC-PT*1*-control	0.59	0.05
19		DIC-PT*2*	0.43	0.05
20		DIC-PT*2*-control	0.26	0.07
21	**MILL B PENTO** 30%H pre-DIC	Control	0.12	0.04
22		DIC-PT*1*	0.14	0.01
23		DIC-PT*1*-control	0.17	0.04
24		DIC-PT*2*	0.13	0.01
25		DIC-PT*2*-control	0.13	0.02
26	**WM 04**	500g bran + 2L hot water (ratio 1:4), *4.4bar*, 10 min	1.00	0.05
27	**WM 05**	500g bran + 2.5L hot water (ratio 1:5), *3.2bar*, 10 min	0.78	0.03
28	**WM 06**	500g bran + 1.5L hot water (ratio 1:3), *6.9bar*, 10 min	0.44	0.04

DIC-PT*1*(135 °C for 20s, 5 bar) and DIC-PT*2* (100 °C for 40s, 2 bar) followed by sudden decompression.
DIC-PT*1*-control and PT*2*-control without decompression.

Table 27. Effect of DIC and steam explosion pretreatment on the bran enzymatic hydrolysis with Pentopan Mono GB® (1% w/w) and feruloyl esterase (0.25 U/g).

The DIC process allowed to release higher amounts of ferulic acid as compared to untreated controls. In general, the use of higher temperature and pressure for shorter time, i.e., of pressure and temperature conditions PT*1* (135 °C for 20s, 5 bar), were more effective. On the other hand, the effect of the decompression step, the percentage of humidity in the samples before DIC treatment and the milling fractions on the enzymatic release of ferulic acid remains unclear. The higher ferulic acid (~ 0.6 g of ferulic acid per kg of wheat bran, which corresponds to ~ 10% of original ferulic acid content in whole bran) was obtained from the Mill A sample having 10% initial humidity under the PT1 conditions.

The results about "Mill B Pento" samples showed instead that no more ferulic acid was released after DIC as compared to the untreated control, suggesting that the residual ferulic acid in the sample remains unavailable to the activity of feruloyl esterase.

Finally, the highest amount of ferulic acid was obtained using bran samples subjected to steam explosion before enzymatic hydrolysis. This thermal pre-treatment allowed the enzymatic release of 1.0 g of ferulic acid/kg dry bran, i.e. approximately 20% of the ferulic acid occurring in whole wheat bran, when treating the sample at 4.4 bar for 10 minutes.

4.3.4 Final protocol for ferulic acid enzymatic release

The complete protocol to obtain the highest ferulic acid recovery from wheat bran is shown in Figure 24.

Wheat bran was first suspended in water at 10% (w/v) and incubated at 90-100 °C for 20 min in order to gelatinise starch. The suspension was then cooled to 60°C before destarching with Termamyl (2 U/g bran) for 60 min. pH was then corrected to 8.3-8.5 before deproteination treatment with Alcalase (20 µl/g bran) for 3-4 h. After filtration and washing, the recovered destarched/deproteinated wheat bran was suspended at 5% (w/v) in water at 50°C for 5 min, followed by enzymatic hydrolysis

of ferulic acid with Pentopan Mono BG® (1% w/w) and feruloyl esterase (0.25 U/g) for 3.3 h. Insoluble fiber residue was separated from the crude hydrolysate containing ferulic acid by centrifugation. The amount of ferulic acid released at the end of this phase was approximately 0.9 g/kg of whole bran, corresponding to approximately 15% of the ferulic acid occurring in whole bran. A similar release of ferulic acid could be obtained using lower concentrations of feruloyl esterase for longer incubation time or higher concentrations of feruloyl esterase for shorter incubation time.

Alternatively wheat bran could be pre-treated with steam explosion at pressure of 4.4 bar for 10 minutes, followed by enzymatic hydrolysis with Pentopan Mono BG® and feruloyl esterase, as described above. In this case, the highest amount of 1g/kg ferulic acid, corresponding to ~ 18% of whole content in wheat bran, was extracted.

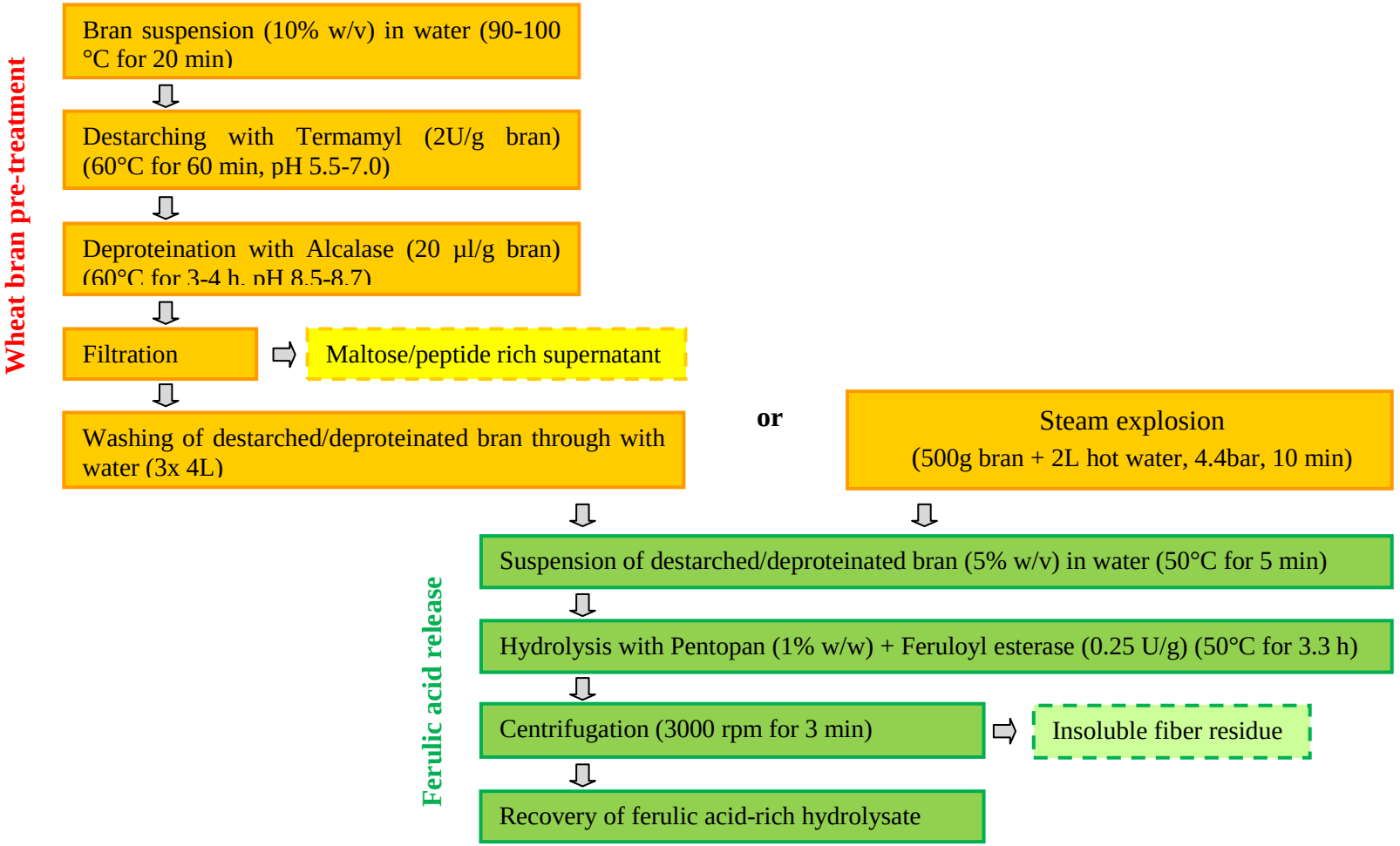

Figure 24. Final protocol for ferulic acid release from wheat bran.

4.4 Bioconversion of ferulic acid in wheat bran hydrolysates

To assess the suitability of the *P. fluorescens* BF13-1p4 strain to bioconvert ferulic acid occurring in wheat bran hydrolysates, a preliminary bioconversion experiment was performed in flasks using wheat bran hydrolysate obtained with the final protocol for bran hydrolysis based on destarching/deproteination (*Method 2*) pre-treatment and the enzyme cocktail Pentopan Mono BG® (1% w/w) + Feruloyl esterase (0.25 U/g) (for details, see paragraph 3.7.4) (Table 28).

Bran hydrolysate + ferulic acid	**Ferulic acid (mg/L)**	**Reducing sugars (g/L)**	**Recovery of ferulic acid (%)**	**Recovery of reducing sugars (%)**
Crude	115.9±2.3	3.46±0.09	-	-
Purified	107.5±0.4	0.46±0.01	92.7 %	13.3%

Table 28. Ferulic acid and reducing sugars occurring in wheat bran hydrolyzate before and after purification with ISOLUTE ENV$^+$ column.

Since the bran hydrolysate contained a limited amount of ferulic acid, i.e.,115.9±2.3 mg/L, corresponding to 0.55 mM ferulic acid, the hydrolyzate was spiked with food-grade ferulic acid at the final concentration of approximately 1 mM, in order to better monitor its bioconversion into vanillin and possible by-products. Bioconversion with 1 mM initial concentration of food-grade ferulic acid in buffer pH 7.0 was carried out under the same conditions for comparison (Figure 25).

Food-grade ferulic acid (1.05 mM) was bioconverted into 0.66±0.03 mM vanillin and 0.14±0.0 mM vanillic acid after 8 h of incubation, corresponding to a vanillin molar yield of 63.2% and a selectivity of 71%. Conversely, when bran hydrolysate was used as substrate, vanillin produced was rapidly oxidised to vanillic acid. This resulted in the accumulation of only 0.16±0.11 mM vanillin and of 1.01±0.17 mM vanillic acid after 8 h of incubation, which corresponded to a significant reduction of selectivity (from 71% to 15%) and of vanillin molar yield (from 63.2% to 13.6%, Figure 25).

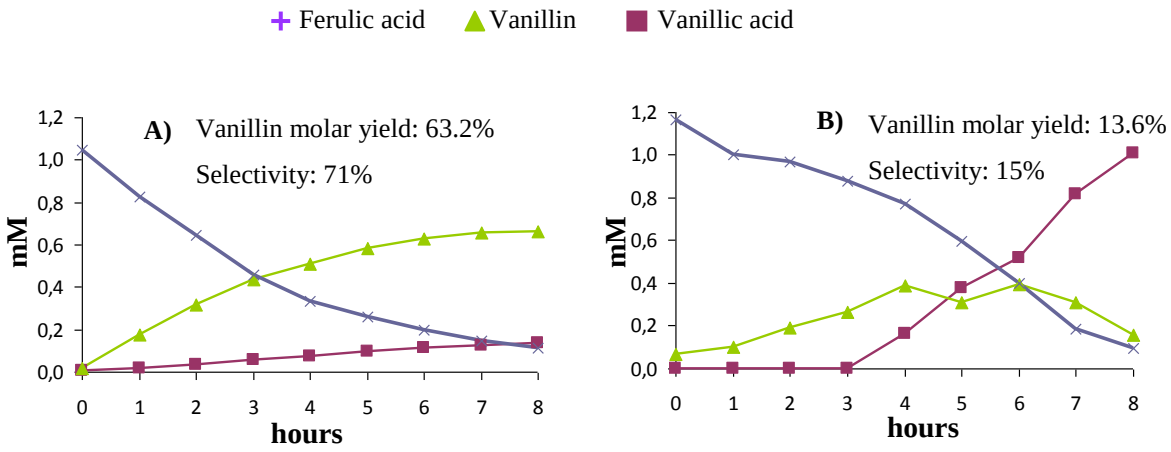

Figure 25. Bioconversion of food-grade ferulic acid 1 mM in bioconversion buffer pH 7.0 (A) and of ferulic acid-spiked wheat bran hydrolysate (B). Bioconversions were carried out in flasks with 6 g/L cells.

In order to assess to what extent the lack of pH control affected the bioconversion efficiency, an additional bioconversion was performed using bran hydrolysate (spiked with ferulic acid to the final concentration of 1 mM as above) amended with phosphate buffer pH 7.0 (by adding a 10X stock solution of phosphate buffer to the hydrolysate). Only a limited increase in vanillin molar yield (from 13.6 to 21.0%) and selectivity (from 15% to 28%) was obtained under these conditions, indicating that the lack of pH control during bioconversion of bran hydrolysate was not the major factor affecting the bioconversion efficiency (Figure 26).

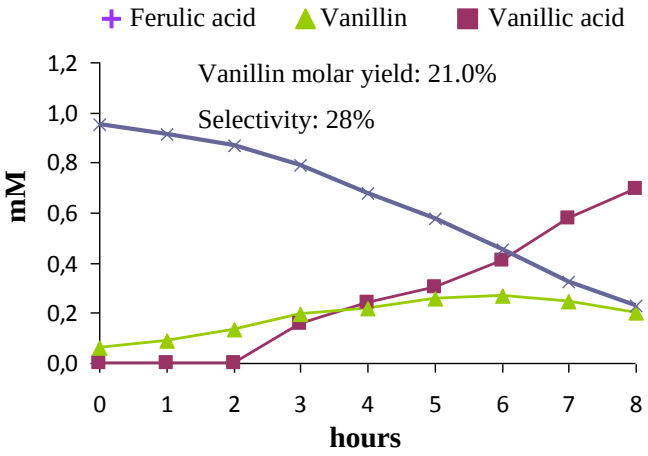

Figure 26. Bioconversion of wheat bran hydrolysate amended with bioconversion buffer pH 7.0. Bioconversion was carried out with 6 g/L cells.

Crude bran hydrolysate also contained a remarkable amount of reducing sugars (Table 28), which might stimulate the oxidative metabolism of the strain during bioconversion, thus leading to the oxidation of vanillin to vanillic acid. Ferulic acid occurring in bran hydrolazate was thus purified from reducing sugars with ISOLUTE ENV+ columns (see paragraph 3.7.5). The protocol allowed the recovery of 93% of the ferulic acid and the removal of 87% of the reducing sugars initially occurring in the crude hydrolysate (Table 28). The purified bran hydrolysate (spiked with ferulic acid to the final concentration of 1 mM and amended with phosphate buffer pH 7.0 as above) was then used as bioconversion substrate. Vanillin 0.60±0.01 mM and vanillic acid 0.17±0.01 mM were obtained after 8 h of incubation, corresponding to vanillin molar yield (55.1%) and selectivity (68%) comparable to those observed with food-grade ferulic acid (Figure 27). This indicated that the presence of reducing sugars was the major factor adversely affecting the bioconversion efficiency of bran hydrolysate, probably as they acted as carbon source for the microorganism, thus favouring growth and production of nonspecific oxidoreductases responsible for vanillin oxidation.

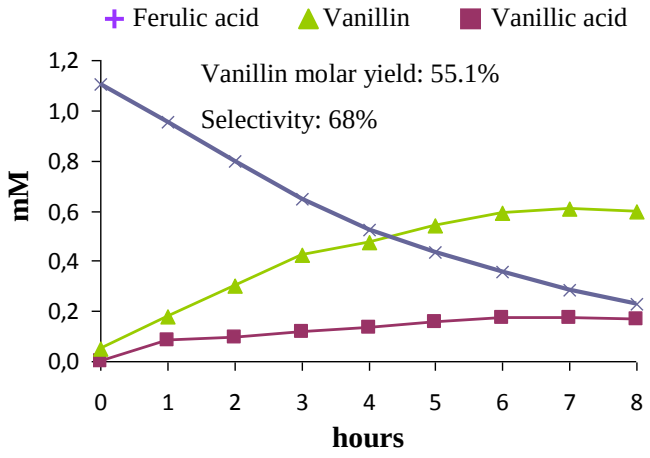

Figure 27. Bioconversion of ferulic acid-spiked wheat bran hydrolysate after purification with ISOLUTE ENV+ column and re-suspension in bioconversion buffer. Bioconversion was carried out with 6 g/L cells.

5 DISCUSSION AND CONCLUSIONS

The biotechnological production of natural vanillin as a feasible alternative to the traditional isolation from *Vanilla planifolia*, is a topic of high interest, as demonstrated by the number of reviews published in the last years. In spite of this, only few works reported remarkable production of vanillin in the literature, that is by employing actinomycetes of the genera *Amycolatopsis* and *Streptomyces* (Rabenhorst & Hopp, 1997; Müller *et al*, 1998).

A major drawback in all most the studies described so far is the vanillin reactivity, which exhibits a toxic effect to most microorganism, leading to a transient formation of the end product and, thus, its rapidly conversion to other by-products.

On the other hand, growth of filamentous actinomycetes results in highly viscous broths, unfavorable pellet formation, and uncontrolled fragmentation and lysis of the mycelium. This may complicate the rheology of the production processes, reduce their productivity, and increase hence the downstream processing costs (Barghini et al, 2008).

The increasing knowledge of the enzymes involved in the catabolic pathway of the substrates, such as ferulic acid and guaiacol, as well as the identification and characterization of corresponding genes, offered new opportunities for metabolic engineering and for recombinant vanillin-producing strains.

In this respect, a major aim of the present work was to investigate the capability of converting ferulic acid to vanillin by resting cells of the recombinant *Pseudomonas fluorescens* strain BF13-1p4(pBB1). Unlike widely engineered *non native E. coli* strains, the native ferulate-converting *P. fluorescens* BF13-1p4 was found to be able to produce, and hence to accumulate vanillin from ferulic acid, by *vdh* gene inactivation via target mutagenesis and concurrent expression of structural genes *fch* and *ech* under the control of their native P_{fer} promoter on a low copy number plasmid (Di Gioia *et al*., 2011).

Initially, the research work focused on the optimization of either the biomass producing phase or the next step of bioconversion in order to obtain cells with the highest ferulic acid (of food grade) ability to produce vanillin, and to achieve the

highest vanillin production, respectively. Several parameters and conditions were optimized by the classical method of changing one variable while fixing the others at a certain level. For the cell growth phase, in particular, the effect of cell concentration (OD_{600}) at the time of induction with ferulic acid and inducer concentration, as well as pH of medium and duration of induction, was investigated. On the other hand, the pH of buffer, cell concentration and ferulic acid concentration were investigated in order to maximize the bioconversion of ferulic acid to vanillin. The highest biomass efficiency of converting ferulic acid to vanillin was found when *P. fluorescens* BF13-1p4 was grown in a 2L stirred tank reactor containing LB medium of food grade (30°C, 500rpm, 4NL/min aeration, and pH 6.8) and induced after 4.5 h of growth (~1.5 OD_{600}) with ferulic acid 2.5 mM for 1 hour before collecting. Biomass was hence recovered by centrifugation, washed and suspended in the bioconversion buffer at desired cell concentration. Biomass suspension was either immediately used for ferulic acid bioconversion to vanillin or stored overnight at 4°C. Optimization of the bioconversion step in flasks at 30°C allowed to find the best ferulic acid concentration (5 or 10 mM), cell concentration (6 g/L) and pH (7.0). Under these conditions, similar vanillin molar yields of 82.9% and 80.2% (in terms of produced vanillin on initial ferulic acid) were obtained with initial ferulic acid concentrations of 5 and 10 mM, respectively. On the other hand, the highest productivity (0.61 mmoles L^{-1} h^{-1}) was obtained with ferulic acid 5 mM within 7 h of bioconversion, whereas the highest vanillin concentration of 8.4 mM (corresponding to approximately 1.4 g/L) with ferulic acid 10 mM within 24 h. The flexibility of the developed protocol enables hence selection between higher product concentration and higher productivity, relying on the same high yield and selectivity, by changing the initial substrate concentration.

Only another mutant strain belonging to genus *Pseudomonas*, i.e. strain HR199, was found capable of accumulating vanillin at higher concentration up to 2.9 mM (0.44 g l–1) from 6.5 mM eugenol within 17 h. However, the accumulated vanillin was further oxidized due to the unspecific dehydrogenase activity (Overhage et al., 1999b). On the contrary, the recombinant strain *P. fluorescens* BF13-1p4 used in this study combines the ability to tolerate relatively high concentration of vanillin with low rate of end product degradation.

In particular, the use of resting cells is required to avoid end product degradation (Di Gioia et al., 2011). This requires additional biomass washing steps; however, it allows the use of a simple buffer solution as reaction medium in the bioconversion step, which might facilitate and reduce costs of end product recovery and purification. In addition, the possibility of storing biomass between the growth and bioconversion phases increases protocol flexibility, while the possibility of reusing the biomass for two consecutive bioconversions without any remarkable effect on the bioconversion efficiency might allow to reduce the volume and/or the number of the biomass production vessels required and thus cots of the overall process.

The opportunity of further optimizing either the biomass-producing step or the conversion of ferulic acid to biovanillin by *P. fluorescens* BF13-1p4 in batch experiments, was investigated by response surface methodology (RSM) using a central composite design. A three-variables (concentration of ferulic acid as inducer, OD_{600} of culture at the time of induction, duration of induction) response surface model for the biomass growth optimization and a five-variables model for the bioconversion experiments were designed. The latter included the initial substrate concentration, cell concentration, pH of bioconversion buffer, reaction temperature and reaction time. RSM proved to be very effective and time saving technique for studying simultaneously the influence of several parameters on response factor by significantly reducing the number of experiments and hence facilitating the optimum conditions. The optimum values of tested variables for the biomass-producing phase were found to be 3.25 mM ferulic acid for induction, 1.62 $(OD)_{600}$ cell concentration at the time of induction and duration of induction of 1.91 h. The response surface results showed that maximum vanillin production of 8.81 mM would be obtained from 12.9 mM of initial ferulic acid concentration, after 13.2 h of bioconversion by using 12.67 g(wet weight)/L of cells at 28.48°C temperature and buffer pH 7.77. The optimal conditions of the process by RSM were thus in agreement with previous experiments, statistically suggesting the reproducibility and strength of the developed protocol for the ferulic acid bioconversion to vanillin.

A major drawback of using ferulic acid as substrate for vanillin production is its cost. Therefore an economic ferulic acid recovery from agro-industrial wastes via enzymatic methods is of interest for several reasons. Vanillin produced by

microorganism can be labeled as natural under the current EU and US regulations, if ferulic acid is obtained from a natural source (such as agro-industrial wastes) and the recovery method is mild (such as enzymatic hydrolysis). The threat to ecological systems and the consumer preference for natural products have always favored the environment-friendly processes. Last but not least, the agro-food industries generate annually large volumes of waste, which raise serious disposal issues and, consequently, considerable costs to various industries. Therefore, the most attractive alternative is the possibility of using agro-industrial residues such as wheat bran as low-cost feedstock in the production of value-added compounds (Priefert *et al*., 2001; Mathew & Abraham, 2006; Bicas *et al*., 2010).

Although the bioconversion of ferulic acid to vanillin by several microorganism was intensely studied, only few papers described the use of ferulic acid obtained from agro-industrial wastes and, in particular, no work has been reported with *Pseudomonas* strains. In addition, an applicable process for ferulic acid extraction from biomass material has not yet been developed, although this phenolic compound occurs widely in plant world (Fazary & Ju, 2008; Li et al., 2008).

In this respect, further aims of the present study were i) to develop a protocol for the pre-treatment of wheat bran and the enzymatic release of ferulic acid from it and ii) to test the suitability of the previously developed protocol for the production of vanillin from ferulic acid obtained by wheat bran hydrolysis.

Wheat bran was selected since wheat (*Triticum aestivum*) is among the most extensively cultivated crops in the world (~ 653 million tons in 2010) and, in consequence, is a major source of agro-industrial residues. In addition, European Union is the world's largest producing region with approximately 140 million tons in the year 2010 (FAOSTAT 2013), and, consequently, a major producer of wheat bran.

A great limitation of ferulic acid recovery from bran is the complexity of the cell wall matrix, in which ferulic acid either acts by cross-linking mainly arabinoxylans or is covalently linked to lignin monomers (Buanafina, 2009).

A number of commercial enzyme cocktails were tested in a tailored combination for the release of ferulic acid from wheat bran, i.e. xylanases Depol 740L and Pentopan Mono GB®, as well as the α-amylase Fungamyl® 800L and Feruloyl esterase (rumen microorganism). In addition, several bran processing approaches, such as the

thermo-physical steam explosion and DIC technology (in french: Détente Instantanée Contrôlée) and/or enzymatic pre-treatment (destarching/ deproteinating), were combined with enzymatic hydrolysis to improve and thus to facilitate the availability of the complex biomass matrix for enzyme activity. Moreover the possibility of improving the amount of ferulic acid recovered from such milling fractions, which may be enriched in ferulate-rich aleurone tissue, was investigated (Lempereur *et al.*, 1997).

Within these experiments, higher amounts of ferulic acid were obtained from bran samples, which were previously destarched/deproteinated with Termamyl (2 U/g bran) and Alcalase (20 µl/g bran), respectively, and hence digested with Pentopan Mono BG® (1% w/w) and Feruloyl esterase (0.5 U/g) for 3.3 h. Under these conditions of enzymatic pre-treatment and hydrolysis, approximately 0.9 g ferulic acid per kg of whole bran was recovered that is approximately 15% of the ferulic acid occurring in whole bran (~ 5.5 g/kg). A similar release of ferulic acid can be obtained using lower concentrations of Feruloyl esterase for longer incubation time or higher concentrations of Feruloyl esterase for shorter incubation time, suggesting that, the use of feruloyl esterase is a major but not the only limiting factor of ferulic acid release from complex biomass matrix, such as wheat bran (Shin et al., 2006).

Thus, additional preliminary experiments were performed by pre-treating the bran with DIC and steam explosion at different temperature and pressure. In addition, for DIC technology, two kind of milling fractions within the company were tested.

Both DIC and steam explosion led to release higher amounts of ferulic acid as compared to untreated controls. The highest concentration of ferulic acid up to 1.0 g/kg of wheat bran (~ 18% of ferulic acid in whole bran) was recovered using bran samples subjected to steam explosion at preliminary operation conditions (bran:hot-water ratio 1:4, 4.4 bar, 10 min) and then digested with Pentopan Mono GB® (1% w/w) and feruloyl esterase (0.25 U/g). This suggests that further improvement of the release of ferulic acid from wheat bran might be achieved *via* rational optimization of the temperature and pressure conditions, as well as time of reaction of the treatment.

Lastly, batch experiments were performed for bioconversions of bran hydrolysates (obtained by enzymatic hydrolysis with xylanase Pentopan and feruloyl esterase) by employing resting cells of *P. fluorescens* BF13-1p4.

Results showed that vanillin production from wheat bran hydrolysate is feasible, but a preliminary purification of ferulic acid from carbohydrates is required. The purification protocol consists in solid phase extraction with ISOLUTE ENV+ columns, washing with water, elution with absolute ethanol, solvent evaporation and ferulic acid re-suspension in a saline phosphate buffer (Di Gioia *et al*., 2009). Following purification, ferulic acid in wheat bran hydrolysates was bioconverted to vanillin with vanillin molar yield of 55.1% and selectivity 68%, i.e., comparable to those obtained with food-grade ferulic acid.

This is the first work in the literature, which demonstrated the ability of a *Pseuodomonas* strain to produce vanillin from ferulic acid obtained by enzymatic hydrolysis of wheat bran.

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