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BIOTECNOLOGIA DEGLI ALIMENTI XXIII Ciclo

BIOTECHNOLOGICAL PRODUCTION OF VANILLIN FROM NATURAL FEEDSTOCKS

(CHIM/11)

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Abstract

This PhD research project was focused on innovative biotechnological production and recovery of vanillin and vanillin precursors. Nowadays, flavours cover over a quarter of the world market for food additives. Flavouring compounds are mainly produced *via* chemical synthesis or by extraction from natural materials. Flavours obtained by chemical synthesis of starting natural substances cannot legally be labelled as natural and the environmentally unfriendly production processes are subject to various problems such as lacks substrate selectivity, which may cause the formation of unwanted compounds thus reducing process efficiency and increasing downstream costs. On the other hand, the extraction processes from plants is often expensive because of the low concentrations of the molecule of interest in the raw material. Moreover the cost depends on uncontrollable factors such as plant diseases and weather conditions. The drawbacks of both methods and the increasing interest of consumers in natural product, reported in recent market survays, have led to the search for other strategies to produce natural flavours. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most widely used flavoring in food and pharmaceutical industries. Chemically synthesized vanillin accounts nowadays for more than 99 % of the total market share. Extraction from vanilla beans is expensive and limited by plant supply, curing time and labour cost. Those factors make vanillin a promising target for biotechnological flavour production. As the Regulation (EC) no 1334/2008 of the European Parliament and of the Council of 16 December 2008 specify, vanillin produced in biotechnological processes starting from natural substrates can be classified as natural flavouring on condition that the natural starting material is specified. In recent years a large number of studies have been made on natural vanillin biosynthesis using microorganisms or isolated enzymes. However, these bioconversions are not yet economically feasible.

The high chemical activity and toxicity of vanillin cause low yield from ferulic acid. Moreover little vanillin was accumulated due to the higher degrading rate of this molecule than that of ferulic acid.

Biovanillin can be synthesized using cells or enzymes starting from different natural compounds, such as ferulic acid, eugenol or capsaicin. The latter, ((6E)-N-(4-hydroxi-3-metoxibenzil)-8-metil-6-nonenamide) is the pungent compound in chili pepper related plants of the *Capsicum* family. It can be hydrolyzed to vanillylamine, (4-hydroxy-3-methoxybenzylamine), a natural precursor of vanillin, by cleavage of its amine bond using specific microbial acylases. The aims of the thesis were: to enhance vanillin production

from resting cells of *E. coli* engineering strains, starting from ferulic acid and by using XAD-4® resin and a new two/phase system of agarose gels and ferulic acid for the controlled release of the substrate in the bioconversion medium (1), to evaluate different strategies for enhancing the production of the capsaicin acylase from *Streptomyces mobaraensis* DSM40847 strain (2), to identify optimal conditions for capsaicin hydrolysis by using the acylase from *Streptomyces mobaraensis* DSM40847 strain (3), to develop efficient procedures for the recovery of vanillin from diluted aqueous solutions (4).

Experiments carried out using whole cells or crude enzyme preparations demonstrated that: (a) the two phase system of agarose gel and ferulic acid developed in this work was compatible with the bioconversion and it permitted to reduce the toxic effect of the ferulic acid; (b) selective recovery of the product, using macroporous resins in an off-line process, enhances the biological conversion of ferulic acid to vanillin using resting cells of *Escherichia coli* engineering strains; (c) thermal pre-treatment at 55°C of crude PVA acylase from *Streptomyces mobaraensis* DSM40847 improves the conversion of capsaicin to vanillylamine; (d) liquid/liquid extraction with *n*-butyl acetate allowed high recovery and high selectivity and supplied the best result in the recovery of vanillin from aqueous solutions.

Riassunto

Questo progetto di tesi ha riguardato lo studio e l'ottimizzazione di un processo biotecnologico innovativo per la produzione di vanillina e di suoi precursori naturali. La ricerca include lo sviluppo di un'efficiente procedura di recupero della vanillina prodotta.
Oggi, il mercato degli aromi ricopre circa un quarto di quello mondiale degli additivi alimentari. I composti aromatici vengono generalmente prodotti per via chimica e sintetica o per via estrattiva da matrici naturali. Gli aromi ottenuti per via sintetica da materia prima naturale non sono classificabili come "naturali" e i processi produttivi dannosi per l'ambiente, sono soggetti a molti problemi come la perdita di selettività di substrato, che può causare la formazione di composti non voluti, con riduzione nell'efficienza di processo e aumenti nei costi di recupero. D'altra parte i processi di estrazione dalle piante sono spesso costosi a causa delle basse concentrazioni delle molecole di interesse nelle matrici naturali. Inoltre i costi dipendono da fattori non controllabili come le condizioni climatiche e le malattie. Gli svantaggi di entrambe i metodi descritti e il crescente interesse dei consumatori per i prodotti naturali, riportato in recenti indagini di mercato, ha portato alla ricerca di nuove strategie per la produzione di aromi naturali.
La vanillina è il composto carattere dell'aroma di vaniglia che viene largamente utilizzato in campo farmaceutico ed alimentare. La vanillina ottenuta per via sintetica ricopre circa il 99% del mercato mondiale. L'estrazione dai baccelli curati della vaniglia è molto costosa e limitata dalle produzione delle piantagioni, dai tempi del processo di cura e dai costi della manodopera. Questi fattori rendono la vanillina un target commerciale importante per le industrie biotecnologiche. In accordo con la direttiva europea (EC) numero 1334/2008 del parlamento e del consiglio europeo del 16 dicembre 2008, la vanillina prodotta per via biotecnologica a partire da materie prime naturali è classificabile come aroma naturale a condizione che la matrice di partenza venga sempre specificata. Negli anni passati sono stati condotti molti studi sulla produzione biotecnologica di vanillina per via microbica ed enzimatica a partire da materie prime naturali. Questi processi di bioconversione non sono ancora competitivi dal punto di vita economico. Infatti, l'elevata attività chimica e la tossicità della vanillina determinano base rese nel processo di bioconversione.
La biovanillina può essere sintetizzata utilizzando cellule microbiche o enzimi a partire da diverse matrici naturali come l'acido ferulico, l'eugenolo e la capsaicina. Quest'ultima, ((6E)-N-(4-hydroxi-3-metoxibenzil)-8-metil-6-nonenamide), è il composto che conferisce la nota pungente al peperoncino della specie *Capsicum*.

La capsaicina può essere idrolizzata da un'acilasi di origine microbica che rompe il legame amminico e determina la formazione di vanillil ammina (4-hydroxy-3-methoxybenzylamine) un precursore naturale della vanillina.

Gli obiettivi della tesi di dottorato sono stati i seguenti:

ottimizzare la produzione di vanillina a partire da acido ferulico utilizzando cellule resting di ceppi ingegnerizzati di *E.coli*, effettuando un recupero in situ del prodotto con la resina XAD-4® e impiegando un nuovo sistema bifasico di gel di agarosio e acido ferulico per il rilascio controllato del substrato nel mezzo di bioconversione (1); ottimizzare la produzione di acilasi attiva su capsaicina dal ceppo DSM40847 di *Streptomyces mobaraensis* (2), stabilire le condizioni ottimali per la reazione di idrolisi della capsaicina utilizzando l'acilasi prodotta dal ceppo DSM40847 di *Streptomyces mobaraensis* (3), sviluppare efficienti metodi di recupero della vanillina da soluzioni acquose (4).

Gli esperimenti condotti utilizzando cellule microbiche o preparati enzimatici grezzi hanno dimostrato che: (a) il sistema bi-fasico di agarosio ed acido ferulico sviluppato in questo studio, è compatibile con la bioconversione e permette di ridurre l'effetto tossico dell'acido ferulico sulle cellule resting di ceppi ingegnerizzati di *E.coli*; (b) l'impiego di una resina macroporosa in un processo off-line di recupero del prodotto permette di aumentare la conversione biologica di acido ferulico usando cellule resting di ceppi ingegnerizzati di *E.coli*; (c) un pre-trattamento termico a 55°C del preparato enzimatico grezzo di acilasi attiva su capsaicina ottenuto dal ceppo DSM40847 di *Streptomyces mobaraensis* aumenta la resa di conversione della capsaicina in vanillil ammina; (d) l'utilizzo della tecnica di estrazione con *n*-butil acetato ha fornito alte percentuali di recupero in vanillina e alta selettività tra acido ferulico e vanillina in soluzione acquose dei due composti.

INTRODUCTION

Food flavour production: market and costs considerations.

The origin of using perfumes and flavours is in early Egyptian times when people used perfumed balms in religious cerimonies. Frankincense and mhyrr derivatives from trees were used to scent atmosphere during rituals, while rose and peppermint were stepped in oils to create an unguent.

During early Christianity perfumes were no longer used, but their use was revived in the medieval period. By 1600's scents were applied to objects and used as bath essence. The advances in organic chemistry knowledge in the late nineteenth century permitted to produce synthetic perfume products that were used in place of certain hard-to-find or expensive ingredients and in textile printing dyes.

But it was only in the twentieth century that scents and designer perfumes were really mass-produced. The foundation of the modern flavor industry was established in 1843 with the synthesis of methyl salicylate (methyl 2-hydroxybenzoate), followed by cinnamic aldehyde ((2*E*)-3-phenylprop-2-enal) in 1856, and benzaldehyde in 1863. The synthesis of vanillin, the key ingredient in flavor creativity, in 1872 represented the explosion for the flavor industry. In those years scientists or business people founded the first flavour and fragrance companies. Many of them still exist, either as such or as the nucleus of larger firms that changed during the decades. This industry has developed into a very profitable market. It includes companies in the food and beverage, cosmetics, household products and the fragrance industries.

The total market for flavours, fragrances, and cosmetic ingredients is estimated at 20 billion of euros. This means 25 % of the total food additives market (1,2). The market shares between the flavour and the fragrance parts are almost equal.

The largest markets are in the Europe, Africa, and Middle East region (36%) and North America (32%), followed by Asia-Pacific (26%) and South America (6%).

Global demand for flavours and fragrances is estimated to reach 23.5 bn of US dollars in 2014 with a growth rate of 4.1% per year. In the Asia–Pacific regions, it will reach 6.480 bn of US dollars in 2014 with a rate of 5.3% per year during 2009-2014. The fastest growth will be registered in China and India where the growth is the fastest and the account cover one-third of total value gains.

In the same period the demand in Asia-Pacific regions will surpass that in Western Europe and become the second largest consumer of flavours and fragrances after North America where the USA cover a quarter of global demand. In Central and South America, in

Africa/Mideast regions and in Eastern Europe the demand growth will be higher than the global average.

There are eight major global companies that share about 60% of the world market. The two largest flavour and fragrance companies are Givaudan and International Flavors & Fragrances, followed by Firmenich and Symrise, Quest International, Takasago, T. Hasegawa, Sensient Technologies, Mastertaste, Danisco, and Mane. The top two companies have a turnover in excess of $2 billion, the next three companies have a turnover in excess of $1 billion each (2,3). The research drives the flavour and fragrance business, in fact the larger companies spend about 7–8% of their total sales per annum on research and development.

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

Food and beverage manufacturers use flavours to change or specify a product taste but also because they can provide several benefits to the end-product. A flavour can often reduce the cost of a product by giving developers the option of replacing more expensive ingredients. In this case a small amount of ingredient can be used for label purposes, whereas natural flavour can be added back to create the fuller taste profile of the product. In the global market during the last years, healthy formulation of food and beverages is a driving force. Flavours can work in concert with healthy ingredients to create a better tasting product, but they can also mask the bad taste associated with many of these additives. Similarly flavours can also be used to replace ingredients that are difficult to source, arduous to handle or are seasonally available.

Extracts derived from floral, fruits and plant sources plays a key part in the flavour industry. Examples of this trend in the world of flavour marketing are floral and fruit flavour mixtures, such as blueberry and lavender, strawberry and passionflower, apple and rose, orange. Similarly, the use of spices and herbs with fruits in manufactured beverages

began a trend thanks to the development of a balanced flavour that incorporates a spice or herb note paired directly with the desired sweet fruit end note such as chocolate cinnamon, berry cardamom, rosemary lime and strawberry basil.

About 10,000 volatiles have been mentioned in food products (4) that are representative of the complexity of the investigations in this field. 2000 synthetic flavors are available on the market and about 400 natural flavors. Beverages and cooked products represent around 70% of the total use of flavor additives. Up to 90% of natural flavour are used in beverages and only 20% in sweets and candies.

From an ecological and environmental point of view the flavour and fragrance industry's weak points are emissions, the use of chemicals and chemical reactions and the production of wastewater. Moreover the use of flavours for foods and beverages is not so easily understood by a certain part of the population. Companies have started sustainable development activities that include: measurable reduction of energy and emissions, optimization of the production processes, financial support for charities, aid organizations, and local cultural activities, equal rights and compensation policies throughout the company, no child labour throughout the company.

Another important point is the search for sustainable raw materials. There are many raw materials used in the flavour industry. They includes a combination of chemicals, extracts, essential oils, distillates, and others. Many ingredients come from tropical countries and from China, Vietnam, Indonesia, Côte d'Ivoire, countries outside of the mainstream business. The supplier companies of these raw materials for the flavour and fragrance industry need to make sure that the supply is sustainable.

In the past decades, the employment of new biotechnological processes for the production of flavours has increased considerably (5). In nature, chiral flavours are often present as single enantiomers. It is possible to synthesize regioisomers or different enantiomers that could show different sensorial properties. In this field, biocatalysis are useful to catalyse a wide range of stereo and region-selective chemical reactions that are not easily carry out by the less selective classical synthetic procedures. For the biotechnological production of flavour compounds about 400 enzymes have been commercialised mainly for stereoselective organic synthesis.

The demand for natural flavours caused a significant shortage of several plant resources such as peppermint and some fruit flavours such as strawberry aroma (6). Isolated aroma compounds are currently only available at prices of more than 5000 $/kg. For instance synthetic 4-decalactone, the flavour compound of peach, costs 150 $/kg, while the same

substance extracted from a natural source is worth 6000 $/kg. Moreover the dependence on the weather and the risk of several plant diseases socio-political trade restrictions represent a problem for the extraction of flavours from natural sources.

To obtain natural flavours or flavours precursors is possible to collect the source from the wild plant population, the agricultural cultivation, and plant tissue culture. The first option is the easiest but it can cause problems with the stocks. The second possibility requires specific conditions for growth of the wild plants which cannot be applied elsewhere. In order to permit the multiplication or the conservation of the plants plant tissue techniques may be employed, to lighten the pressure on the supply of natural flavours, but the agricultural cultivation of the plant remains the most economic solution. Many microbial processes to produce flavours have been described, but their application in the industrial field are limited because of the low yield obtained. However the high costs for down-stream processing for the recovery of the microbial flavours in low concentration from fermentations broths could be compensated by the fact that the market price of natural aromas is 1&l00 times higher than that of synthetic aromas. The price of microbial flavours has to range between 200 and 2000 US $/kg to be competitive (7,8).

Many flavours have high volatility and low solubility in water. It is often required to keep the concentration of the flavour in the broth at low level because of the inhibition and toxicity towards the microorganisms themselves. These problems make the industrial applications difficult. An important challenge for researchers in the biotechnological production of flavours is the development of specific fermentation techniques and recovery methods.

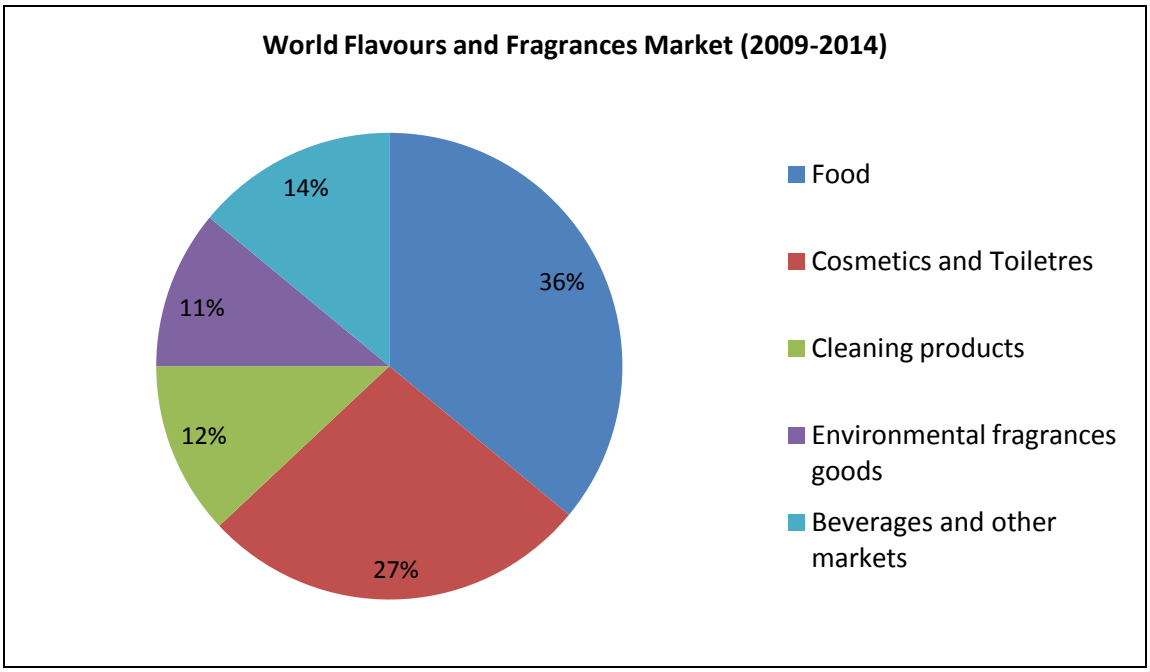


Fig.1 World Flavours and Fragrances Market (2009-2014); source Freedonia group http:www.freedoniagroup.com

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

Table 1 Food Flavour demand (1997-2010) Freedonia group

http:www.freedoniagroup.com

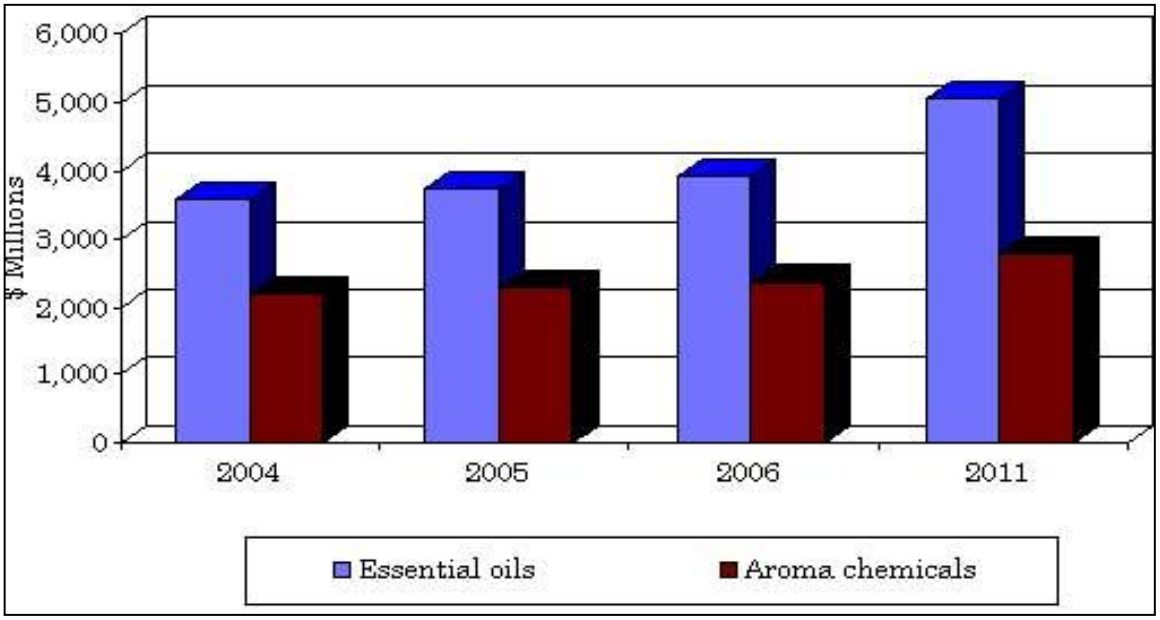


Fig.2 Estimated Worldwide Essential Oil & Aroma Chemical Sales Freedonia Group http:www.freedoniagroup.com

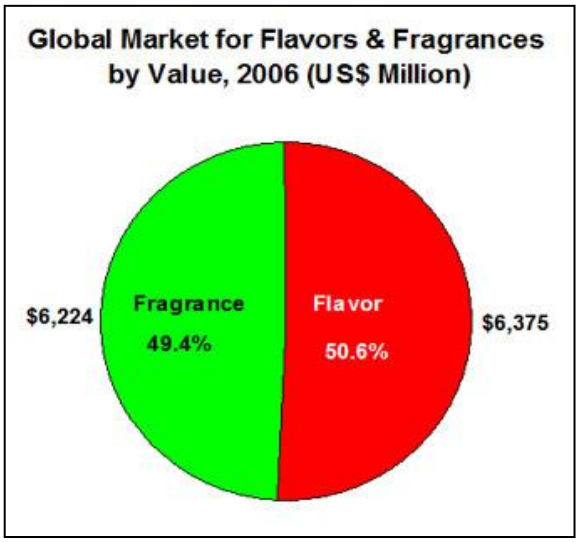


Fig.3 Global Market for flavors and Fragrances 2006; Source IAL Consultants (16 Apr. 2007) Press Release

Market pull	***Technical push***
Increasing consumers' demand for 'organic', 'bio', 'healthy', and 'natural'.	High chemo-, regio- and stereoselectivities of biocatalytic systems.
Industrial dependence on distant (frequently overseas) raw materials, undesired/limited raw materials.	Sustainability of bioprocesses.
Industrial dependence on distant (frequently overseas) raw materials, undesired/limited raw materials.	Improved biocatalysts by evolutionary and rational enzyme engineering and metabolic engineering.
Search for natural character-impact compounds.	Improved downstream processing, especially in situ product-recovery techniques.

Table 2 Driving forces to use biotechnological methods for flavour production

Characteristics	***Biotechnological strategy***	***Exemplary product***
Formation of unwanted by products owing to complex metabolic pathways	Over expression of key genes of the synthetic pathways Heterologous gene expression/use of engineered enzymes Knockouts of genes involved in product degradation 'Precursor approach' instead of de novo biosynthesis Screening; enrichment cultures Subsequent biotransformation converting a by-product to the desired product	3-Methylbutyl acetate Cinnamyl alcohol[31] Verbenol[32,33] Vanillin[34] 2-Phenylethanol[35] raspberry ketone[36,37] Perillyl alcohol[38] 10-hydroxypatchoulol[39] 4-Decanolide[40]
Toxic properties of the flavour compounds produced	In situ product recovery by: Adsorption, e.g. on XAD resins Stripping and adsorption Extraction (two-phase bioprocess) Membrane-based processes Resting cells instead of growing ones Product-tolerant strains	6-Pentyl-á-pyrone C2C5 alkyl esters[40] Furfurylthiol[41] 2-Phenylethanol[43] phenylacetaldehyde 2- sonovalal[60] phenylethyl acetate[43] Acetaldehyde[44] Vanillin[45]
Toxic properties of the precursor molecules	Sequential precursor feeding On line monitoring of precursor/bioactivity Immobilisation of microorganisms Two-phase bioprocess with an organic solvent as the precursor reservoir Resting cells instead of growing ones Precursor-tolerant(solvent-tolerant) strains Fungal spores instead of mycelia	4-Octanolide 3-methylbutyl acetate[46] carboxylic acids[47] Limonene transformation Products[48,49] Propanoic acid[50] phenylacetic acid[51] 5-Decanolide[52] 4-hexanolide[53] Carvone[54,55] Perillic acid[56] Carvone[57,54] Methylketones[58,59]

Table 3 Main drawbacks during microbial flavour production and biotechnological strategies

Product Process	*Precursor*	*Microorganism*	*Process data*	*Remarks*
L-Glutamic acid	-	*Corynebacterium glutamium*	150 g L-1, 60 h, 1.500,000 t	Aerobic cultivation; up to 500-m^3 scale; mutants with highly permeable cell walls
Citric acid	-	*Aspergillus niger*	>200 g L-1 , 912 days, 1,000,000 t year-1; yield >95%	Downstream processing by precipitation as calcium citrate
Acetic acid	Ethanol	*Acetobacter, Gluconobacter*	'Vinegar' with 10 to >20 %, >190,000 t year-1; yield ~98%	Aerobic cultivation at 100-m^3 scale; Frings aerator for high oxygen transfer rates
L-Lactic acid	-	*Lactobacillus*	210 g L-1, 140,000 t year-1; yield >90%	More than 100-m^3 scale; recovery of lactic acid by salt splitting technology
(Z)-3-Hexenol ('leaf alcohol')	Linolenic acid	Soy lipoxygenase + plant hydroperoxide lyase + baker's yeast	4 g kg-1, 510 t year-1(also by isolation from plant oils)	Addition of baker's yeast to obtain the alcohol; without yeast the aldehyde is the major product
Vanillin	Ferulic acid	*Amycolatopsis Streptomyces*	Up to 18 g L-1, 50 h, 110 t year-1	In situ product recovery by crystallisation at more than 10 g L^{-1} possible
4-Decanolide (G-decalactone)	Ricinoleic acid	*Yarrowia lipolytica*	11 g L-1, 55 h, several tons per year	Final acidification and temperature Increase effect cyclisation of all 4-hydroxydecanoic acid to the corresponding lactone
2-Phenylethanol	1-Phenylalanine	Diverse yeasts; e.g. *Saccharomyces* and *Kluyveromyces*	>10 g L-1, 30 h, 0.51 t year-1	Fed-batch cultivation; in situ product recovery by two-phase system with more than 25 g L^{-1} in the organic phase possible
Short-chain carboxylic acids, e.g. 2-, and 3-methylbutyrate	Fusel alcohols	*Acetobacter, Gluconobacter*	Up to 95 g L-1, 72 h	Two-step cultivation: biomass + bioconversion period; used as flavour acids but also for ester syntheses

Table 4 Some microbially produced flavour compounds and corresponding bioprocess feature.

Vanilla flavour

Vanilla is the most popular flavour worldwide. It's widely used in food, beverages and cosmetics and the most important one by both the tonnage and dollar basis. Vanilla extract is obtained by aqueous ethanolic extraction of cured vanilla beans, of *Vanilla planifolia Andrews*, a member of the orchid family (Orchidaceae). Vanilla beans are grown in four main areas of the world and each sites yields vanilla with different flavour characteristics. Madagascar, is the first producer of vanilla beans in the world and its product is known as Madagascar Bourbon vanilla. The term Bourbon applies to beans grown on the Bourbon Islands - Madagascar, Comoro, Seychelles and Reunion. It's the highest quality pure vanilla available, described as having a creamy, sweet, smooth, mellow flavor. Indonesia is the second largest producer of vanilla. Indonesian vanilla is woody, astringent and phenolic. Madagascar and Indonesia produce 90 percent of the world's vanilla bean crop. Mexico, where the vanilla orchid originated, now produces only a small percentage of the harvest. Mexican vanilla is described as creamy, sweet, smooth and spicy. Tahiti is another important vanilla-producing country. In this region vanilla is grown from a different genus of vanilla orchid, and it is flowery and fruity. The cost, supply, and quality of the vanilla beans are subject to fluctuations, because of severe weather episodes and diseases of the plant.

The genus *Vanilla* belongs to the family Orchidaceae, with more than 18,500 species. The *Vanilla* Swartz genus has more than 100 species, amongst which 15 are aromatic. The most important species from an economical point of view are: *Vanilla planifolia* Andrews (previously known as *V. fragrans*), that is the most resistant against diseases; *V. pompona* Schiede and *V. tahitensis* J.W. Moore both cultivated on a small scale. The pods from the former are of an inferior quality while those from the latter are of more expensive (9).

Growing conditions, excessive rain and drought can cause severel diseases. Fungal diseases are caused by, *Calospora vanillae* (anthracnose, whole plant), *Fusarium* sp. (root rot, fruit rot), *Phytophthora* sp. (fruit rot), *Colletotrichum* sp. and *Gloeralla vanilliae* (root rot). *Cymbidium mosaic* virus and the *cucumber mosaic* virus can determine serious demage to the crops.

The plant grows well from sea level to altitudes of more than 760 meters at a temperature ranging from 20 to 30 °C (9). Tree or artificial support are requested for the growth of the Vanilla vine and aerial roots adhere to them. Vanilla height can reach to 10–15 meters.

When the plant is grown by cuttings needs about 2-4 years before flowering and can produce for 5-6 years. In each plant there are about 20 clusters of 15-20 flowers. After hand pollination the 60 per cent of these flowers will develop into pods.

In the wild vanilla plants grow as a green, thick and vine up trees with clusters of colored orchids. These delicate flowers usually bloom for just one day and can only be pollinated by the small specialized Melipona bee, that can live only in Mexico. If the flowers are not pollinated they drop to the ground and no vanilla beans are produced. This means in most places pollination is done by hand. Vanilla beans are harvested green after 9 months of maturation, the vanilla beans undergo an elaborate processing known as curing, under high-heat and high-humidity conditions for about 6 months. It is a sort of fermentation process that produces the flavour thanks to the drying and to the

hydrolysis of the vanillin glucoside, resulting in free vanillin, the most abundant component in the vanilla flavour. Curing usually yields 2.5–4.5% vanillin or less, on a dry-weight basis of cured beans (Bala 2003; Ranadive 2003), corresponding to 1.75–2.1% of vanillin in cured beans containing 30% moisture. During the preparation of vanilla extract cured beans are extracted a part of the vanillin can be lost. This phenomenon appeared also when it is added to foods or to other materials as vanilla extract or pure vanillin. The tendency of vanillin to escape from cured vanilla beans is based on the hydration state and reactivity of the compound and on the acid–base conditions. It's possible to optimize the curing process and obtain around 8–10% of vanillin, on a dry-weight basis. The demand for vanilla flavor cannot be met by vanilla extract, that contains more than 250 compounds (Hartman *et al*. 1992), not only because of the cost of vanilla beans but also for the requirement for large amount of vanillin in many flavours. According to the legislation (ISO 5565-2, 1999), vanillin content in natural vanilla extract should be 1.6-2.4 %.

Most of the vanilla beans produced are used in the US market; the annual consumption is around 1200–1400 tons with a market value of approximately US$100 million. Around 40% of the beans are imported into the USA are used in ice creams. Approximately 300–400 tons of vanilla beans are used in the rest of the world.

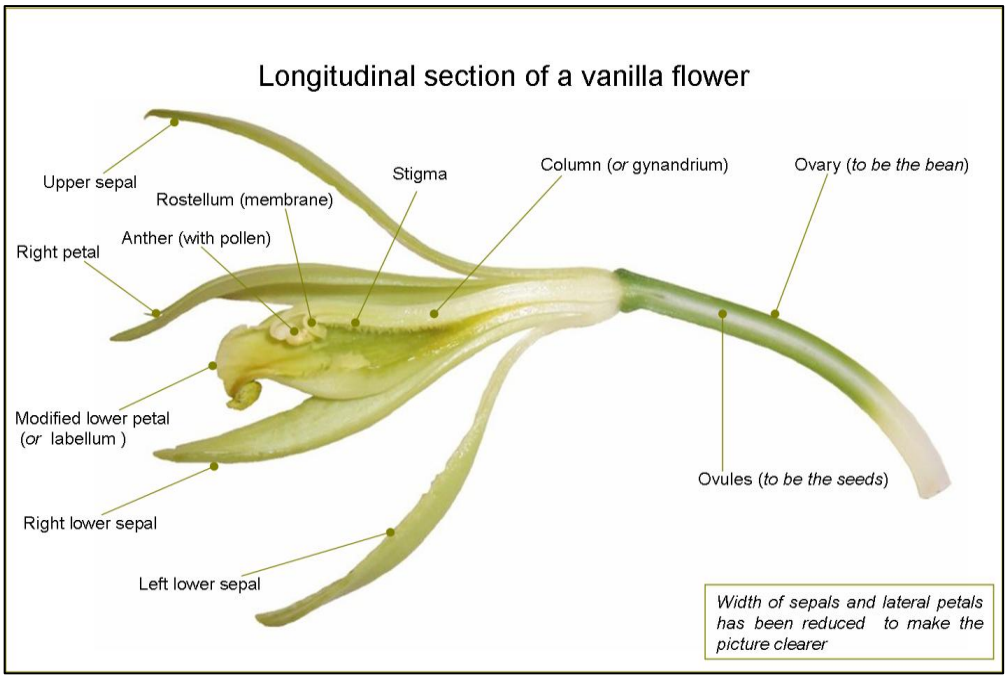


Fig.4 Vanilla flower

Fig.5 A vanilla plantation in a wood on Réunion Island.

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

Table 5 Parameters of laboratory curing processes under traditional Indonesian conditions.

Although vanilla is widely known as a high-value crop, the prices of vanilla beans are also notably volatile and more recently (2007-2009) have dropped sharply to much below the remunerative levels that may lead farmers to switch from vanilla to less labor intensive and more lucrative crops.

The United States is the world's largest consumer of vanilla, followed by Europe (especially France, Germany and Italy) and Canada [Source: FAOSTAT, November 2009; http://faostat.fao.org/].

In vanilla beans more than 250 compounds have already been identified such as fatty acids, monoterpenoids, benzoic acid derivatives, alcohols, phenylpropanoids and other phenolics, esters and ketones.

Major components in cured beans, besides vanillin are hydroxybenzaldehyde and *p*-hydroxybenzylmethyl ether while glucovanillin, bis[4-(*β*-D-glucopyranosyloxy)benzyl-2-isopropyltartrate] (glucoside A) and bis[4-(*β*-D-glucopyranosyloxy)benzyl-2-(2-butyl)tartrate] (glucoside B) are the major compounds in green beans (10, 11, 12). More than 95% of the volatile components are present at very low level (below 10 ppm) (13).

Fig.6 Vanilla beans production and curing. Manual pollination (a,b), cultivation (c), harvesting (d) and curing (e,f).

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

Table 6 Natural vanilla extract; the table reports the ratio values quantity of the major component in vanilla flavour. Source: "Note d'information" N° 2003-61 (June 16th, 2003)
Vanillin content should be 1.6-2.4% (ISO 5565-2, 1999)

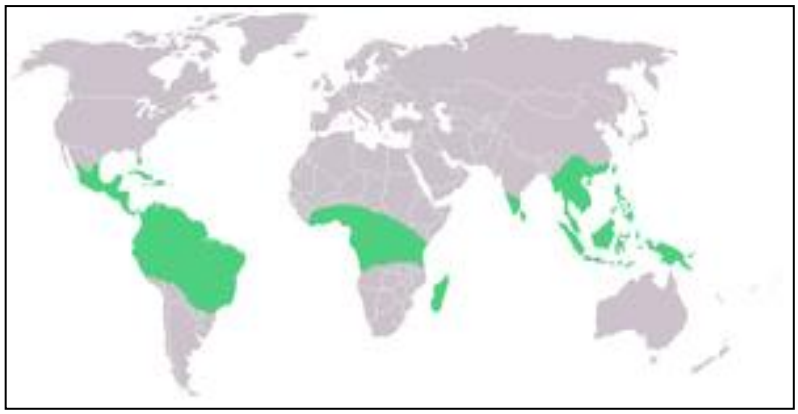


Fig. 7 Vanilla producers country.

Vanilla history

The ancient Totonaco Indians of Southeastern Mexico were the first people to cultivate vanilla. They believed that the tropical orchid was born when Princess Xanat, forbidden by her father from marrying a mortal, fled to the forest with her lover. The lovers were captured and beheaded. Where their blood touched the ground, the vine of the tropical orchid grew. Aztecs conquered the Totonacs in the fifteenth century and soon developed a taste for the vanilla bean. The Totonacs sent vanilla beans to the Aztec capital, Tenochtitlan as tribute. They named the bean *tlilxochit*, or black flower, because the mature bean has a dark colour. In 1518, the Spanish conquistadores, led by Herman Cortez, came to Mexico and observed the Aztec emperor, Montezuma, drinking *Choclatl,* a beverage of ground cocoa and corn flavored with *tlilxochitl* (cured vanilla beans) and honey. Spanish chefs started making *Vainilla* (little sheath) flavored chocolate and for 100 years it was consumed by nobility. In the Mexico 18th century, Mexico was the sole producer of vanilla. In early 1800's the French took vanilla cuttings from Mexico to Reunion Island, the *Ile de Bourbon* (the surname of the Kings of France). The plants flourished and flowered, but because of the absence of Melipona bees on the island, no beans were produced. In 1837 the Belgian botanist Morren succeeded in artificially pollinating the vanilla flower but unsuccesfully the process was attempted in Reunion Island. In 1841 Edmond Albius, a 12 year old slave discovered the correct technique of hand pollinating the flowers. Vanilla was taken to the neighboring French possessions of Madagascar, Comoro and Santa Maria. By 1898 about 200 tons of Vanilla beans a year were being produced by these islands. In 1930 the control cartel for vanilla prices and distribution was repealed. In the late 1970s a tropical cyclone destroyed the most important croplands and this caused a rise of the vanilla price. In 1980s prices remained

high although of the introduction of Indonesian vanilla. In the next few years prices decreased, to nearly US$20 per kilogram, but in 2000 another tropical cyclone Hudah struck Madagascar. This fact led in three years, the vanilla price to US$500 per kilogram in 2004 and has brought new countries into the vanilla industry. The market price down to the $40 per kilo range in the middle of 2005 because of good crops and production of imitations of vanilla.

Fig.8 Drawing of Vanilla from the Florentine Codex (ca. 1580) and description of its use and properties written in the Nahuatl language

Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde), represents the major component of natural vanilla. In addition to being an important flavor molecule, vanillin is valued also for other properties, including anti-oxidant, antimicrobial, and anti-inflammatory properties. The antimicrobial effects on the fungi *Aspargillus flavus, A. niger, A. ochraeus* and *A. parasiticus* and the bacteria *Escherichia coli*, *Bacillus subtilus* and *Staphylococcus aureus* were reviewed by Tipparaju et al. (14). Thanks to this property it can be used as food preservative for a wide variety of products like diary products, soft drinks and fruit juices (15, 16). Its odour threshold for humans is 11.8 x 10-14 M (17). Its flavour is pleasant also at very high concentration. Because of the large consumption of vanilla-flavoured products, vanillin is also made by other routes, such as via synthesis or by biotechnological routes. Only 0.2% of the approximately 6,000 t of vanillin used in the flavour market is derived from plants, for which vanilla is the major source (17,18). Most vanillin is synthetic and it is produced on a scale of more than 10 000 tons per year. some several tons comes from microbial processes (19,20). About 60% of the vanillin goes into food and beverages, 33% into perfumes and cosmetics and 7% into pharmaceuticals. The price of natural vanillin extracted from vanilla is estimated to be between $1,200 and $4,000 per

kilogram (17,18). Natural vanillin derived from microbial production has a price of about $1,000 per kilogram (21).

Fig. 9 Vanillin molecule; Ethyl vanillin molecule.

Synthetic vanillin

Food industries generally use synthetic vanillin. It is produced at an estimated rate of 13,000 tons annually. It is used in combination with ethyl vanillin, in many applications. In the past, vanillin was obtained from lignin by alkaline hydrolysis, in fact conifer wood contains up to 30% of its lignin as coniferil alcohol derived from ferulic acid (Hocking 1997). Vanillin obtained from lignin, a natural polymer is considered synthetic because many chemicals modifications have to carry out. Today, only 10–15% of synthetic vanillin is produced from lignin, most is chemically synthesized from guaiacol, a petrochemical product, in a cleaner process for the environment (fig.10).

Synthetic vanillin costs about $11–15 per kilogram (21,22). It's an important intermediate in the production of various chemicals, herbicides and medicines. Natural and synthetic vanillin are chemically identical. Because of the price differences between natural vanillin and synthetic vanillin and in order to avoid the counterfeiting analysis of the ource from which the vanillin is derived are carried out by NMR and spectrometry.

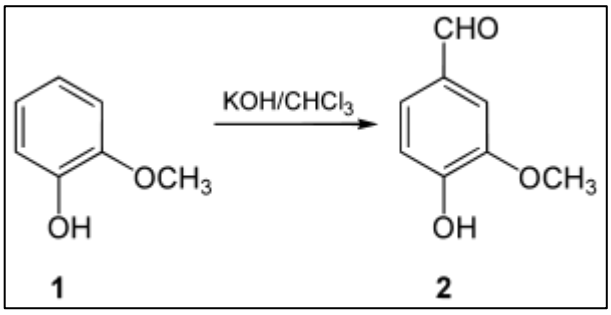


Fig.10 Synthesis of Vanillin from guaiacol (Reimer)

Biosynthesis of vanillin

Vanillin from Vanilla planifolia: biosynthetic pathway and vanillin extraction from vanilla beans

Vanillin is found in trace amounts in many plants. It is present in many essential oils, such as clove, cinnamon, and mace (Clark 1990). In the table 3 are listed the plant species that produce detectable amounts of vanillin. The compound is in high quantity only in plants from the genus *Vanilla*. There are around 130 species of *Vanilla*, but only two species, *Vanilla planifolia* and *Vanilla tahitensis*, are allowed to be used in food.

Vanillin is specifically present in the non-photosynthetic white parenchyma cells of the endocarp in the white inner fruit portion (Joel *et al*. 2003.), but in the outer green fruit exocarp there is 95% of the total vanillin found in the vanilla pod. Vanillin and intermediates in the vanillin biosynthetic pathway are present in the placenta and the adjacent endocarp parenchymatic cells. Vanillin accumulation begins after 3 to 4 months of fruit development. The molecule is sparsely water-soluble, particularly in acidic plant vacuoles (Frenkel and Havkin-Frenkel 2006) and it accumulates predominantly in the intercellular space.

Many studies suggested that vanillin is produced by the shikimate pathway and the phenylalanine (phenylpropanoid) pathway. In the first phenylalanine or tyrosine undergo deamination to a C6–C3 phenylpropanoid, which then serves as a precursor for the biosynthesis of vanillin. A general view on the metabolic origin of vanillin is reported in (fig. 12,13). Two possible pathways explaine the biosynthesis from phenylpropanoid. Zenk (1965) suggested the 'ferulate pathway' in wich the aromatic ring on C6–C3 compounds (*trans*-cinnamic, *p*-coumaric acids) undergoes hydroxylation and methylation giving rise to ferulic acid. This compound undergoes chain shortening to vanillin. Podstolski (*et al*. 2002), argued the 'benzoate pathway'; in this case the chain shortening of a phenylpropanoid is the first metabolic event, followed by hydroxylation and methylation of the aromatic ring to yield vanillin. In 2001 Wildermuth demonstrated that an early intermediate in the shikimic acid pathway gives rise directly to the benzoate pool, and there is no production of phenylpropanoids and their degradation to benzoate-pathway intermediates. In various experiments carried out using *V. planifolia* plant cell cultures do not produce vanillin in any significant amount and this means might be due to the presence of different biosynthetic pathways operate in the beans and in the cell culture. The figure 14 shows that vanillin can be formed through different ways in a complex network of compounds.

Vanillin is generally extracted from vanilla cured beans in water–alcohol mixtures. The alcohol is removed and the aqueous solution is brought to alkaline pH to increase vanillin solubility. This mixture is next extracted in a non-polar solvent to remove impurities, such as lipids, followed by acidification to attenuate the affinity of vanillin to the solvent. Under these conditions, in which vanillin is not soluble, it can be removed by sublimation, resulting in a highly purified product.

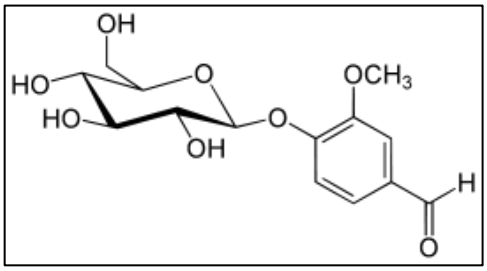


Fig.11 These green seed pods contain vanillin only in its glycoside form(b), and lack the characteristic odor of vanilla.

Natural occurrence of vanillin in plants		
Species	***Tissue***	***Percentage of dry weight***
Unicorn plant (*Proboscidae cuisianica*)	Roots, pod	0,01
Potato (*Solanum tuberosum*)	Tuber skin	0,01
Clove (*Syzygium aromaticum*)	Dry flower buds	Trace
Sian benoin	Vascular tissue exudates	Trace
Narcissus (*Triandrus narcissi, Tazetta arsissi*)	Roots, basal plate	0,01–0,60
Hyacinth (*Hyacinthus orientalis*)	Roots, basal plate	0,20–0,50
Vanilla planifolia	Pod (cured)	1,00–8,00
Vanilla tahitensis	Pod (cured)	0,50–2,00
Vanilla pompona	Pod (cured)	0,01–2,00

Table 7 Natural occurrence of vanillin in plants

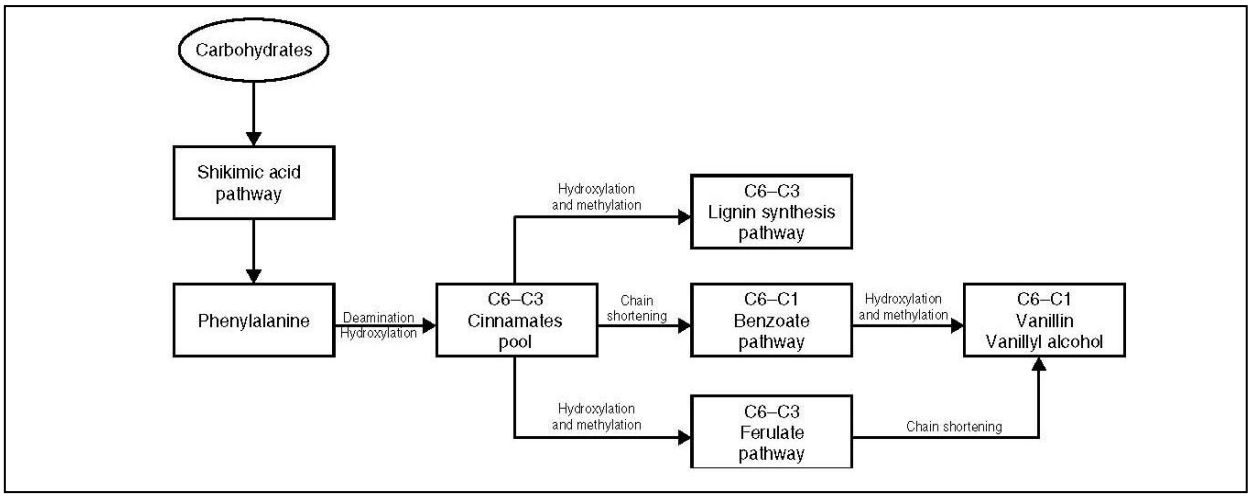


Fig. 12 Possible biosynthetic route to vanillin in Vanilla planifolia showing the ferulate and benzoate pathways

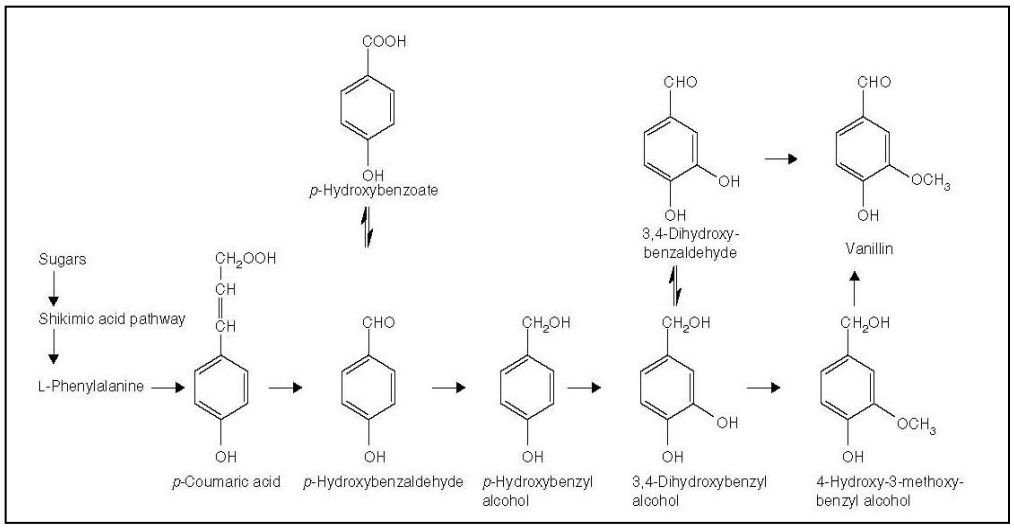


Fig. 13 Proposed vanillin biosynthetic pathway in Vanilla Planifolia

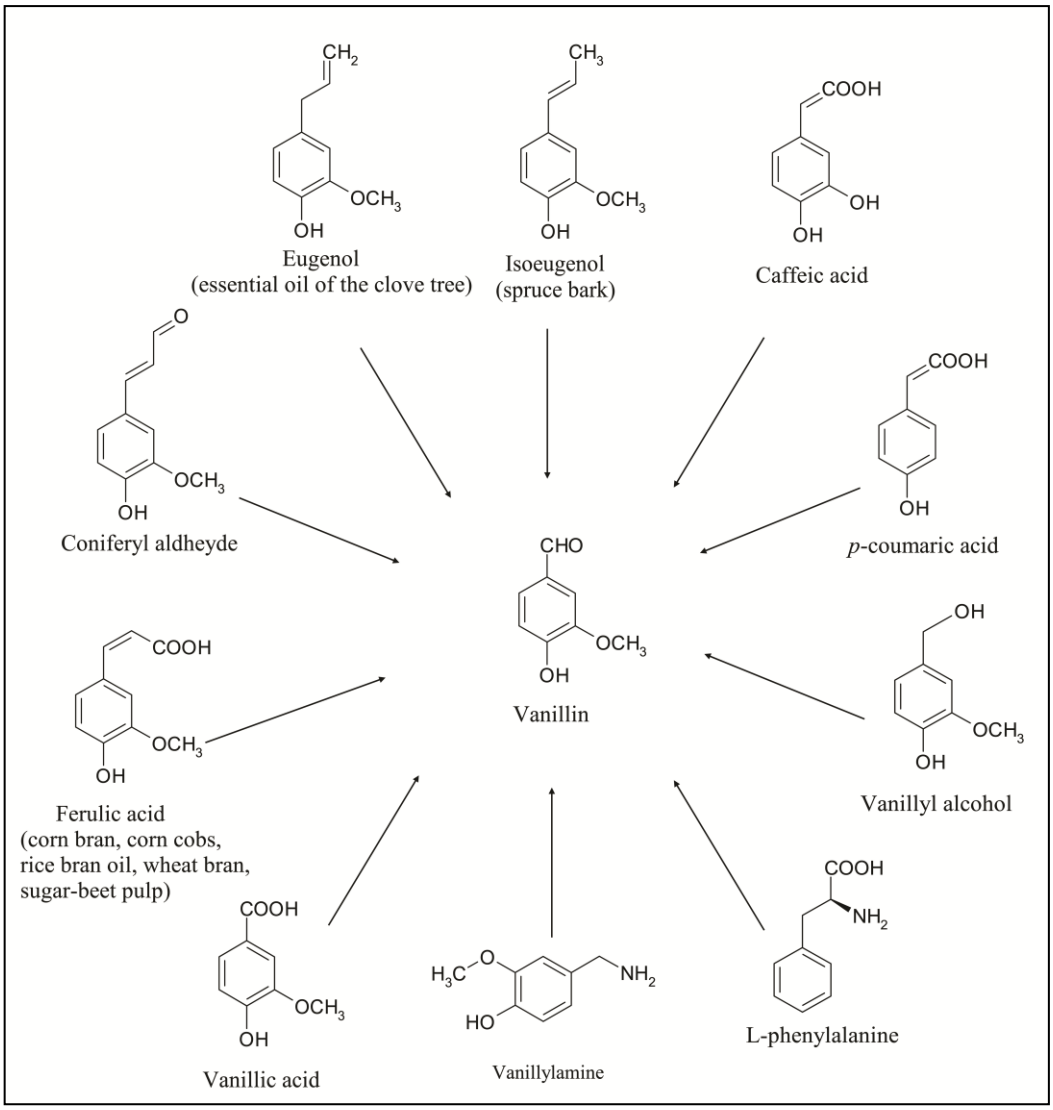


Fig.14 Major substrates for biotechnological production of "natural" vanillin.

Biotechnological production of vanillin

With the increasing interest in natural products, alternative processes are being developed to produce natural vanillin at a lower price. Information regarding vanillin biosynthesis is only from *V. planifolia* and the process is not exactly known.

Natural vanillin can be obtained from cell or tissue culture or tissue culture for bioconversion of natural precursors to vanillin. Vanillin can be released from lignin by enzymatic degradation or obtained by using microbial or fungal cultures for the bioconversion of natural precursors to vanillin. An artificial pathway for the synthesis of vanillin, from glucose was constructed by Frost *et al*.in 1998.

Vanillin production from *V. planifolia* cell cultures is not economically feasible (16, 23, 24, 25). *Capsicum frutescens* and *Capsicum annuum* cell cultures were able to produce some vanilla flavour compounds and these process can be enhanced by treating the cultures with methyl jasmonate or by feeding with exogenous ferulic acid respectively (26,27). Enzymes from the clone genes could be employed for the production of vanillin or vanillin intermediates starting from readily available precursors.

The knowledge of these systems are useful to control unwanted side reactions such as vanillin conversion to vanillyl alcohol or vanillic acid, which often occurs when microorganisms are fed with precursors. Enzymes from soybean are able to convert isoeugenol into vanillin and a soybean lipoxygenase can produce vanillin from esters of coniferyl alcohol.

The microorganisms *Bacillus fusiformis*, *Pseudomonas fluorescens*, *Pseudomonas acidovorans*, *Penicillium simplicissimum*, *E.coli*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae*, *Pycnoporus cinnabarinus*, *A. niger* are able to convert fed natural phenylpropanoids precursors, such as ferulic acid, eugenol, isoeugenol, coniferyl alcohol, vanillyl alcohol and vanillylamineisorhapotin (a stilbene), into vanillin. These precursors require a chemical modification in the aliphatic carbon side chain because they have the same aromatic substitution pattern as vanillin.

High yields of vanillin (more than10 g l^{-1}) in the conversion of ferulic acid was obtained by using *Amycolatopsis* species and *Streptomyces setonii* cells. Both the microorganisms had a high tolerance for vanillin, that is toxic and high reactive, and led to a molar yields were about 75% .

Ferulic acid is an expensive feedstock while eugenol is a much cheaper alternative, but the vanillin yields are lower. Ferulic acid is the most abundant hydroxycinnamic acid in the plant cell wall.

A wide range of microorganisms produce a feruloyl esterases that causes the releasing ferulic acid from the plant (28). Sugar beet pulp and maize bran can be source for ferulic acid.

Eugenol can be efficiently converted to ferulic acid, with a molar yield of 93.3% (29), by an *E. coli* XL1-blue strain expressing the *vaoA* gene from *Penicillium simplicissimum* encoding vanillyl alcohol oxidase, which converts eugenol to coniferyl alcohol, together with the genes *calA* and *calB* encoding coniferyl dehydrogenase and coniferyl aldehyde dehydrogenase of *Pseudomonas*.

The enzyme 4-hydroxycinnamoyl-CoA hydratase/lyase from *Pseudomonas fluorescens* converted ferulic acid CoA into vanillin. This gene in combination with 4-hydroxycinnamoyl-CoA ligase was overexpressed in *E. coli.*

E. coli has been genetically engineered to convert shikimate into vanillin by introducing the genes encoding a shikimate dehydrogenase yielding 3-dehydroshikimic acid, a dehydratase converting this into protocatechuic acid and a catechol-*O*-methyltransferase converting this acid into vanillic acid. Finally a reductase yielded vanillin (30). The high quantity of vanillin in the producing tissues of vanilla plants cause no good results in genetic engineering of vanilla plants to overexpress these enzymes. In the other hand genetically engineered organisms will be successful for the production of vanillin. The cost of vanillin from a microbial production was estimated to be $1,000 per kilogram.

Degradation of lignin to vanillin

Lignin, an abundant by-product of the paper industry, is a cell wall constituent in plants and contains vanillin subunits in its polymeric structure (Janshekar and Fiechter 1983).

The vanillin yield from the chemical breakage of lignin is around 4%. The enzyme-catalyzed oxidative degradation of lignin is carried out by extracellular enzymes from the white rot fungus *Phanerochaete crysosporium* (Tien 1987). They include heme-containing peroxidases (including lignin peroxidase) and manganese-dependent peroxidase as well as laccase, a copper-containing phenol oxidase. The enzymatic process can degrade only lignin fragmentation products obtained by chemical treatment with sulfuric acid. Moreover the activity of lignin-degrading enzymes depends on supplementation with co-factors including a redox mediator for lignin peroxidase, the veratryl alcohol (3,4-

dimethoxybenzoyl alcohol) and on Mn^{2+} ions that mediate the activity of the manganese-dependent peroxidase. The process yields around 1% vanillin as well as a vast array of other by-products.

Bioconversion of ferulic acid and eugenol to vanillin

Scientific literature report that microbial and fungal fermentation used for the production of biovanillin is based on degradation or bioconversion and not on the novo synthesis like happen in plants. Only a few companies produce vanillin on large scale by biotechnological route with a price of 500–1000 US dollars per kg although there are many studies on the subject. This fact is due to the high production costs, .

For the biotechnological production of vanillin many C6–C3 source compounds, such as eugenol and ferulic acid, are investigated. They are currently employied in fermentations (Benz and Muheim 1996; Priefer *et al*. 2001; Lesage-Meessen *et al*. 2002; Walton *et al*. 2003; Desmurs *et al*.2004; Mathew and Abraham 2006).

Eugenol, is the principle component of clove oil (80%), is cheap and readily accessible. It can be transformed into ferulic acid by a *Pseudomonas* strain in a series of reactions that involve the formation of coniferyl alcohol, coniferyl aldehyde, and finally ferulic acid (De Jong *et al*. 1992; Fraaije *et al*. 1995; Furukawa *et al*. 1998; Priefert *et al*. 1999; Van den Heuvel *et al*. 2001).

There are problems related to this process; the biotransformation can start only after an isomerization of eugenol, not soluble in water, to isoeugenol and eugenol at low concentration can be toxic to microorganisms. The use of strains able to grow in non-acqueous media and more resistant to eugenol toxicity could be agood strategy to adopt in order to optimize the process.

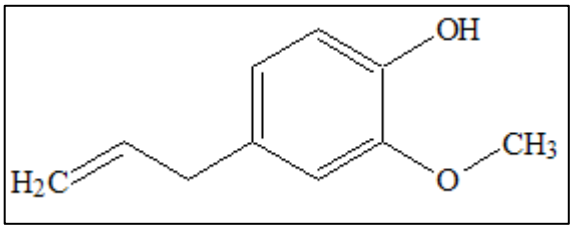


Fig.15 Eugenol molecule

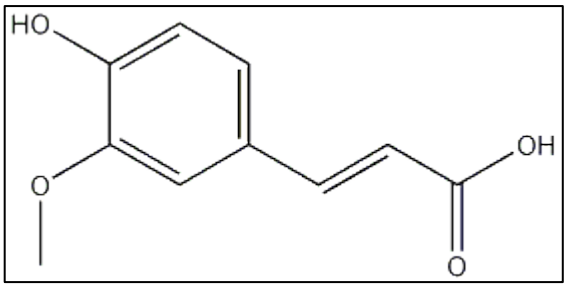


Fig.16 Ferulic acid molecule

Ferulic acid is the most abundant hydroxycinnamic acid in the plant cell wall and it is present in several cereal crops (Clifford 1999).This compound is esterified to arabinose moieties in plant cell walls and may be cross-linked to diferulate or other polymeric forms of the compound. Crosslinking may be to another wall-bound ferulic acid or to other cinnamic acid derivatives. On a dry-weight basis, the content of ferulic acid in the cell walls, is around 0.4–0.7% of the cell wall material of wheat, 1.2% in rice endosperm, 3% in maize bran, and 0.5–1.0% in sugar beet, (Walton *et al*. 2000).

Cell wall hydrolyzing enzymes and cinnamoyl esterase from *Aspergillus niger* can be used to obtain the release of high portion of ferulate from the cell wall matrix of cereal bran or sugar beet pulp by hydrolyzing the ester bond (Faulds and Williamson 1995, Kroon and Williamson 1996).

Ferreira *et al*. in 1993 and Bartolome *et al*. 1997demonstrated that the cinnamoyl esterase from *Pseudomonas* can release both monomeric and dimeric forms of ferulic acid from cereal bran and spent barley grain.

In various plant materials diferulate is released together with free ferulic acid. (Parr *et al.* 1996; Saulnier and Thibault 1999). In maize bran there is around 24% of the free-form ferulic acid (Lapierrea *et al*. 2001), 21% in maize sheath (Santiago *et al*. 2006) and 14% of the ferulate content in wheat flour (Vansteenkiste *et al*. 2004). All the compounds released with ferulic acid can interfere with the biotransformations. The content of free ferulic acid can reach to 1% of the content in the parent materials (Ferreira *et al*. 1993; Faulds and Williamson 1995; Kroon and Williamson 1996; Bartolome *et al*. 1997; Couteau and Mathaly 1997).

Priefert et al. (2001) found four different mechanisms for the shortening of the side chain of ferulic acid: non-oxidative decarboxylation, side chain reduction, and coenzyme A (CoA) dependent as well as independent deacetylation.

The most known CoA-dependent non-oxidative chain-shortening mode of action is an enzyme system, part of the hydroxycinnamate- degradation process in *Pseudomonas* (Walton *et al*. 2000). The starting reaction is ligation of ferulic acid to CoA, and it is catalyzed by 4-hydroxycinnamate: CoA ligase. An enzyme termed 4-hydroxycinnammoyl-CoA hydratase/lyase (HCHL) next catalyses the hydration and cleavage of feruloyl-CoA to vanillin and acetyl-CoA (Gasson *et al*. 1998). Microorganisms degradate the compounds formed for energy and intermediary metabolites and produce apart from vanillin, vanillic acid, protochatechuic acid, and products of ring cleavage.

In order to mantein the phenylpropanoid content in plants, compounds useful for the formation of vanillin the pathway described, was introduced to plants. Expression of HCHL in *Nicotiana tabacum* plants (Mayer *et al*.2001) and in hairy root culture of *Datura stramonium* L. (Mitra *et al*. 1999, 2002), was successfully obtained and it increased redirection of phenylpropanoid metabolism.

The plants accumulated no vanillin but other products such as glucose esters of 4-hydroxybenzoic acid, β-D-glucosides, vanillic acid glucoside and 4-hydroxybenzyl alcohol glucoside. In the plant tissues not specializing in vanillin biosynthesis stopped the reactions at *p*-hydroxybenzyl alcohol or *p*-hydroxybenzoic acid formation (Herz 2000; Havkin-Frenkel and Belanger 2007).

The second important pathway from ferulic acid is a CoA-dependent oxidative chain-shortening enzyme catalyzing the degradation of ferulate to vanillic acid, but it has no biotechnological approach because it no lead to the desidered compound.

Vanillin biosynthesis in *Vanilla* species occurs in specialized cells, where vanillin is glycosylated and expelled from the cellular interior and accumulated in intercellular spaces around the seeds (Havkin-Frenkel *et al*. 2005). Because of the reactivity and toxicity of the carbonyl group in the vanillin or its glycosylated form, feeding vanilla tissue cultures with 3,4-dihydroxybenzaldehyde resulted in the accumulation of glycosylated vanillyl alcohol.

The control of the curing process can lead to produce beans containing 10% of vanillin. This means that 10 tons of purified vanillin can be obtained from 100 tons of cured beans. This product is economically competitive with biotechnologically produced vanillin and it can be labeled as natural.

The *in vitro* enzymatic degradation of C6–C3 compounds to C6–C1 products, such as the production of benzoic acids and aldehydes from hydroxycinnamic acids could explained the formation of vanillic acid by a non oxidative mechanism involving a hydrolyase activity coupled to hydration of the side-chain 2,3 double bond of 4-coumaric acid, with

subsequent cleavage of the side chain to yield acetate and 4-hydroxybenzaldehyde. Podstolski *et al*. in 2003 purified a chain-shortening enzyme from *V. planifolia*, which catalyzes the cleavage of coumaric acid to 4-hydroxybenzaldehyde. Vanilla tissue transform ferulic acid to vanillin in a similar way and cloning enzymes from this family represent a possible route for the biotechnological production of vanillin in cell-free extracts. The biotechnological process starting from ferulic and by using microorganisms acid is affected by several problems such as the degradation of the vanillin to vanillic acid or to vanillyl alcohol. In fact the enzymes that oxidize or reduce vanillin are non-specific and are difficult to control. Cell-free systems require co-factors and are too expensive.

A number of microorganisms, including gram-negative bacteria of the *Pseudomonas* genus, actinomycetes of the genera *Amycolatopsis* and *Streptomyces*, and the basidiomycete fungus *Pycnoporus cinnabarinus*, have been proposed for the production of vanillin from ferulic acid. In several ferulic-degrading microorganisms, ferulic acid is first activated to feruloyl-CoA by a feruloyl-CoA synthetase (encoded by *fcs* gene), and then the CoA thioester is subsequently hydrated and cleaved to vanillin and acetyl-CoA by a enoyl-CoA hydratase/aldolase (encoded by *ech* gene; Fig. 17). However, the vanillin produced from these microorganisms is either rapidly converted to other products or utilized by the microorganism as a source of carbon and energy. Recently, genetic engineering has been applied to produce vanillin from ferulic acid using metabolically engineered *Escherichia coli*. The latter is a non-native vanillin producer and needs to be transformed with the genes encoding for the bioconversion of ferulic acid to vanillin. A major drawback of *E. coli* vanillin-producing systems is the genetic instability of the recombinant strains that causes rapid declines in levels of vanillin production. Our studies demonstrated that these problems can be overcome by the use of integrative or low-copy number vectors. Resting cells of an *E. coli* strain (JM109/pBB1) harbouring a low-copy number plasmid including the ferulic catabolic genes from *Pseudomonas fluorescens* gave a final product concentration of 2.5 kg vanillin m^{-3} (1). The industrial strain was claimed to yield more than 10 kg m^{-3}. Other strategies that allow for increasing the production of vanillin in *E. coli* include isolation of vanillin resistant mutant and enhancement of acetyl-CoA consumption through TCA cycle with amplification of *gltA*, the gene encoding citrate synthase.

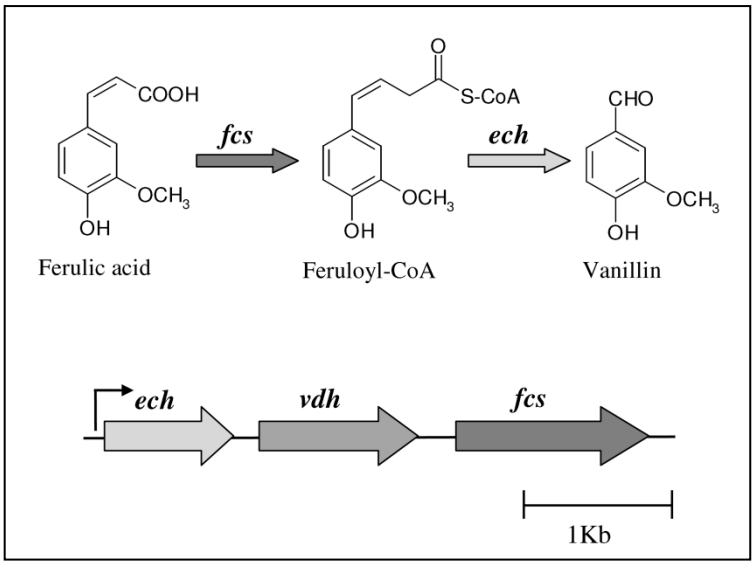


Fig. 17 Pathway for the biotransformation of ferulic acid to vanillin in Pseudomonas strains and organization of the ferulic catabolic genes. The genes encoding feruloyl-CoA synthetase (fcs) enoyl–CoA hydratase/aldolase (ech) and vanillin dehydrogenase (vdh) are organized in an operon.

Use of enzymes

Biotechnological production of vanillin can include could potentially be applied to clone genes for relevant enzymes that could be an *in vitro* enzyme-based system. The principle advantage is to avoid the vanillin conversion to vanillyl alcohol or vanillic acid, which often occurs when microorganisms are fed with precursors.

Vanillin can be released from vanillylamine in capsaicin. Van den Heuvel *et al.* (2001) used the flavoprotein vanillyl alcohol oxidase (VAO) from the ascomycete *Penicillium simplicissimum* to convert both creosol and vanillylamine to vanillin with high yield. For the creosol conversion the reactions proceeds via a two-step process in which the initially formed vanillyl alcohol is further oxidized to vanillin. The problem correlated to this route is the low amount of capsaicin in pepper or other plant sources. Creosol, also found to be converted to vanillin by the same enzyme, is a major component obtained from heating wood or coal tar but may not be considered a natural precursor.

Beta-glucosidase, can be used to improve the yield of vanillin because catalyzes the hydrolytic release of vanillin from glucovanillin, the natural parent compound that accumulates in vanilla beans. Commercial enzyme preparations from almonds can be used to increase the production of vanillin in curing beans (Havkin-Frenkel *et al*. 2005; Dignum *et al*. 2001).

Lignostilbene-dioxygenase (EC 1.13.11.43), from *Pseudomonas* sp. TMY1009, catalyze the oxidative release of vanillin from stilbenes, found in wood bark (Kamoda *et al*. 1989). Synthetic enzymes, produced by DNA cloning were used for the production of coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, and vanillic acid (Markus *et al*. 1992).

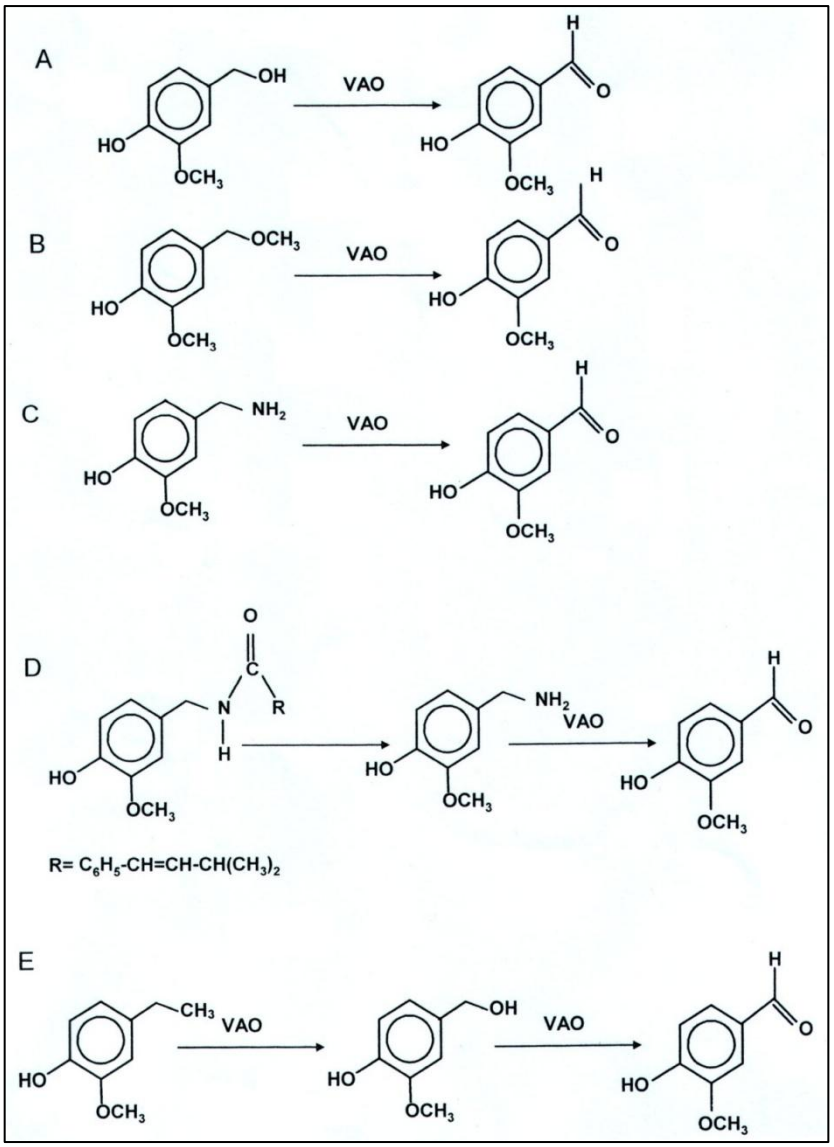


Fig.18 Enzymatic production of vanillin from natural feedstocks: A Vanyllil alchol; B. 2-methoxy (4-methoxymethyl) phenol; C Vanyllil amine; D Capsaicin; E Creosol; VAO Vanillyl alcohol oxidase.

Penicillin V acylase

Penicillin acylases (EC 3.5.1.11) are produced by a wide range of microorganisms, including bacteria, yeasts, and fungi and generally catalyze the hydrolysis of the side amide bonds in β-lactam compounds like as penicillin G (Pen G), penicillin V (Pen V), and ampicillin. In particular, penicillin G acylase (PGA) and penicillin V acylase (PVA) hydrolyze Pen G and Pen V specifically, producing 6-aminopenicillanic acid (6-APA), whose commercial importance in industrial synthesis of various semi-synthetic penicillins has led to the development of penicillin amidase research and application. Their high efficiency has resulted in the replacement of conventional chemical process in favour of enzymatic ones by the industry. Penicillin acylase have been categorized as β-lactam acylase. This kind of enzymes are generally characterized as an N-terminal-nucleophile (Ntn) hydrolase superfamily, which is composed of enzymes that share a common fold around the active site and contain a catalytic serine, cysteine, or threonine residue at the N-terminal end (Brannigan et al., 1995). These enzyme are initially produced in the cytoplasm of the cells as a single-chain precursor with four distinct segments (signal sequence, small (α) subunit, linker peptide, and large (β) subunit. After the removal of several polypeptides through posttranslational autocatalytic processes, the enzymes are then converted to the mature form of a heterodimer composed of an α subunit and a β subunit in the cell periplasm (Kasche etal., 1999; Shizmann et al., 1990; Kim and Kim, 2001), (fig.20). *S. mobaraensis* has been shown to produce a capsaicin-hydrolysing acylase, (*Sm*-PVA) that is secreted in the culture medium (Koreishi et al. 2006).

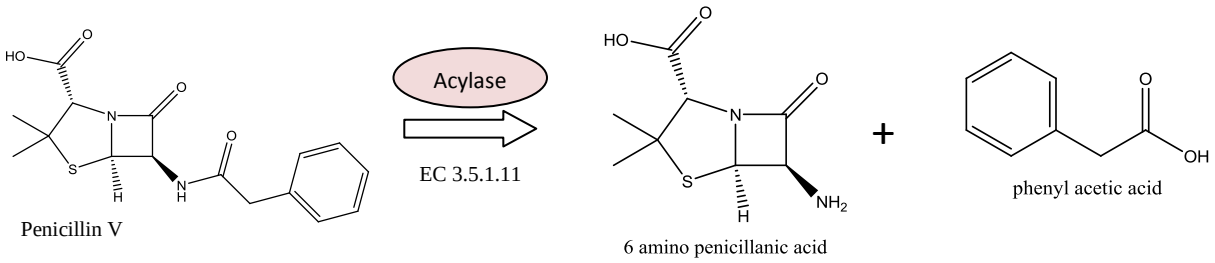
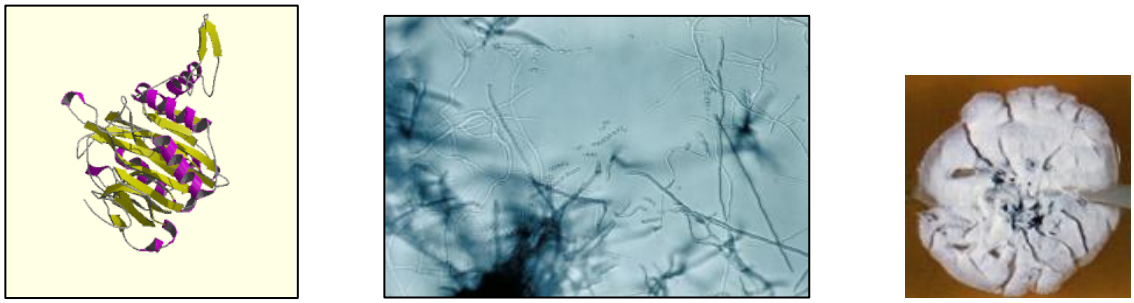


Fig.19 Penicillin hydrolysis is catalized from PV Acylase.



1 2 3

Fig. 20 Penicillin V Acylase structure (1); Streptomyces mobaraensis (2, 3).

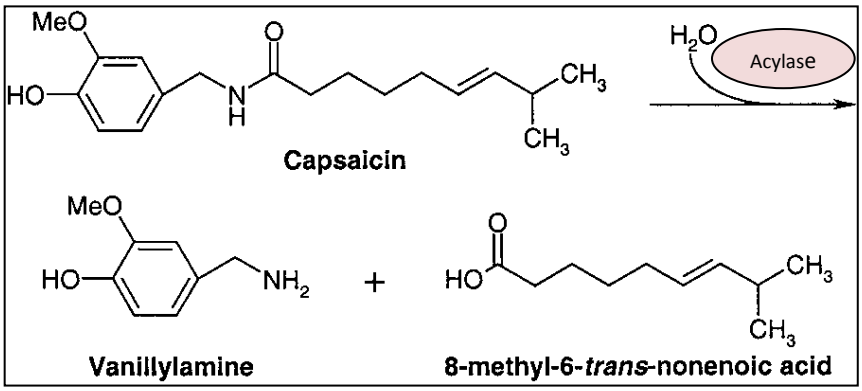


Fig. 21 Enzymatic hydrolysis of capsaicin

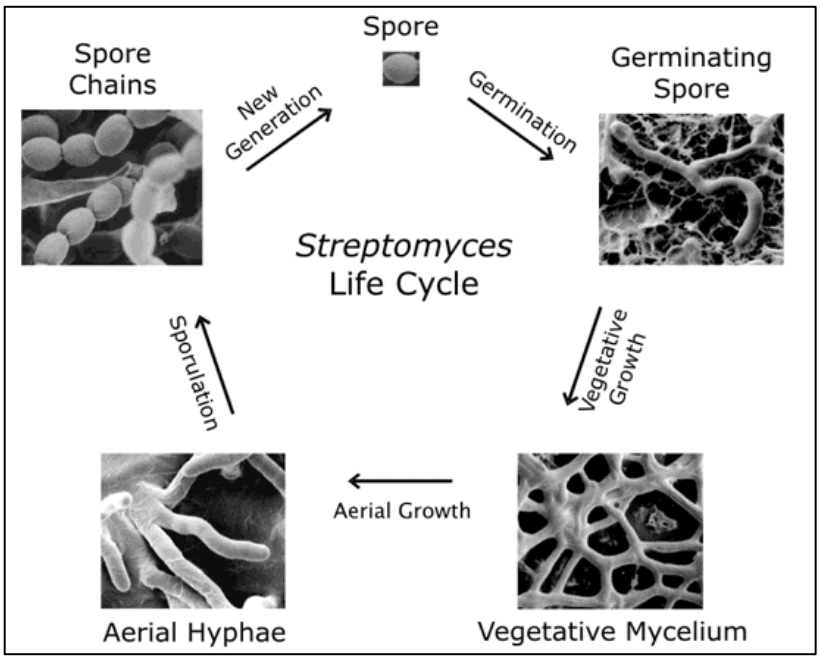


Fig.22 Streptomyces life cycle

Recovery of vanillin from acqueous solutions

Recovery of the product plays a fundamental role in a biotechnological process. The recovery of vanillin from bioconversion broth is influenced by several problems.

Bacterial cells were affected by the presence of vanillin because of its toxic effect. During fermentation vanillin can be transformed into unwanted products such as vanillyl alcohol or vanillic acid with the higher chemical reactivity and toxicity.

Pervaporation technique was studied by K.W. Boddeker *et al*. in 1997 (61), but the low vanillin volatility at the bioconversion temperature represented an important disadvantage.

The in situ recovery of vanillin can improve the productivity of the biotechnological process. Macroporous resins can be used to adsorb vanillin and reduce its concentration in the fermentation liquor. In large-scale separation process from aqueous solutions adsorption resins are usually employed. They were used for recovering aromatic compound pollutants such as aniline (63), naphthalene derivatives (64), phenolic compounds (67–69), salicylic acid and p-hydroxybenzoic acid (70) from wastewater but also erythromycin (71), red pigment (72), catechol (73) and licorice flavonoids and glycyrrhizic acid from fermentation liquor or water.

Hua et al. in 2007 demonstrated that the addition of adsorbent resins to the culture medium during the biotransformation of ferulic acid to vanillin by a *Streptomyces sp*. strain in a fed-batch process increased the vanillin yield. The resins have no functional groups and the adsorption phenomenon is based on van der Waals forces, which forms weak bonds with molecules (74) and on the large surface areas. The reversibility of the adsorption process, allows the easy recovery of the wanted products and the regeneration of resins by washing with common organic solvents such as like ethanol.

The use of solvent extraction for the recovery of bio-products has been well documented (75,76).

Solvents can allow the selective recovery of the produced vanillin from the bioconversion medium without removing the substrate. This operation cannot be performed in the presence of the living cells because of the solvent toxicity. In order to solve this problem the membrane based, dispersion-free, solvent extraction technique can be used to recover vanillin from dilute aqueous solutions (77).

In particular, we investigated the performance of adsorption-regeneration techniques, using macroporous resins with cross linked-polystyrene framework or active carbon powder and liquid/liquid extraction with *n* butyl-acetate and ethyl-acetate.

Amberlite XAD-4® Resin

It's a macroporous resin with cross linked-polystyrene framework, and derives its adsorptive properties from its patented macroreticoular structure containing both a continuous polymer phase and continuous pore phase, high surface area and the aromatic nature of its surface. These characteristics gives resin physical-chemical and thermal stability and its pore size distribution allow the recovery of organic hydrophobic substances at low molecular weight from polar solvents.

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

Table 8 XAD-4® Resin properties

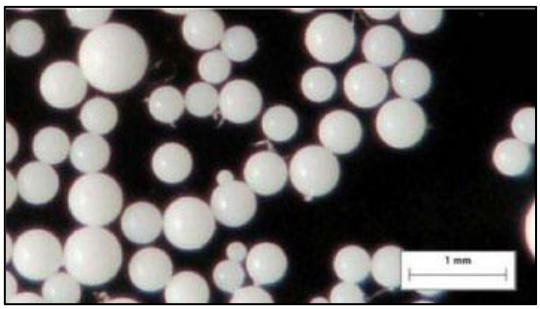


Fig. 22 XAD-4® resin particles

Regulations

US and Europe, are considered the principal producers and users of flavors in manufactured products. Their legislation influence the use of natural or synthetic flavors.
In the USA, in 1965 a positive list of flavoring substances was adopted. The parameter used for the classification as GRAS (generally recognized as safe) are: long usage without toxicity or by undertaking the safety evaluation of novel flavoring ingredients. The only alternative designations for the consumers are "natural flavor" and "artificial flavor".
According to the US FDA (Food and Drug Administration), regulation CFR 21, the only vanillin that can be labeled as 'natural vanillin' is vanillin that comes from vanilla beans. That obtained from a natural source and by a natural process, can be classified as 'natural flavor' in a non-vanilla product. In Europe the regulatory body is different and, follows principally the direction taken by the French regulatory authority (the DGCCRF). The latter ruled that natural vanillin must have an isotopic deviation equal to or greater than –21.2 0/00 PDB. The Regulation (EC) no 1334/2008 of the European Parliament and of the Council of 16 December 2008 established criteria and properties on flavourings and certain food ingredients with flavouring properties for use in and on foods. It'll be into force on 20th January in 2011 and provides for:

- community list of flavourings and source materials approved for use in and on foods;
- conditions of use of flavourings and food ingredients with flavouring properties in and on foods;
- rules on the labeling of flavourings.

In according to this regulation," ***flavourings*** are not intended to be consumed as such, which are added to food in order to impart or modify odour and/or taste and are made or consisting of the following categories: flavouring substances, flavouring preparations, thermal process flavourings, smoke flavourings, flavour precursors orother flavourings or mixtures thereof ".
Flavouring substance is defined chemical substance with flavouring properties while ***natural flavouring substance*** is flavouring substance obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes. ***Natural flavourings*** correspond to substances that are naturally present and have been identified in nature. Vanillin

produced in biotechnological processes starting from natural substrates can be classified as natural flavouring on condition that the natural starting material is specified.

AIMS OF THE THESIS

Aim of the thesis

In recent years a large number of studies have been made on natural vanillin biosynthesis using microorganisms or isolated enzymes. However, these bioconversions are not yet economically feasible. Van den Heuvel *et al*., (2001) described the process to obtain vanillin from capsaicin using a bi-enzymatic process with mammalian and bacterial enzymes. The enzymes used are too expensive and the process is not competitive from an economic point of view. The discovery of microbial acylases that efficiently hydrolyze capsaicin provides a valuable opportunity to develop a cost-effective process for enzymatic synthesis of vanillin.

Previously studies on the biotechnological production of vanillin using resting cells of *E.coli* engineering strains demonstrated how the high chemical activity and toxicity of vanillin caused low yield from ferulic acid. Moreover high concentration of ferulic acid had a negative effect on the cells; Barghini *et al*. in 2007 demonstrated that the pulse additions of ferulic acid could determine a further increase of the final vanillin production.

The aims of this thesis were: to enhance vanillin production from *E. coli* engineering strains (resting cells) starting from ferulic acid and by using XAD-4® resin and by a new two/phase system of agarose gels and ferulic acid for the controlled release of the substrate in the bioconversion medium (1); to evaluate different strategies for enhancing the production of the capsaicin acylase from *Streptomyces mobaraensis* DSM40847 strain (2), to identify optimal conditions for capsaicin hydrolysis by using the acylase from *Streptomyces mobaraensis* DSM40847 strain (3), to develop efficient procedures for the recovery of vanillin from diluted aqueous solutions (4).

MATERIALS AND METHODS

Bioconversion of ferulic acid to vanillin by using *Escherichia coli* resting cells.

Bioconversion of ferulic acid to vanillin was carried out in phosphate saline buffer (M9), at different pH values 6.80 and 9.00, using resting cells of *E. coli* JM109(pBB1) and FR13 strain, Cells were grown in LB medium (Composition per liter: tryptone 10 g; NaCl 5 g; yeast extract 5g) containing kanamicin (25 μg/ml; FR13 strain) or ampicillin (50 μg/ml; JM109(pBB1) strain). Conversion of ferulic acid to vanillin and was monitored by HPLC (as decribed below).

Release of ferulic acid from agarose gels plugs

In this work a two phase (solid-liquid) system for the modulated release of ferulic acid in the liquid phase was developed. For this purpose ferulic acid was entrapped in gels containing different concentrations of agarose. All gels were prepared by dissolving agarose powder at 2% in 15 ml of M9 phosphate buffer by boiling 2-3 minutes in a microwave oven. The agarose solutions were mixed and cooled at 50°C prior adding ferulic acid. The solutions were placed in 100 ml flasks and cooled at room temperature for 24 hours in static conditions. The diffusion process was studied employing the response surface methodology. The two parameters of concentration were entered into the design Modde 5, which generated a full-factorial screening design with a total of 12 experiments including three triplicate centre points. The amount of ferulic acid released in the bioconversion medium was the response studied using central composite design (CCD). Range of variation of concentration of ferulic acid was set from 4 to 5 mg/ml and that of agarose in the gels varied from 1.7 to 1.8 %.

In situ recovery of vanillin using XAD-4® resin.

Bioconversion experiments using *E.coli* FR13 strain, were carried out emending the bioconversion medium with XAD-4® resin. The resin was first soaked with ethanol for 24 h, 2% hydrochloric acid (v/v) and 2% (w/v) sodium hydroxide for 2 h, respectively. Conversion of ferulic acid to vanillin and hydrolysis of capsaicin were monitored by HPLC

Production of acylase active on capsaicin from *Streptomyces mobaraensis* DSM40847 strain and optimization

Microorganism cultivation and culture media

The acylase utilised for the conversion of capsaicin to vanillylamine was produced by *Streptomyces mobaraensis* DSM40847 strain. Cultivation was carried out at 28°C in shaken Erlenmeyer flasks (120 rpm), or in STR-type reactor (aeration rate, 1 vol/vol min-1; stirrer speed, 450 rpm). DSM40847 strain was grown on medium containing: glucose (10.0 g/L), dextrin (10.0 g/L), N-z amine (5.0 g/L), yeast extract (5.0 g/L), $CaCO_3$ (1.0 g/L). For capsaicin acylase production, the strain was grown on the following media

Medium MA: soluble starch (40g/L), polypeptone (20.0 g/L), beef extract (40.0 g/L), $MgSO_4$ (20.0 g/L), K_2HPO_4 (2.0 g/L);

Medium MTG: glucose (5 g/L), soy bean meal (10 g/L), beef extract (4 g/L), peptone (4 g/L), yeast extract (1 g/L), $MgSO_4$ (20.0 g/L), K_2HPO_4 (2.0 g/L);

Medium MTA: soluble starch (40g/L), soy bean meal (10 g/L), beef extract (4 g/L), peptone (4 g/L), yeast extract (1 g/L), $MgSO_4$ (20.0 g/L), K_2HPO_4 (2.0 g/L);

Medium MG: glucose (5 g/L), polypeptone (20.0 g/L), beef extract (40.0 g/L), $MgSO_4$ (20.0 g/L), K_2HPO_4 (2.0 g/L);

Medium C: glucose (20.0 g/L), yeast extract (5.0 g/L), asparagine (1.5 g/L), $CaCO_3$ (5.0 g/L), NaCl (1.0 g/L), $MgSO_4 \times 7\ H_2O$ (0.5 g/L), $CaCl_2 \times 2H_2O$ (0.1 g/L), 1 ml of mineral solution (boric acid 0.5 g/L; $CuSO_4 \times 5\ H_2O$ 0.04 g/L; KI 0.1 g/L; $FeCl_3 \times 6\ H_2O$ 0.2 g/L; $MnSO_4 \times H_2O$ 0.4 g/L; $FeSO_4 \times 7\ H_2O$ 0.4 g/L; ammonium molybdate 0.2 g/L);

Medium S/BIS: glucose (10.0 g/L), peptone (4.0 g/L), yeast extract (4.0 g/L), $MgSO_4 \times 7\ H_2O$ (0.5 g/L), K_2HPO_4 (4.0 g/L).

Medium M8: meat extract (2.0 $g \cdot l^{-1}$), yeast extract (2.0 $g \cdot l^{-1}$), casein hydrolysate (4.0 g/L), glucose (10.0 g/L), soluble starch (20.0 g/L), $CaCO_3$ (3.0 g/L).

AF/Ms: glucose (20.0 g/L), soybean meal (8.0 g/L),yeast extract (2.0 g/L), NaCl (1.0 g/L).

AUR/M: maltose (20.0 g/L), dextrin (10.0 g/L), yeast extract (2.0 g/L), meat extract (4.0 g/L), peptone (4.0 g/L), soybean meal (15.0 g/L),$CaCO_3$ (2.0 g/L).

Medium T: glucose (5.0 g/L), meat extract (4.0 g/L),yeast extract (1.0 g/L), peptone (4.0 g/L), soybean meal (10.0 g/L), $CaCO_3$ (1.0 g/L), NaCl (2.5 g/L).

Batch fermentation were carried out in duplicate in Erlenmeyer flasks on an orbital shaker at 180 rpm or in stirrer tank reactors Applikon (Schiedam, The Netherlands) and in air lift

reactors, unless indicate otherwise, under the following conditions: incubation temperature 30°C, aeration rate, 1vol/vol min^{-1}; stirrer speed, 450 rpm; silicone antifoam 1ml/L.

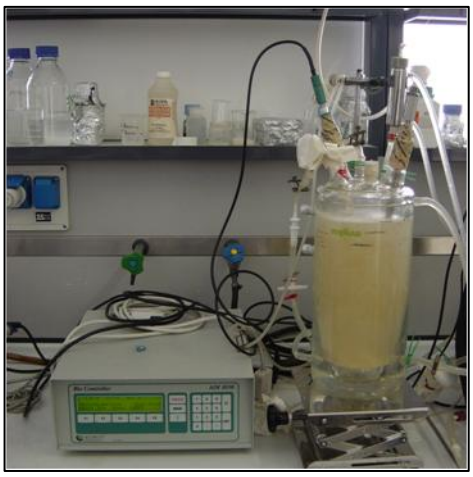


Fig.23 CSTR reactor and Air lift reactor used in this study.

Determination of enzymatic activity

Acylase activity was determined either by a spectrophotometric method, (Varian Cary 50 MPR), using a coupled enzymatic reaction with DAO, (diamine oxidase EC 1.4.3.6) and POD, (peroxidase EC 1.11.1.7) or by a specifically developed HPLC method using capsaicin (130 mM) as substrate. In the latter case the consumption of the substrate and the production of vanillylamine were monitored. HPLC reverse phase system was equipped with a C-18 column (250 x 14,6 mm I.D; S-5µm) and UV detection at 235 nm. The mobile phase was composed of acetonitrile and phosphate buffer pH 8 (1:1). In adsorption and liquid/liquid extraction experiments vanillin and ferulic acid concentrations were measured by HPLC analysis using a C-18 column (50x 2 mm I.D; S-2,5µm) and UV detection at 235 nm. The mobile phase was composed of water and methanol with 1% acetic acid (1:1).

Preparation of the crude enzymatic extract

Enzymatic raw preparations used in capsaicin hydrolysis reactions were obtained from fermentation broth of *Streptomyces mobaraensis* DSM40847 strain in the following way: ammonium sulfate at 65% was added to the broth recovered after filtration. After 10 minutes at 4°C, the sample was centrifugated at 4000 rpm for 15 minutes , and the supernatant was removed. An isovolume of Tris HCl 50 mM a pH 7.80 and ammonium sulfate at 65% was added. After centrifugation at 4000 rpm for 15 min the supernatant was removed and the sample was concentrated in 1/10 of initial volume of Tris/HCl 50 mM, pH 7.80.

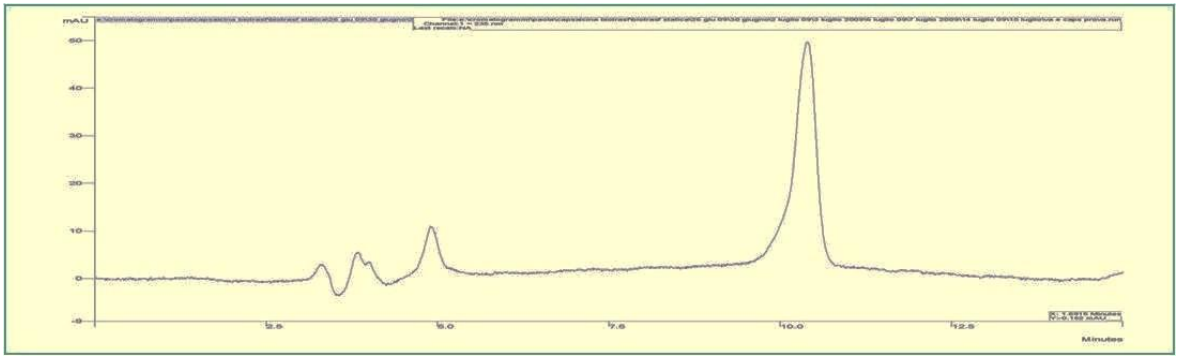


Fig.24 Chromatogram (vanillylamine 5.2 minutes and capsaicin 10.3 minutes)

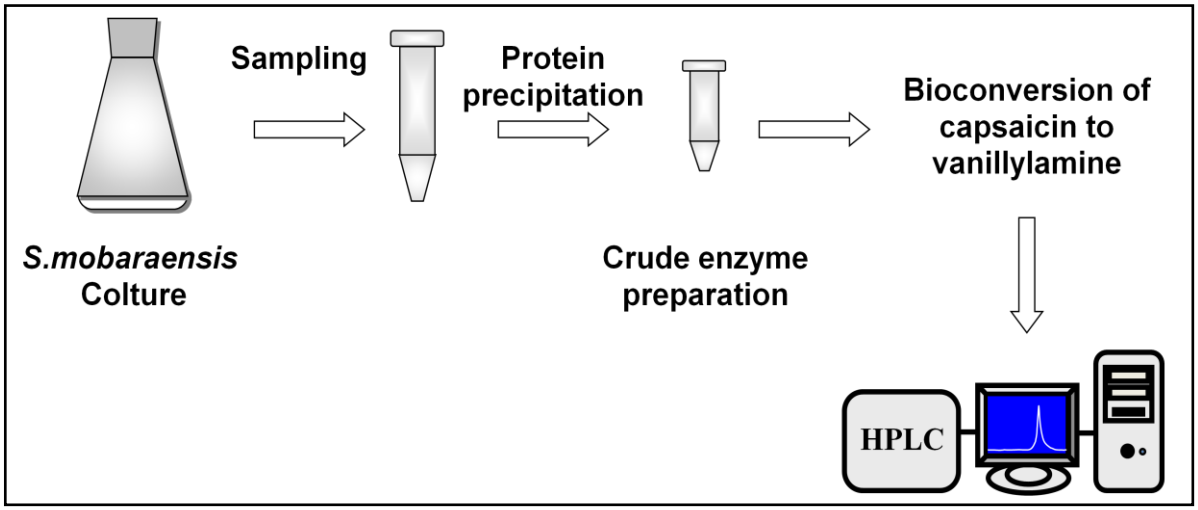


Fig. 25 Experimental strategy

Recovery of vanillin from acqueous solutions

Adsorption experiments

Analytical reagent grade of vanillin, ferulic acid and XAD-4® macroporous adsorption resin with crosslinked styrene-divinylbenzene framework were purchased from Sigma®. The physical properties of the resin were listed in Table 8. The resin was first soaked with ethanol for 24 h, 2% hydrochloric acid (v/v) and 2% (w/v) sodium hydroxide for 2 h, respectively. Active coal was washed in M9 buffer with a volume ratio 1:2 for 24h at 37°C in shaken tubes. Finally, the resin and active coal were washed to neutral with deionized water and dried at 80 °C.

Quantification of vanillin and ferulic acid in the liquid phase was determined by HPLC chromatography. The HPLC reverse phase system was equipped with a C-18 column (250 x 14,6 mm I.D; S-5μm) and UV detection at 235 nm. The mobile phase was composed of acetonitrile and phosphate buffer pH 8 (1:1). In adsorption and liquid/liquid extraction experiments vanillin and ferulic acid concentrations were measured by HPLC analysis using a C-18 column (50x 2 mm I.D; S-2,5μm) and UV detection at 235 nm. The mobile phase was composed of water and methanol with 1% acetic acid (1:1).

Kinetic studies

The kinetic studies were carried out at 30°C, with resin or active coal in solutions of vanillin in M9 buffer. The solutions were shaken at 180 rpm. An aliquot of the mixture at 15-min intervals was taken and the content of residual vanillin and ferulic acid in the supernatant solution was monitored by HPLC analysis.

Static adsorption equilibrium

A set of adsorption tests in mixtures of vanillin and ferulic acid, have been performed at different pH value in the range 4.00-11.50, and at different temperature with resin at 0.1 g/ml. Adsorption experiments in vanillin solution with active coal at different concentration, have been carried out at 30°C and at pH 5.50 or 7.50.

The mixtures were shaken at 180 rpm, for 2 hours to reach the equilibrium, and the amount of remaining vanillin and ferulic acid in the supernatant solution were determined by HPLC analysis.

Column adsorption studies

A glass column with internal diameter of 0.5 cm and length of 10 cm was used. The adsorbent resin was supported in the column over a glass wool plug. The amount of 0.2 g of resin was filled into the column. A total of 50 ml of vanillin solution at 0.6 mg/ml and pH 7.50 or 5.00 was fed to the column. The experiments were carried out at room temperature The flow rate of liquor was kept constant at 2 ml/min, in over night. An aliquot of the effluent solutions were collected at the end of adsorption and analyzed for the vanillin content.

Liquid/liquid extraction by using *n*-butyl acetate

Liquid/liquid extraction experiments with *n*-butyl acetate were carried out with mixture of vanillin, (0.5 mg/ml) and ferulic acid (0.5 mg/ml), at 20°C with a total volume ratio of solution and organic solvent of ½ in a double extraction process.

Chemicals

All chemicals and HPLC solvents were of the highest purity commercially available and were purchased from Sigma Aldrich®.

RESULTS AND DISCUSSION

In recent years a large number of studies have been made on natural vanillin biosynthesis using microorganisms or isolated enzymes. However, these bioconversions are not yet economically feasible.

The first part of the section reports the results of the experiments carried out to optimize the bioconversion process from ferulic acid using resting cells of *E.coli* engeneering strains. These cells are able to convert ferulic acid into vanillin thanks to the presence of the genes that codify the key enzymes for the bioconversion. Vanillin and ferulic acid have toxic effects on the bacterial cells and this caused low yield in vanillin and the production of unwanted products such as vanillyl alcohol or vanillic acid. In order to control the release of ferulic acid in the bioconversion medium an innovative two phase system of agarose and substrate was developed. The in situ recovery of vanillin from bioconversion medium using XAD-4® resin and the possibility to carry out the bioconversion at pH values, at which the highest selectivity of the resin was obtained, have been evaluated.

The second part of this section reports the results obtained from the experiments of optimization of the production of vanillylamine starting from capsaicin and using an extracellular acylase, (*Sm*-PVA) from *S. mobaraensis* DSM40847 strain. The vanillin production from capsaicin as natural source using a bi-enzymatic process (with mammalian and bacterial enzymes) has been previously described (van den Heuvel *et al*., 2001), but it was never translated into a commercial process because the mammalian enzyme used to obtain vanillylamine was too expensive. The discovery of microbial acylases that efficiently hydrolyze capsaicin provides a valuable opportunity to develop a cost-effective process for enzymatic synthesis of vanillin. In particular we studied the production of this enzyme under batch fermentation conditions in stirred tank (STR) and airlift (AR) bioreactors; the effect of the composition media, the agitation speed, the dissolved oxygen tension on acylase production was examined. The optimal conditions for capsaicin hydrolysis by using the acylase from *Streptomyces mobaraensis* DSM40847 strain have been set up.

The third part of the section contains the results of the developing of efficient procedures for the recovery of vanillin from diluted aqueous solutions. The final bioconversion media contain low amount of vanillin and sometimes residual ferulic acid that could be re-utilised in an off-line system. In order to develop a cost-effective process for the biotechnological production of vanillin, the performance of different extraction procedures on the recovery of vanillin and ferulic acid from diluted aqueous solutions were evaluated. In particular we investigated the performance of: adsorption-regeneration techniques, using macroporous

resins with cross linked-polystyrene framework or active carbon powder and liquid-liquid extraction techniques, with *n*-butyl acetate.

Production of Vanillin from Ferulic Acid using Escherichia coli resting cells

Production of vanillin at alkaline pH

In order to develop an in situ process for the recovery of vanillin using XAD-4® resin, it was evaluated the possibility to carry out the bioconversion experiments at pH values at which the resin exhibited higher selectivity (i.e. pH=9.00). The results, reported in Fig. 26, showed that *E. coli* FR13 cells efficiently convert ferulic acid to vanillin when bioconversion experiments are performed at pH=9.00.

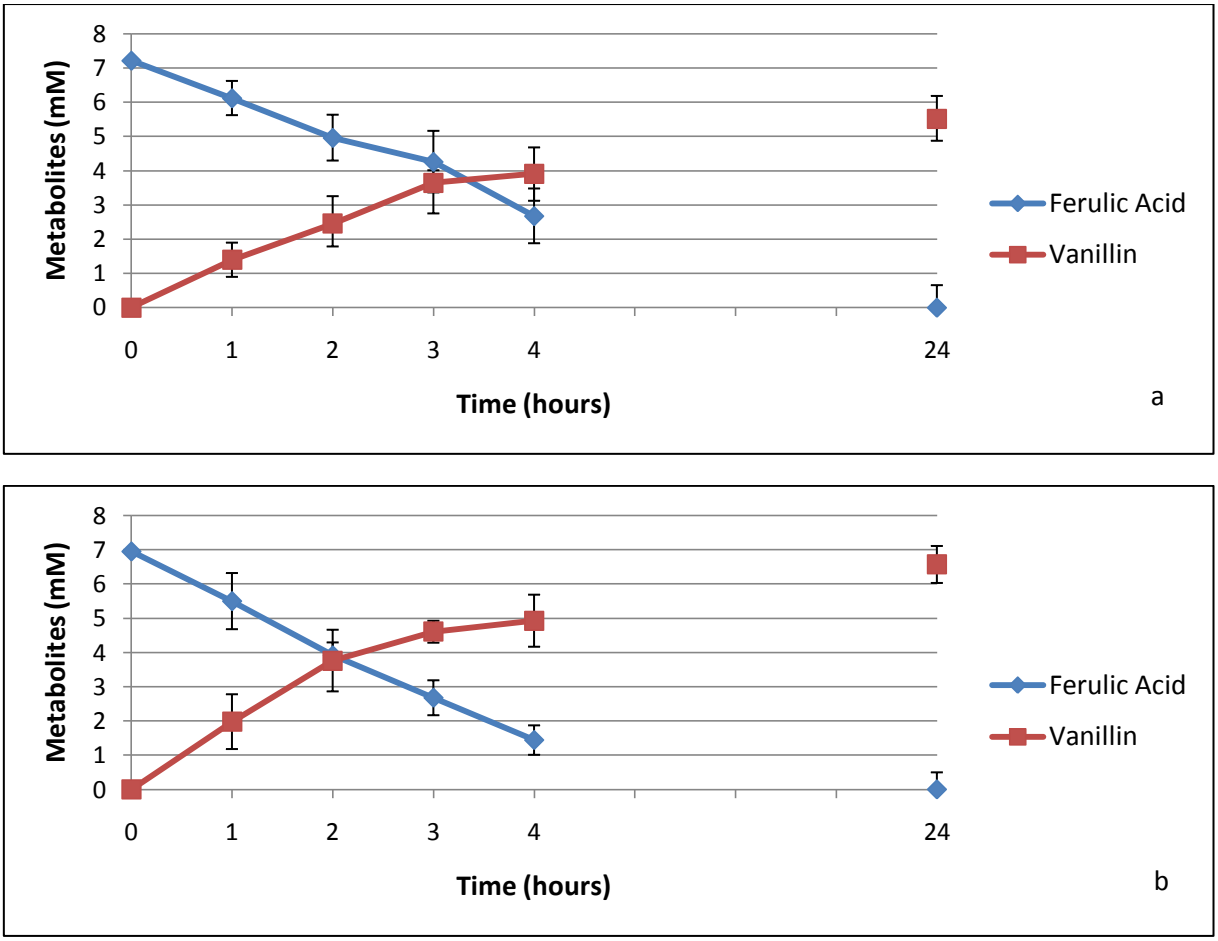


Figure 26 Bioconversion of ferulic acid to vanillin using E.coli FR13.
Experiments were carried out at 30°C at pH 6.80 (a) and pH 9.00 (b). Error bars are standard deviations.

The charge and the arrangement of the molecules of ferulic acid and vanillin depends on the pH. At pH 9.00, 95% of the molecules of vanillin and the totality of those of ferulic acid are deprotonated and, therefore, negatively charged. The hydrophobic nature of the fatty acid tails of phospholipids of the plasma membrane makes the diffusion of polar

molecules and charged ions difficult. They can be transported across membranes with consumption of energy.

The process occurs with a neutral electronic balance in which one negatively charged molecule of ferulic acid enters into the cell and one negatively charged molecule of vanillin goes out, without altering the pH inside the cell and avoiding further oxidation/reduction of vanillin.

In situ recovery of vanillin using XAD-4® resin.

Preliminary bioconversion experiments carried out using *E.coli* FR13 cells incubated in phosphate saline buffer containing XAD-4® resin indicated (Fig. 27) that the presence of the resin into the bioconversion buffer has a negative effect on the production of vanillin. In the presence of the resin, only about one third of the substrate was consumed and a strong reduction in the ferulic-to-vanillin conversion rate was observed after a 24-h incubation.

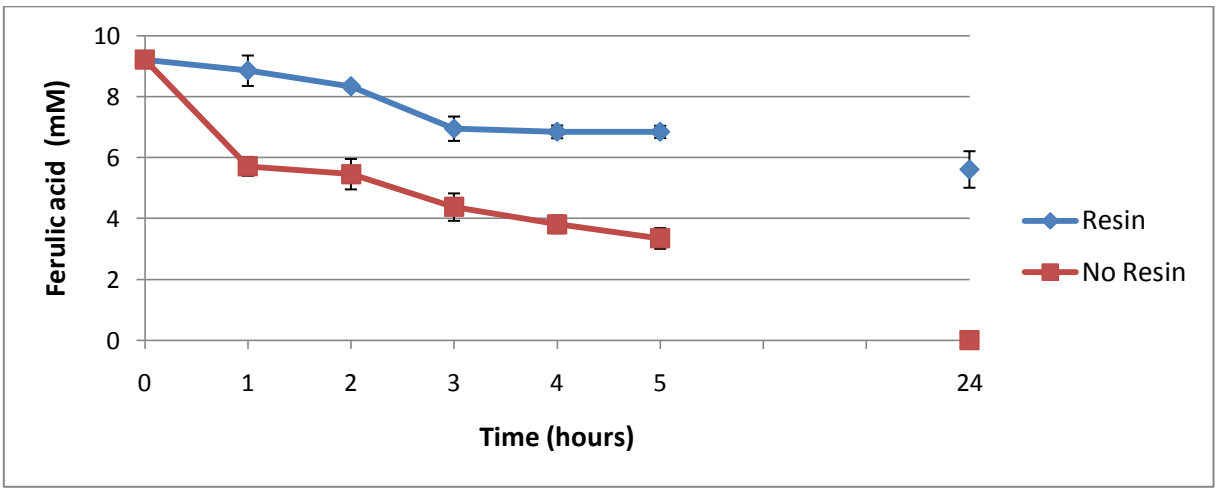


Figure 27 Bioconversion of ferulic acid to vanillin.

Experiments were carried out at 30°C using *E.coli* FR13. Bioconversion buffer was emended with XAD-4® resin at 0.1 g/ml (blue line). Error bars are standard deviations.

Release of ferulic acid from agarose gels plugs

Diffusion experiments

In this study was developed a two phase system formed by a matrix of agarose in which the ferulic acid was entrapped. The matrix enables the diffusion of the substrate in the bioconversion medium. At high concentration ferulic acid could be toxic for the cells and in order to obtain a controlled release of ferulic acid during the bioconversion, a set of experiments of diffusion at 30°C were carried out. Gels plugs at different concentration of agarose and of ferulic acid, were used in diffusion experiments in M9 buffer to evaluate the effect of the matrix structure on the release of the substrate. The concentration of the ferulic acid released in the medium increased with the amount of the acid in the gels plugs (fig. 30), while decreased with the percentage of agarose (fig. 31).

In gels, diffusion phenomena are explained by different models essentially linked to the nature of the diffusing macromolecule (rigid or flexible). The agarose matrix obtained after the ageing process entrapped the ferulic acid molecules. In order to evaluate the influence of the contact surface of the two phase liquid/gel on the diffusion of the ferulic acid through the agarose structure, experiments were carried out using different volumes of gels in the range from 10 to 25 ml. The results reported in the figure 32 suggested that the contact surface has not a relevant effect in the first hours of diffusion, while the difference in ferulic acid concentrations after 18 hours, when equilibrium was reached, were reasonably sensibile.

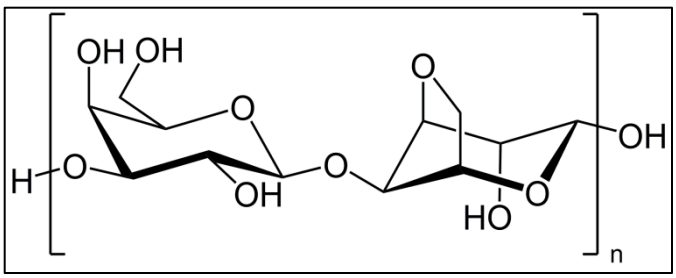


Fig.28 Agarose monomer

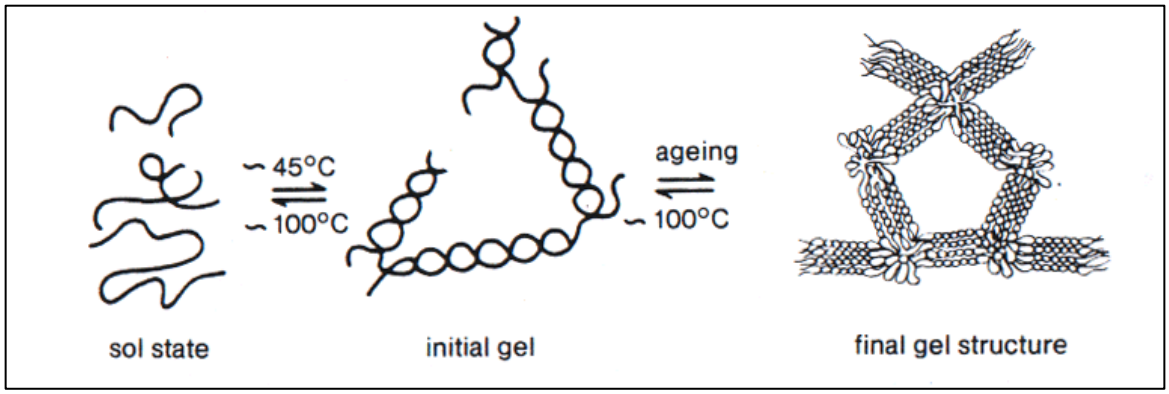


Fig. 29 Agarose gel structure

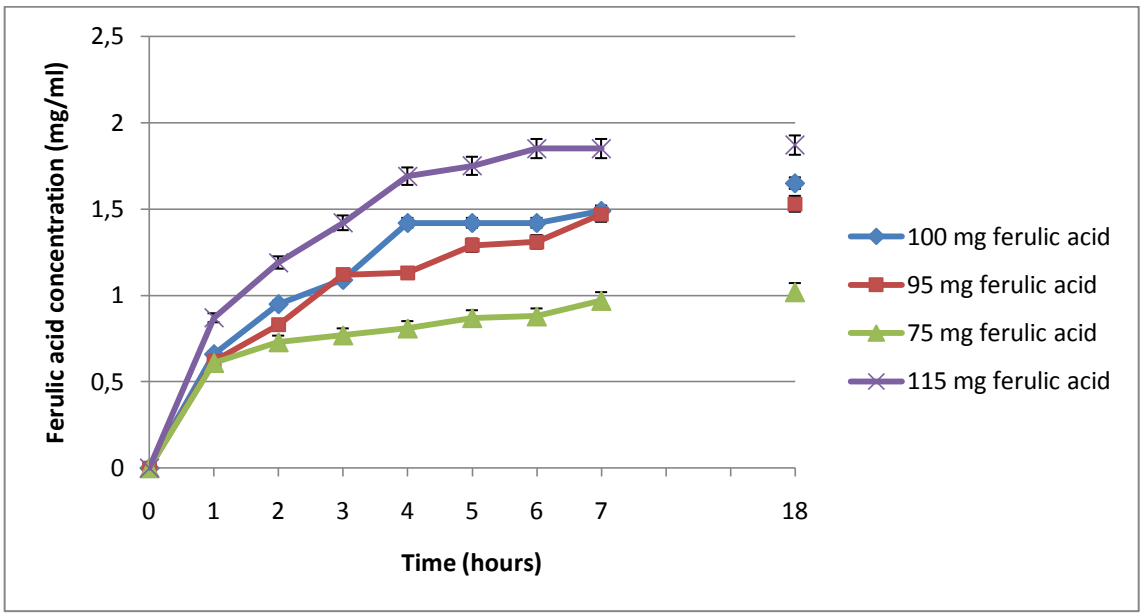


Fig. 30 Diffusion of ferulic acid from agarose gels plugs.

Experiments were carried out at 30°C using gels at 1.5 % of agarose and at different ferulic acid concentration. Error bars are standard deviations.

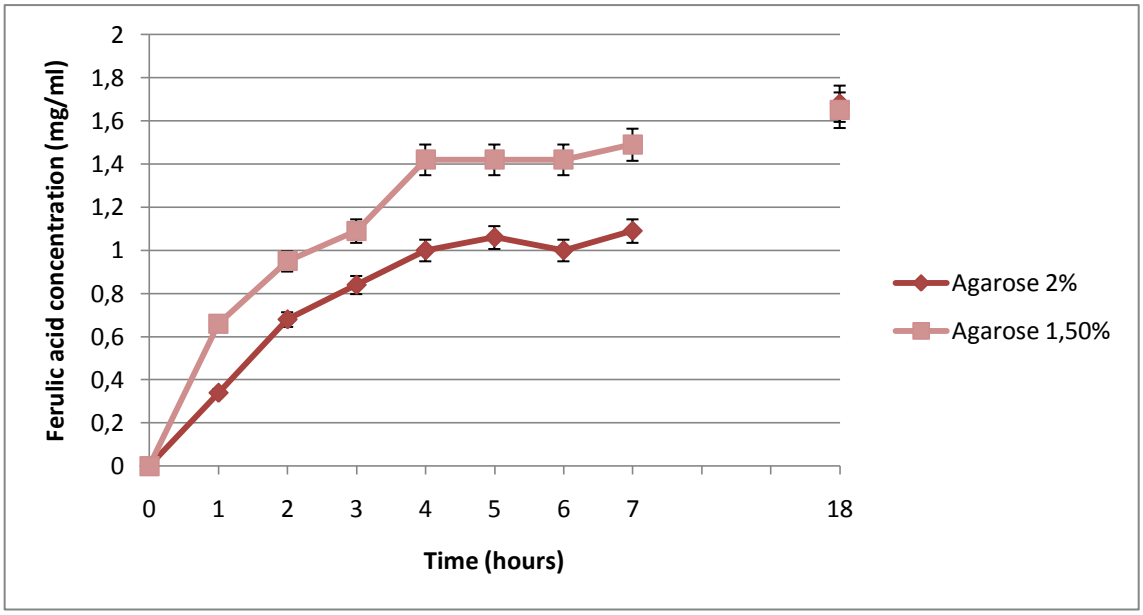


Fig. 31 Diffusion of ferulic acid from agarose gels plugs.

Experiments were carried out at 30°C using 15 ml of gel plug at different concentration of agarose and with ferulic acid at 4.5 mg /ml. Error bars are standard deviations.

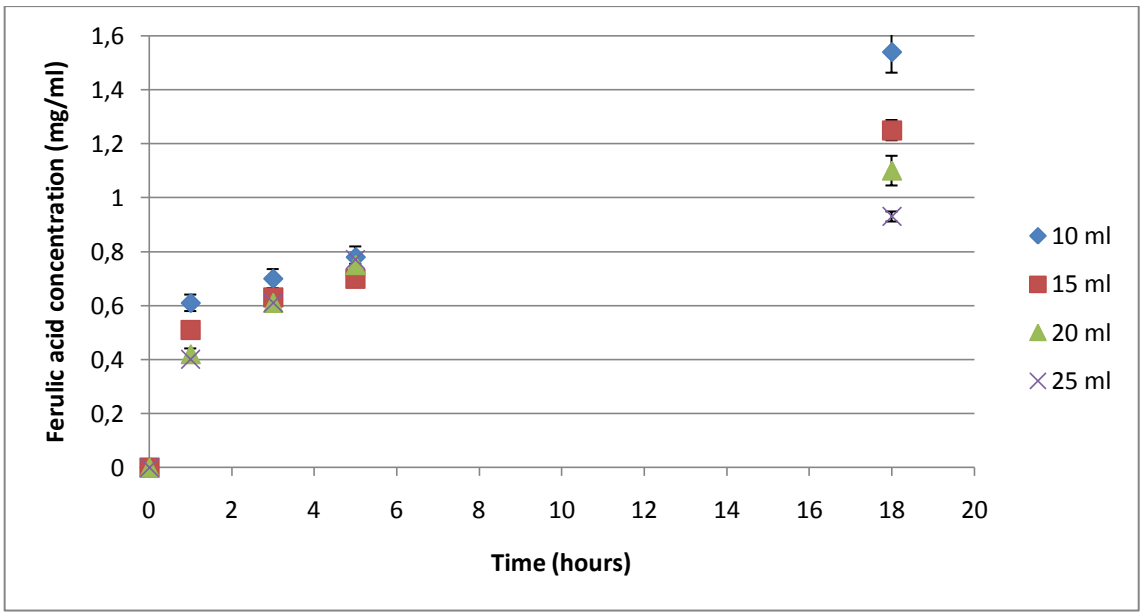


Fig. 32 Diffusion of ferulic acid from agarose gels plugs.

Experiments were carried out at 30°C using different volumes of gels plugs containing 4 mg /ml of ferulic acid. Error bars are standard deviations.

To evaluate the influence of the volume of the liquid phase on the diffusion process, using the two phase system developed, experiments were carried setting up volumes in a range of variation from 20 to 40 ml of bioconversion buffer. The results obtained suggested that the diffusion of the ferulic acid in the medium was not influenced by the volume of the liquid phase during the first two hours while became more relevant in the rest of the diffusion experiments (fig.33).

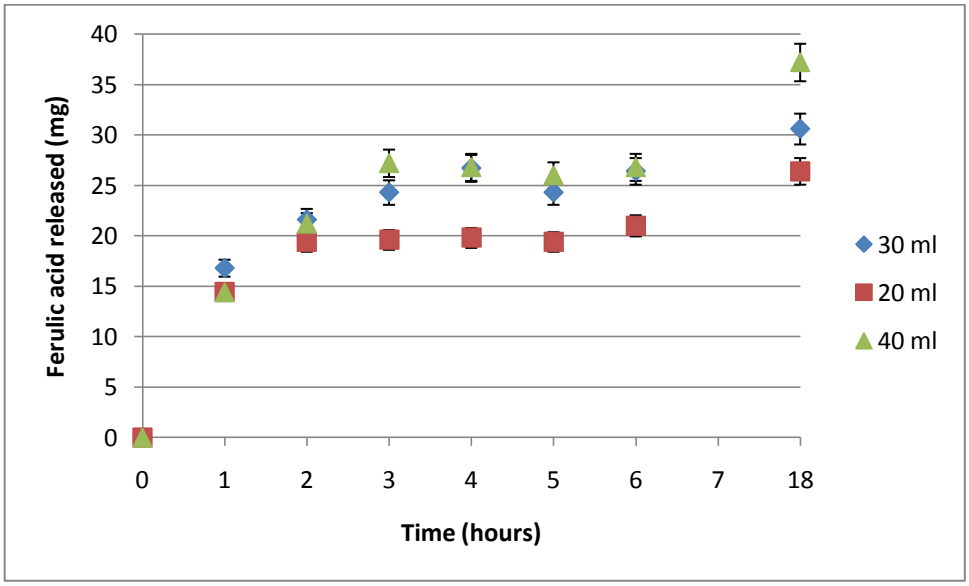


Fig.33 Diffusion of ferulic acid from agarose gels plugs.
Experiments were carried out at 30°C using different volumes of liquid phase. Error bars are standard deviations.

To optimize the release of ferulic acid during the bioconversion process the data collected after 18 hours of diffusion were analysed using the RSM methodology; the results showed that the highest amount of ferulic acid released (22 mg) was achieved at high ferulic acid concentration (5 mg/ml) and at a low agarose concentration (1,7%) (fig.34).

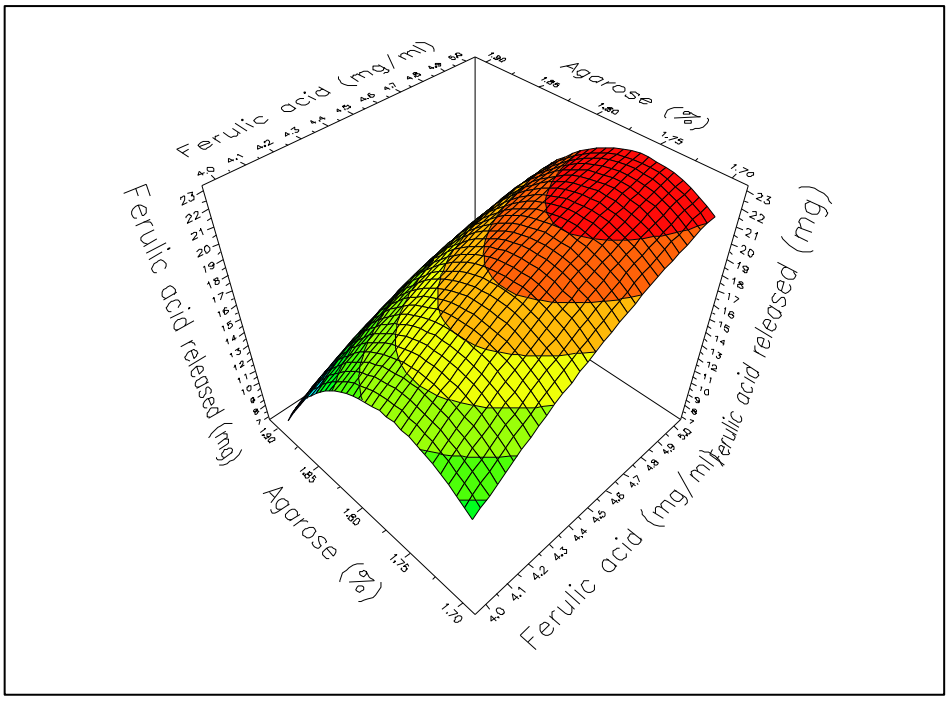


Fig.34 Mutual effect of ferulic acid and agarose concentration inside gels on the releasing of ferulic acid in the bioconversion medium evaluated as surface response calculated by the use of Modde 5.0.

Bioconversion experiments with agarose gels entrapped ferulic acid.

Bioconversion experiments using resting cells of *E.coli* JM109(pBB1) strain were carried out to test the possibility to modulate the release of the substrate into the bioconversion buffer using a two phase system of agarose gels plugs containing ferulic acid. The system developed in this work was compatible with the bioconversion (fig. 35), and it permitted to reduce the toxic effect of the ferulic acid.

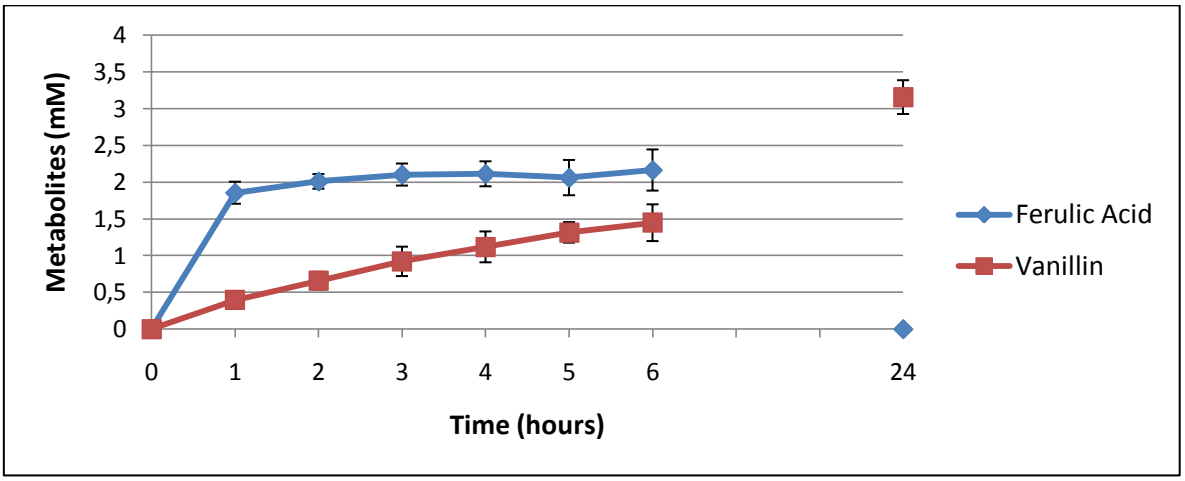


Fig. 35 Bioconversion of ferulic acid to vanillin using agarose gels entrapped ferulic acid.

Experiments were carried out at 30°C and pH 7.00 using *E.coli* JM109(pBB1). Error bars are standard deviations.

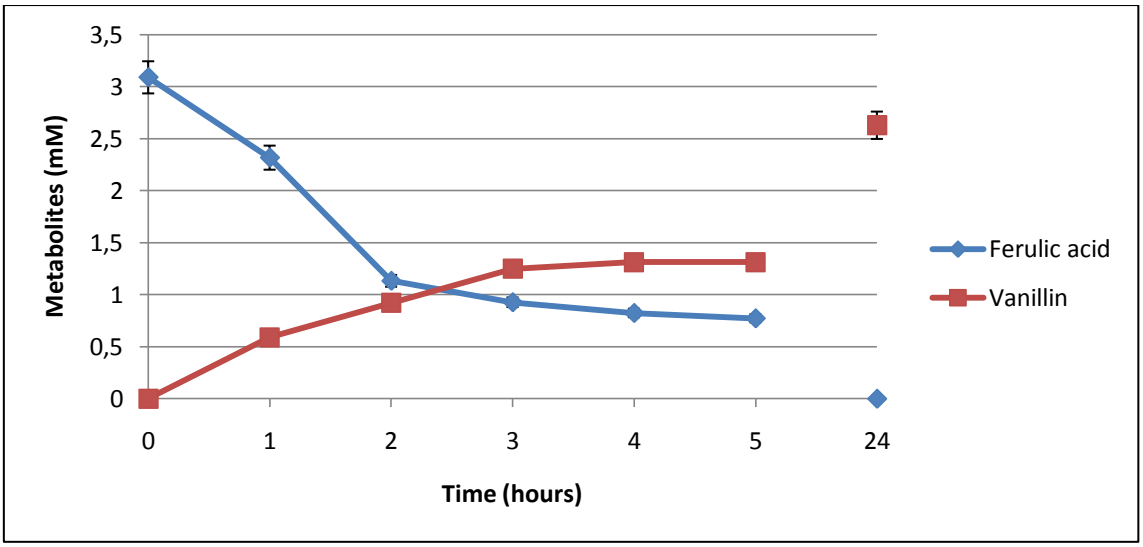


Figure 36 Bioconversion of ferulic acid to vanillin.

Experiments were carried out at 30°C at pH 7.00 using *E.coli* JM109(pBB1) without agarose gels plugs for the controlled release of ferulic acid. Error bars are standard deviations.

Production of a microbial acylase active on capsaicin

Effect of media composition on acylase production

It has been previously demonstrated that *S. mobaraensis* NBRC13819 strain produced an extracellular acylase able to convert capsaicin into vanillylamine, a vanillin precursor. In order to identify the best conditions for acylase production by *S. mobaraensis*, DSM40847 strain was grown on different media. The results obtained from fermentations carried out using shaken flasks suggested that the medium containing soluble starch, polypeptone and beef extract, produced the highest titres of biomass and capsaicin acylase, while using the medium containing glucose, soybean meal and yeast extract the lowest titres of biomass and not production of capsaicin acylase were obtained.

	Biomass (g/L)	**Acylase (U/ml)**
Medium MA	32 ± 0.9	0.02
Medium C	9.29 ± 0.2	0.00053
Medium S/BIS:	8.4 ± 0.3	0.003
Medium M8	12.9 ± 0.7	0.0081
AF/Ms	8.12 ± 0.5	0
AUR/M:	9.94 ± 0.2	0.008
Medium T	4.93 ± 0.06	0.015

Table 9: Effect of medium composition on biomass and acylase production by S. mobaraensis DSM40847 in shaken cultures after 144 hours of growth.

The results obtained suggested to evaluate the effect of different sources of carbon (soluble starch or glucose) and organic nitrogen (polipeptone and meat extract, or meat extract, yeast extract and peptone soybean meal) on the production of the acylase from *Streptomyces mobaraensis* DSM40847. Batch fermentation were carried out at 30°C in duplicate either in shaken flasks (180 rpm) or in stirrer tank reactors. The results indicated that the extracellular enzymatic activity profile (fig.37) and cell growth (fig.38) were strongly affected by the use of different organic nitrogen sources in media enriched with glucose.

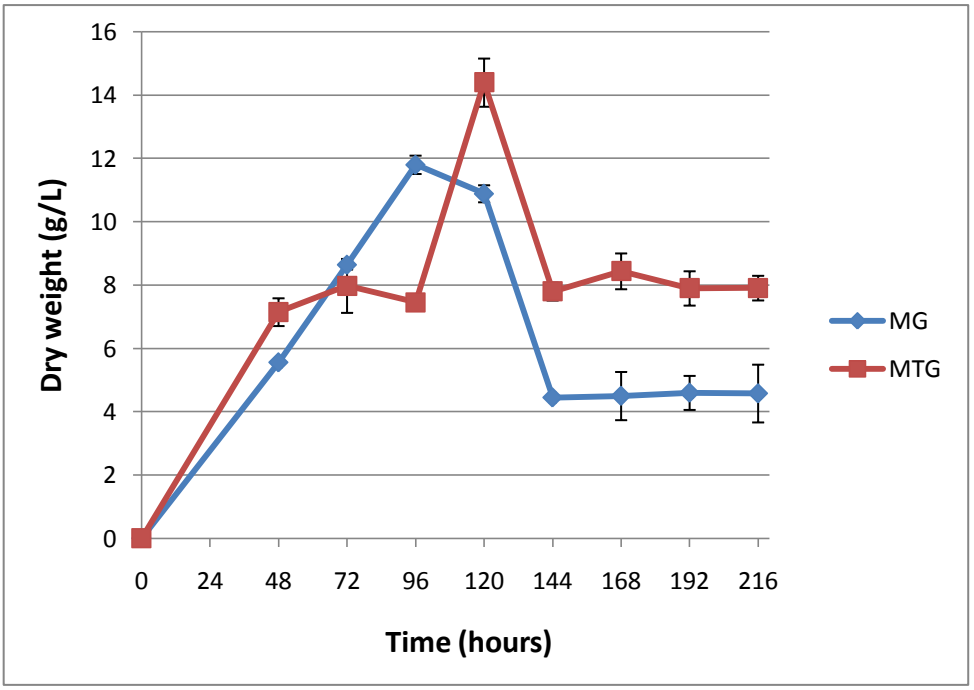


Figure 37 Biomass production (dry weight) of Streptomyces mobaraensis DSM40847 grown on media containing glucose (5 g/L) as carbon source.

Error bars indicate standard deviations.

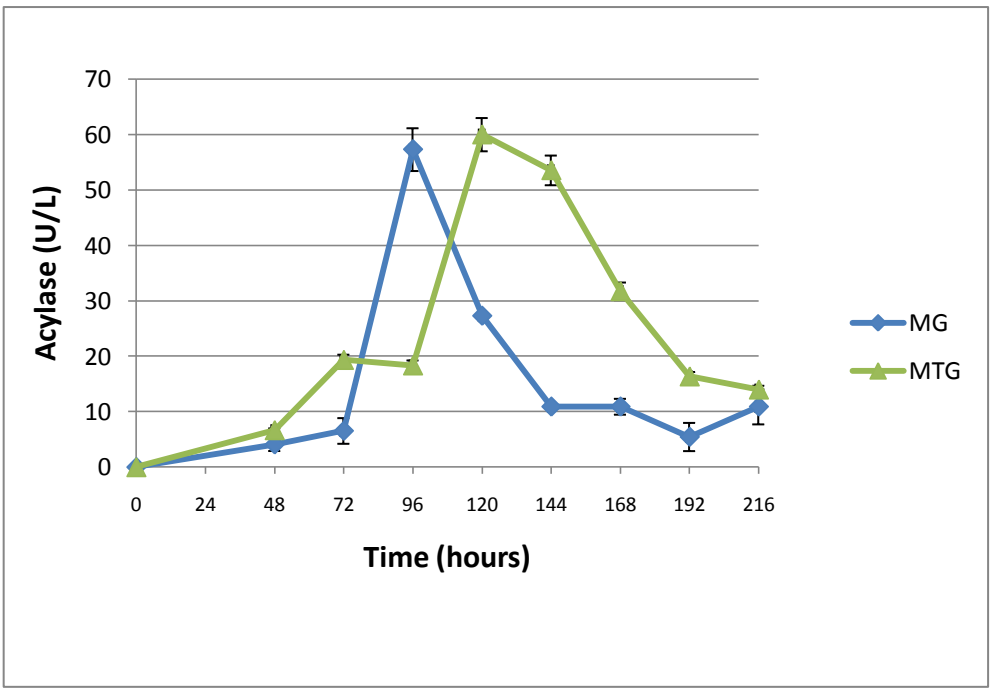


Figure 38 Acylase production of Streptomyces mobaraesins DSM40847 grown on medium M and MT containing glucose (5 g/L) as carbon source.

Error bars indicate standard deviations.

The highest value of acylase activity was detected at hour 96 on medium M, when the higher quantity of biomass was obtained, and at hour 120 on medium MT. After 96-120 hours of growth, a decrease in the amount of biomass, (dry weight) (fig.37) and of the acylase activity (fig. 38) were observed. The first phenomenon is caused by cell lysis in both of the investigated nitrogen sources.

In experiments of growth carried out in stirred reactor, time course profiles of capsaicin-hydrolysing acylase production were affected by the use of different nitrogen sources, and from nutritional factors (fig.39,40). The highest level of acylase activity (140 UI/L) was obtained when strain DSM40847 was grown on medium M enriched with soluble starch, (fig.39). Media containing soluble starch a similar profile of production of the biomass was observed, (fig.40). Cell lysis phenomenon was limited in CSTR experiments; at the end of the fermentation process, were obtained 18 g/L (medium M) and 23 g/L (medium MT), respectively.

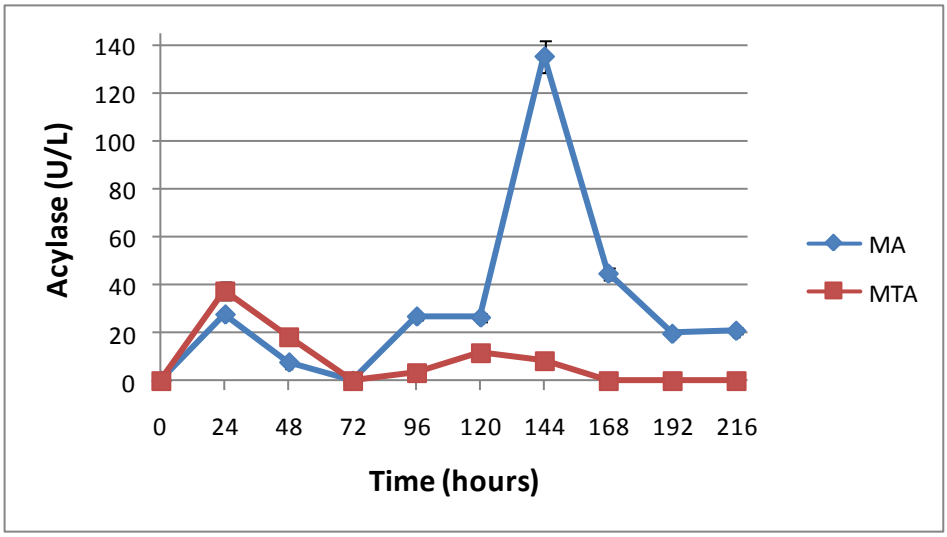


Figure 39 Acylase production of Streptomyces mobaraesins DSM40847 grown on medium M and MT containing soluble starch as carbon source.

Error bars indicate standard deviations.

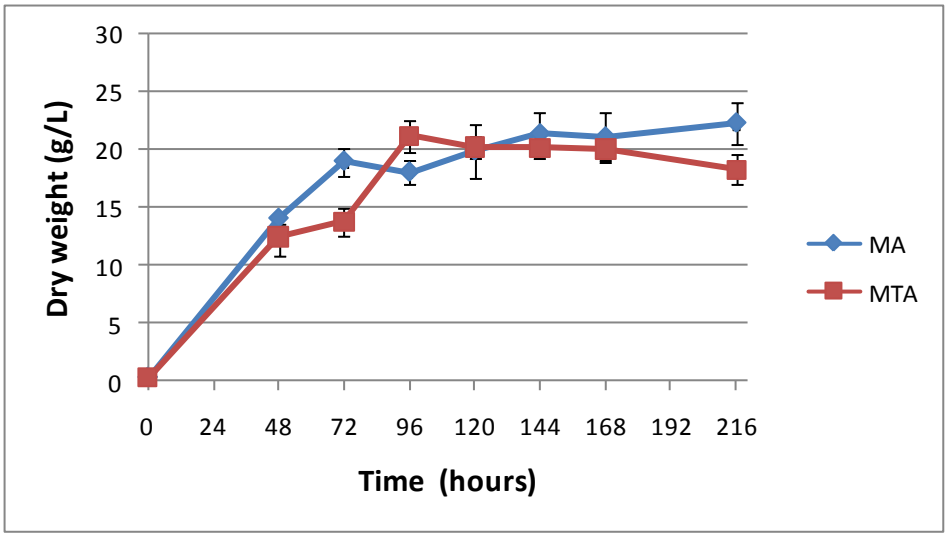


Figure 40 Biomass production of Streptomyces mobaraesins DSM40847 grown on medium M and MT containing soluble starch as carbon source.

Error bars indicate standard deviations.

Effect of agitation speed

The effect of agitation speed on the production of capsaicin acylase by *S. mobaraensis* in a stirred tank reactor (STR) was tested because there was no studies in literature. The fermentations were carried out at the constant temperature of 30°C and aeration rate of 1.0 vvm, with different agitation speeds of 300, 450 and 600 rpm. The maximum level of acylase activity was achieved at 450 rpm after 168 hours of fermentation (Fig.41). When the agitation speed was higher or lower than 450 rpm, we observed a reduction in the bacterial growth that was attributed to the effect of shear stress at the higher agitation speed (600 rpm) and of oxygen limitation at the lower speed (300 rpm). Time profiles of biomass dry weight, secreted proteins and enzyme production at 450 rpm are shown in figure 41. Biomass concentration increased rapidly (up to 7.3 g [dry weight]/L) during the first 24 hours of fermentation and continued to increase, albeit at a lower rate, until the end of the experiment. Dissolved oxygen concentration rapidly dropped at values near zero during the first 24 hours, remained unaltered for the next 120 hours and then increased till the end of the fermentation. Surprisingly, production of acylase activity rapidly increased between 144-168 hours of fermentation, when an increase in dissolved oxygen levels was measured, and reached a maximum level at the end of the fermentation. Under optimal culture conditions in STR the production of acylase could be increased 8-fold when compared with shaken flask.

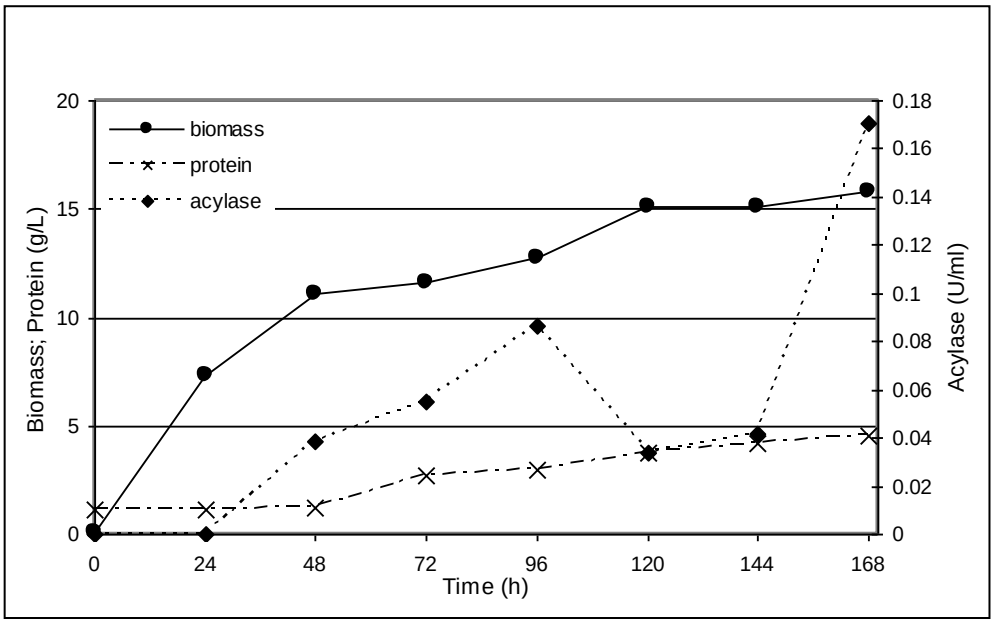


Figure 41 Time courses of biomass, proteins and acylase production during STR (450 rpm) fermentation of S. mobaraensis DSM40847.

Comparison of different bioreactor systems

The negative effect of the shear force arising in STR bioreactors on the growth of *S. mobaraensis*, suggested to evaluate the possibility to cultivate the microorganism in air lift (AR) reactors. Biomass and acylase production in the AR were higher than that achieved in the STR (Table 10). However, the increase in acylase levels was not proportional to changes in biomass concentration and consequently the specific yield (enzyme activity/biomass content) in the AR (5.7 mU/g of biomass) was lower than that achieved in the STR (7.8 mU/g of biomass).

	Acylase (U/ml)	**Biomass (g/L)**
STR	*0.14*	*16*
AR	*0.16*	*28*

Table 10: Maximal acylase and biomass production obtained in STR and AIR LIFT (AR) reactors.

Strain maintenance of Streptomyces mobaraensis DSM40847 strain and inocolum preparation

The effect of the maintenance conditions on the production of acylase was evaluated. Three different clonal lines of DSM40847 strain were analysed: Two selected clonal lines were stored in agar medium GYM plates at 4°C (Line 1 and 2) and one clonal line was stored in a frozen stock in medium GYM and glycerol at -80°C. The three clonal lines were used to prepare inoculums in MA medium The results reported in figure 42 shows that clonal lines 1 and 2 produced similar levels of activity at 192 hours of cellular growth, while clonal line 3 provided a profile of activity similar to the line 1 during the first 144 hours of growth but at the end of the fermentation permitted to obtain higher levels of activity. At 192 hours line 3 provided 2-fold more acylase than clonal lines 1 and 2. These results suggest that the best storage condition to maintenance DSM40847 strain is at -80°C on GYM and glycerol medium.

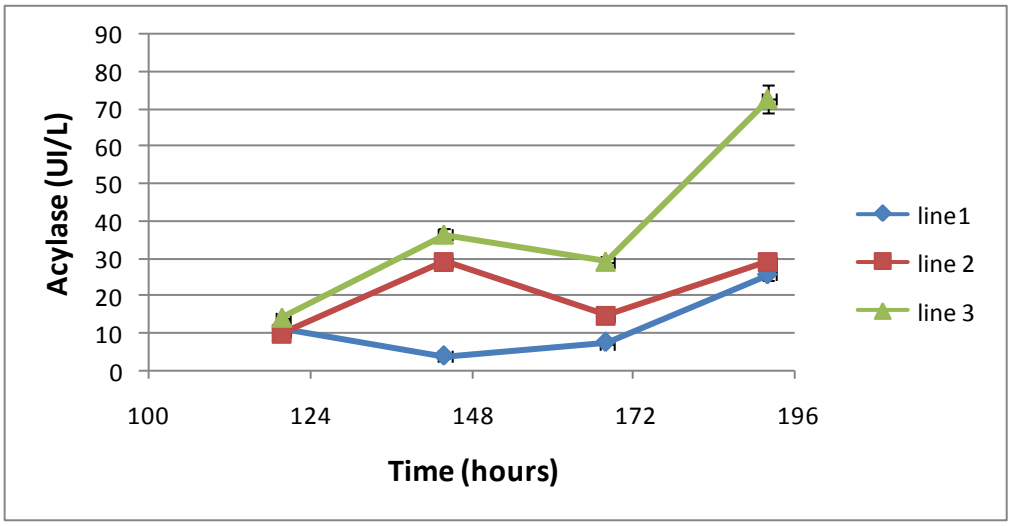


Figure 42 Capsaicin Acylase production from three lines of Streptomyces mobaraensis DSM40847 strain at different storage conditions. (+4°C on plate, line 1 and 2; - 80°C,on medium GYM and glycerol, line 3).

Acylase activity in the broth was recovered by precipitation with armonium solfate. Acylase activity was monitored by determining the consumption of capsaicin by HPLC chromatography. Error bars are standard deviations.

Effect of the temperature on the conversion of capsaicin to vanillylamine

The effect of the temperature on the hydrolysis of capsaicin using crude preparations of acylase from *Streptomyces mobaraensis* DSM40847 strain was investigated. The range of temperature analysed was from 37 to 60°C, and acylase activity was measured by HPLC assay monitoring the capsaicin consumption in the time. The results, reported in figure 43, indicated that an increase in the acylase activity was obtained increasing the temperature up to 55°C. Therefore, the optimal temperature to carry out the conversion of capsaicin to vanillylamine using the crude preparation of *Sm*-acylase resulted to be 55°C.

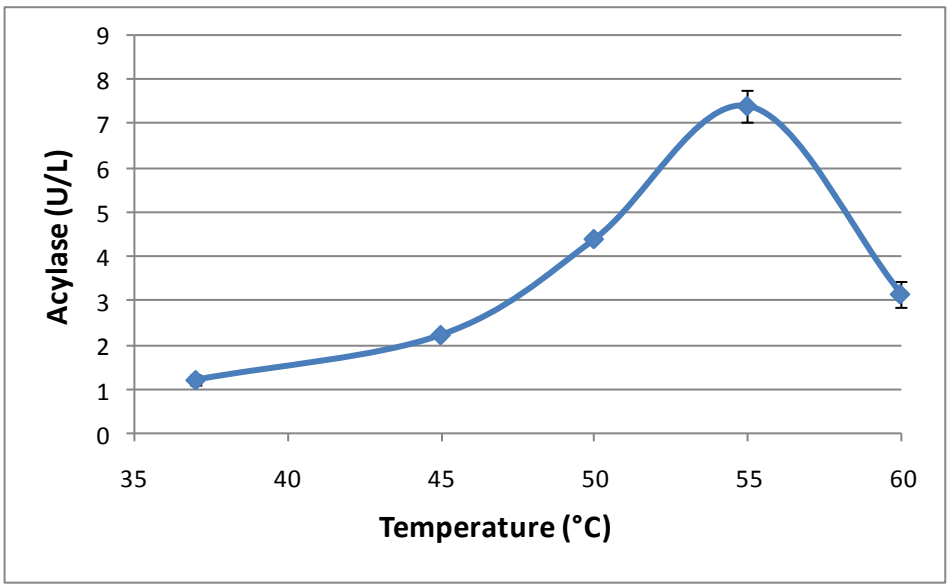


Figura 43 Effect of temperature on acylase activity from S. mobaraensis.
Experiments were carried out using raw enzymatic preparation obtained from fermentation broths of *Streptomyces mobaraensis* strain cultivated on MA medium. Acylase activity was monitored by determining the consumption of capsaicin by HPLC chromatography. Error bars are standard deviations.

Effect of cobalt ions on the hydrolysis of capsaicin

Experiments carried out using the PVA from *S. mobaraensis* 13819 strain indicated that acylase activity of the purified enzyme, increased adding cobalt ions to the reaction buffer (Koreishi et al. 2006). We evaluated if a similar effect could be determined with the crude preparations of acylase from *S. mobaraensis* DSM40847 strain. Hydrolysis reactions were carried out at different temperatures, in Tris HCl 50 mM (pH 7.80), using 800 µg of extracellular proteins recovered by precipitation with ammonium sulphate, containing

~7,5 mUI of acylase activity (measured at 55°C). The results obtained (fig. 44) indicated that the addition of cobalt ions to reaction buffer, caused an increase of 25% in the levels of the acylase activity levels at 37°C, (from 1.2 ±0.1 UI/L [no cobalt ions], to 1.5±0.07 UI/L [with cobalt ions]). In particular at 55°C the activity measured in buffer enriched with cobalt ions was 50% lower than that within cobalt ions (7.5 ± 0.04 UI/L). The addition of cobalt ions to the reaction buffer is not required for the conversion of capsaicin to vanillylamine.

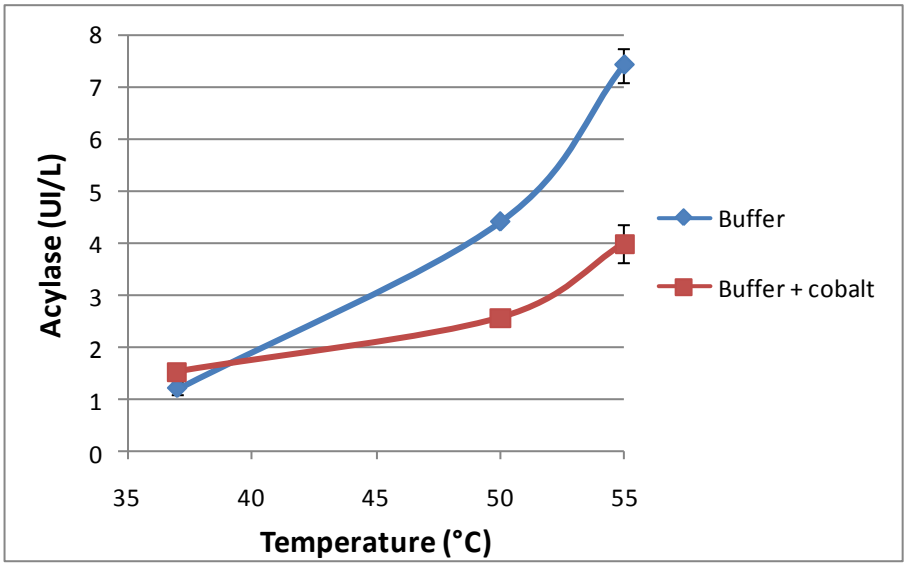


Figura 44 Effect of cobalt ions on the acylase activity

Capsaicin hydrolysis was carried out using crude enzymatic preparations of acylase obtained from coltures of *S.mobaraensis* DSM40847 strain cultivated on MA medium. Acylase activity was monitored by determining the consumption of capsaicin by HPLC chromatography. Error bars are standard deviations. Error bars are standard deviations.

Stability of the enzymatic preparations

The stability of the crude preparations of acylase stored at -20°C, 4°C and 20°C. Acylase activity of the samples was measured on fresh preparations and after 7 and 14 days of storage at different temperatures. Capsaicin hydrolysis reactions were carried out at 55°C using constant volume of enzymatic preparation and in triplicate.

The results reported in fig.45, indicated that the higher activity was obtained maintaining the enzymatic preparation at -20°C. At the latter temperature, the enzymatic preparation is probably affected by the presence of proteases. After 14 days of incubation more than 60%

of the initial activity was retained. At 4°C and 20°C a loss of 75% of the initial activity was registered.

Enzymatic preparations obtained from shaken flasks coltures of *S.mobaraensis* on MG medium, were less stable. The stability could be increased by a purification step or by a pre- incubation at 55°C.

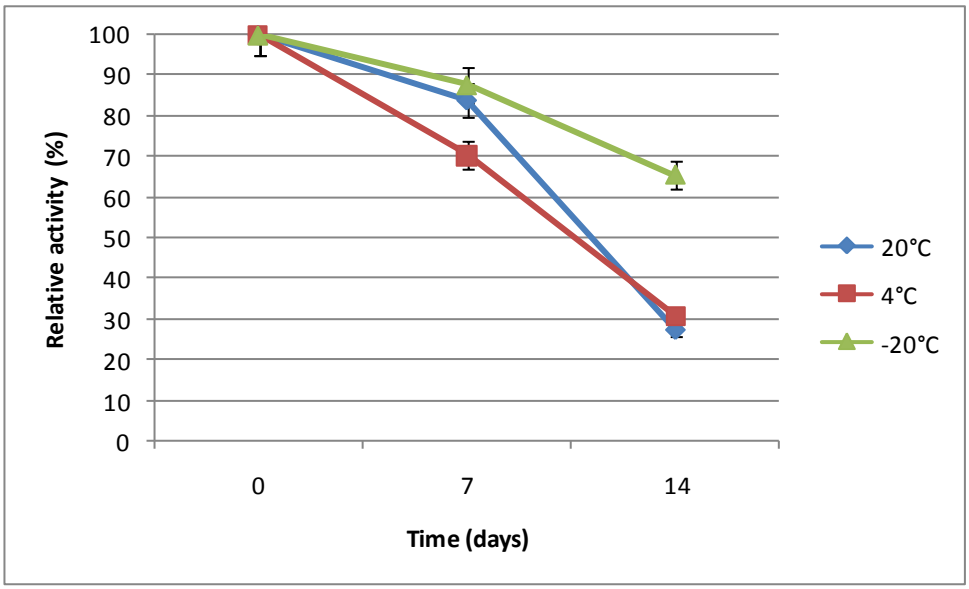


Figura 45 Relative enzymatic activity (%) of enzymatic preparations stored at different temperature for 14 days.

Experiments of capsaicin hydrolysis were carried out using raw enzymatic preparations obtained from *S.mobaraensis* DSM40847 strain cultivated on MA medium. Acylase activity was monitored by determining the consumption of capsaicin by HPLC chromatography. Error bars are standard deviations.

We also evaluated the short time stability of the preparations incubated at 37°C and 55°C. The results obtained (figure 46) indicated that when the enzymatic preparation was incubated at 4°C and 37°C, the acylase activity remained unchanged. On the contrary the incubation of enzymatic raw preparation at 55°C for at least two hours, at least, caused an increase of 5 times in the levels of activity. The same effect was observed after a 24 hours incubation due to the thermal treatment. The increase in the levels of acylase activity at 55°C can be related to the possible precipitation of inhibitors due to the thermal treatment and/or to the thermal inactivation of specified proteases present in the crude preparations.

In conclusion a pre-incubation at 55°C for at least two hours at least, of the crude enzymatic preparations was useful to obtain an efficient conversion of capsaicin in vanillylamine.

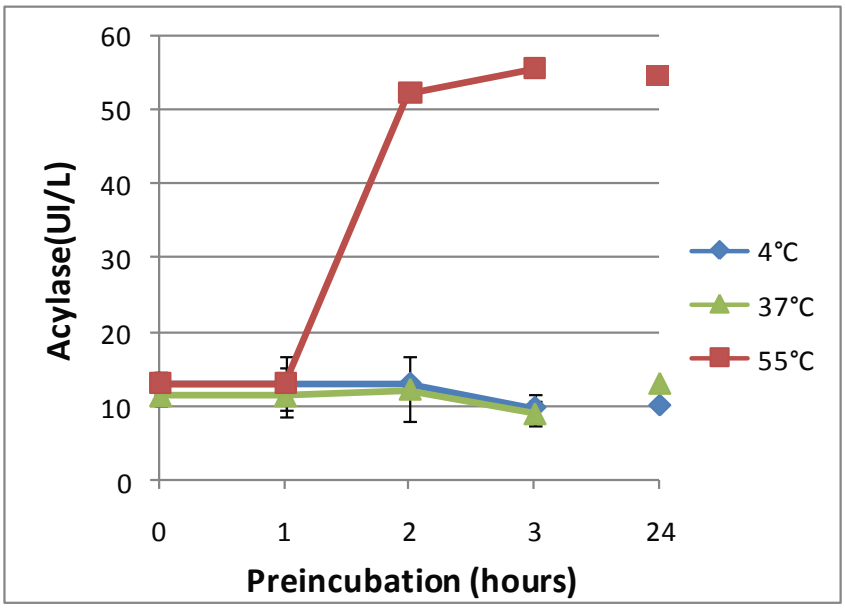


Figure 46 Acylase activity levels of raw enzymatic preparation pre-incubated at different temperature in the range of 0 – 24 hours.

Experiments were carried out using raw enzymatic preparations obtained from fermentation broths of *Streptomyces mobaraensis* strain cultivated on MA medium Acylase activity was determined monitoring capsaicin consumption at 55°C by HPLC analysis.

Error bars are standard deviations.

Cell associated acylases

Previous studies in scientific literature reported that generally *Actinoplanes uthaensis produced* an acylase in a cell associated form. Preliminary data of acylase activity obtained from *S.mobaraensis* DSM40847 suggested that the enzyme was produced mainly in a cell associated form that could be recovered by using a treatment with a KCl 0,8M solution. To verify this hypothesis washed cells recovered from coltures on medium MA, were treated with buffer containing KCl 0,8 M, and the washing buffer was tested for acylase activity. The results indicated that no activity could be recovered using the salt treatment and this suggested that the enzyme is adsorbed on cellular wall and that doesn't exist in associated form.

	Washing	***Esocellular activity***	
		A.utahensis	*S.mobaraensis*
Biomass	Buffer	Absent	Absent
Biomass	Buffer plus KCl 0,8M	Present	Absent

Table 11 Comparison results obtained after washing of biomass of A.utahensis and S.mobaraensis

Development of efficient procedures for the recovery of vanillin from acqueous solutions

Adsorption on XAD-4® Resin and active coal

Effect of pH on the adsorption capacity of XAD-4® resin

The final bioconversion media contain low amount of vanillin and sometimes residue ferulic acid that could be re-utilised in an off-line system. Moreover the recovery of vanillin during the bioconversion can increase the process yield. Ferulic acid and vanillin can be recovered from liquid medium using macroporous resins, such as XAD-4® resin. The selectivity of a resin is defined as the equilibrium distribution of an analyte between the liquid phase and the resin. Previous results of the adsorption experiments using XAD-4® resin suggested that after two hours of incubation the equilibrium was reached. This time was chosen for the successive investigations.

To evaluate the effect of pH on the adsorption capacity and selectivity of XAD-4® resin, adsorption experiments were carried out in acqueous mixture of vanillin and ferulic acid at 30°C. The charge and electronic arrangement of vanillin and ferulic acid change at different pH values in dependence of the hydroxyl group and the carboxyl group respectively. It could be calculated the charge and arrangement of the molecules at different pH values by using the Sparc-on line Calculator® software (table 13,14). Vanillin has a pKa of 7.4, it can be calculated that when pH values are equal to 6.00 and 3.00, the undeprotonated vanillin molecules are 96.2% and nearly 100%, respectively. When pH is 10.00, all vanillin molecules are deprotonated to become negatively charged. The resin is non ionic and it can adsorb weak polar and non polar compounds on its surface, as expected. Results, reported in Figure 47, indicated that the adsorption capacity of XAD-4® resin was affected by pH. In moderate acidic condition (pH 5.50-6.00) the resin had the best capacity to adsorb vanillin (5.98 mg vanillin/g resin) and showed no selectivity. In the same condition the adsorbed vanillin percentage was 95% after two hours of adsorption, (fig. 48). Resin can adsorb ferulic acid but the recovery percentages obtained were lower; the highest value of adsorbed ferulic acid percentage (67%), and adsorption capacity (3.35 mg ferulic acid/g resin) were obtained at pH 6.00 (fig.49).

The highest selectivity (more than 95%), but lower adsorption capacity values were obtained at alkaline pH (9.00-10.50) when vanillin molecules became deprotonated and are negatively charged (fig. 3).

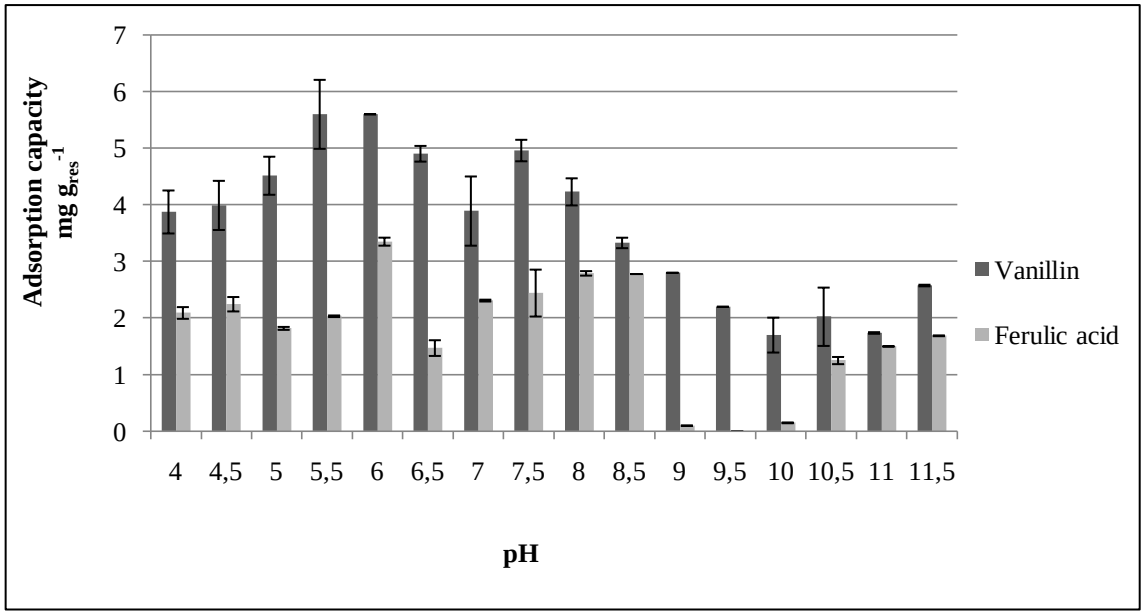


Fig. 47 Adsorption capacity of XAD-4® resin at 30°C after 2 hours-incubation.

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

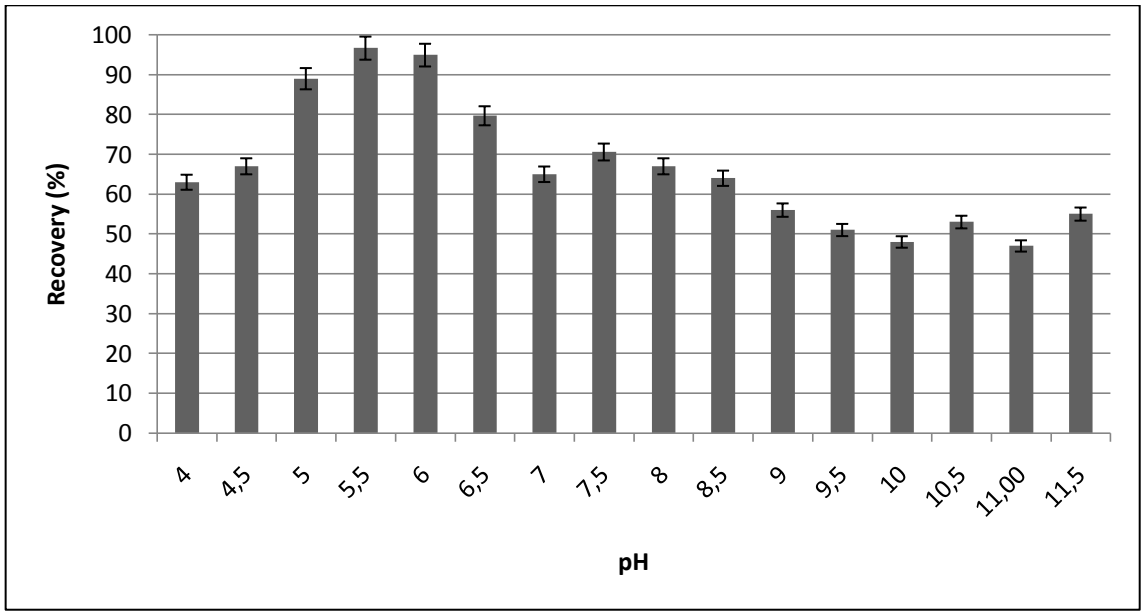


Figure 48 Vanillin recovery (%) after two hours-incubation at 30°C.

Adsorption experiments on XAD-4® resin were carried out on mixture of vanillin and ferulic acid, at different pH, in triplicate. Error bars are standard deviations. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

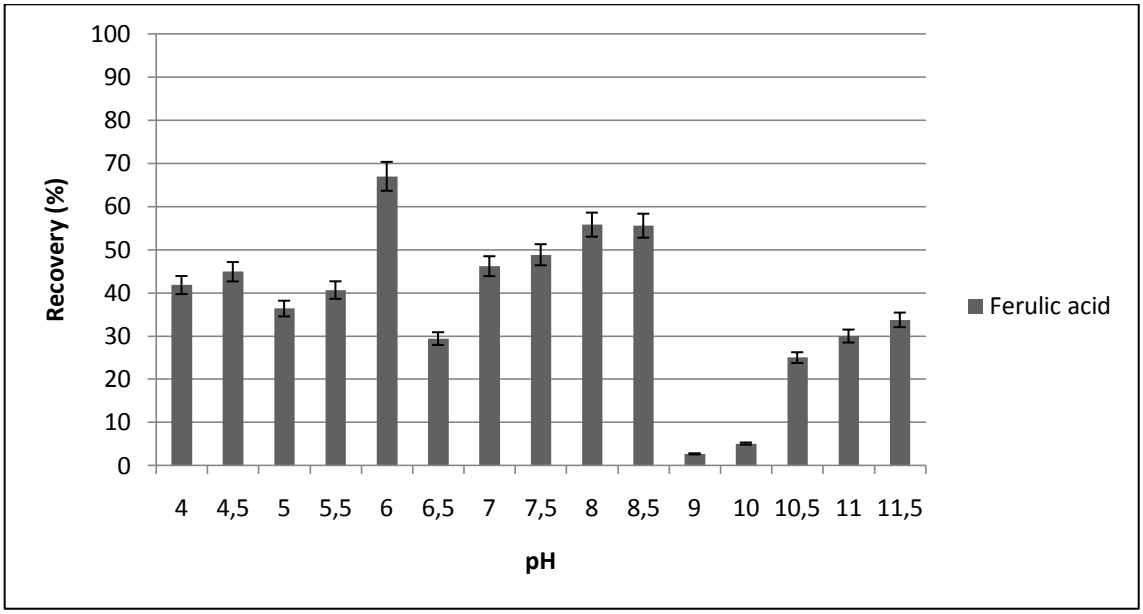


Figure 49 Ferulic acid recovery (%) after two hours-incubation at 30°C.

Adsorption experiments on XAD-4® resin were carried out on mixture of vanillin and ferulic acid, at different pH, in triplicate. Error bars are standard deviations. Quantification of ferulic acid in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

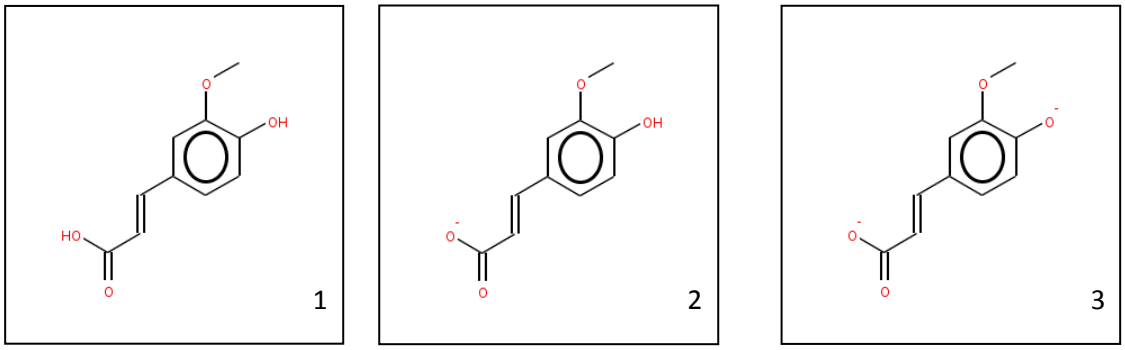


Fig. 50 Ferulic acid possible molecules arrangement and electronic charge.

The possible forms are calculated by using the Sparc-on line Calculator® software.

p H	*1*	*2*	*3*
5.50	8	92	0
9.00	0	38	62
9.50	0	17	83
10.00	0	6	94
10.50	0	2	98
11.00	0	1	99

Table 13 Distribution percentage of the possible forms of the ferulic acid molecule at different pH values. (Sparc-on line Calculator® software).

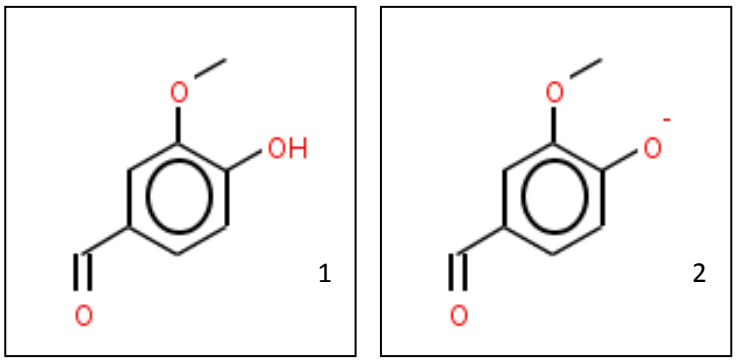


Fig. 51 Vanillin possible molecules arrangement and electronic charge.

The possible forms are calculated by using the Sparc-on line Calculator® software.

p H	*1*	*2*
3.00	96.2	3.8
5.50	100	0
6.00	99	1
9.00	5	95
9.50	2	98
10.00	1	99
10.50	0	100
11.00	0	100

Table 14 *Distribution percentage of the possible forms of the vanillin molecule at different pH values.* (Sparc-on line Calculator® software).

A good description of the adsorption process from solution measurements, when there are different interactions forces can be made using the Freundlich model. It is an empirical equation to suit for multilayer, heterogeneous adsorption sites.

The amount of adsorbed vanillin (Q) was calculated as follows:

$$Q = (G_0 - G_{eq}) \cdot \frac{V}{m}$$

wherein, G_0 was the initial concentration of vanillin, G_{eq} was the equilibrium concentration of vanillin, V was the volume of solution and m was the weight of resin.

The equation used was:

$$Q = K_F C^{1/n}$$

K_F is a parameter according to temperature and 1/n is a parameter reflecting the affinity between solute and adsorbents. The values of K_F and 1/n were shown in Tables 14 and 15 and the plots are shown in Fig. 50. Data analysis suggested that the adsorption between vanillin and the resin may be a multilayer adsorption because there is a linear relationship in the model.

Resin	**K_F**		**1/n**		**R^2**	
pH	5.50	9.00	5.50	9.00	5.50	9.00
	3.0396	0.0132	0.6659	1.1904	0.962	0.9681

Table 15 Fitting parameters of the Freundlich isotherms for XAD-4® resin under different. conditions.

Active Coal	**K_F**		**1/n**		**R^2**	
pH	5.50	7.50	5.50	7.50	5.50	7.50
	22.3560	81.34	1.2262	0.4027	0.8568	0.9338

Table 16 Fitting parameters of the Freundlich isotherms for active coal under different conditions.

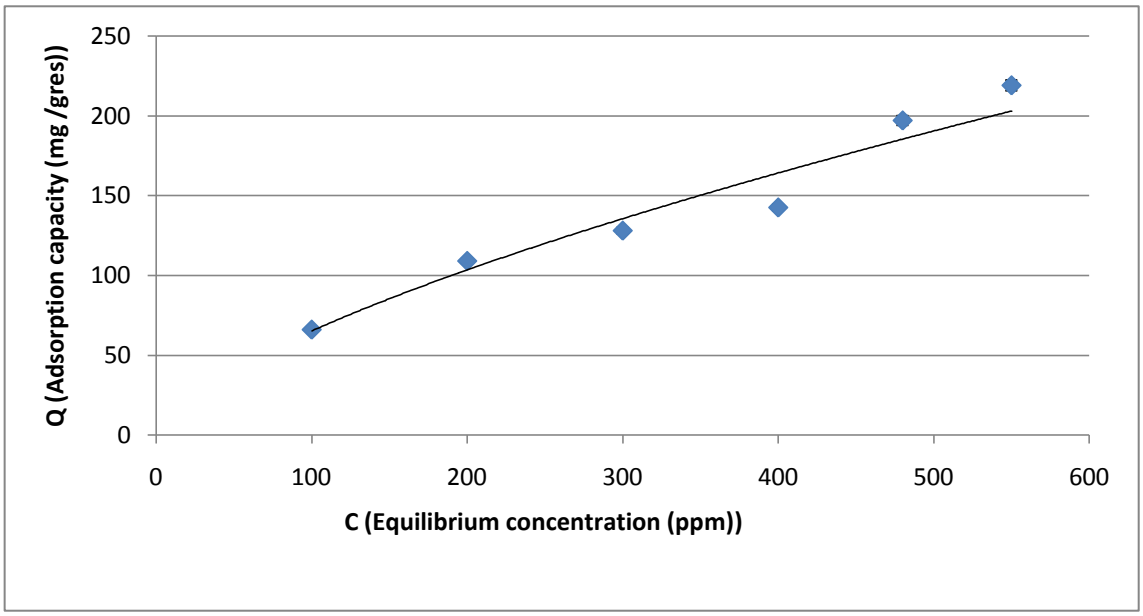


Fig.52 Equilibrium adsorption isotherm of resin at 30°C and pH 5.50. Adsorption experiments were carried out in solution of vanillin (0.55 mg/ml) in M9 buffer using different amount of resin. Adsorption time was 2 hours to reach the equilibrium. Adsorption capacity was expressed as mg of metabolites adsorbed per gram of resin. Quantification of vanillin and ferulic acid in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

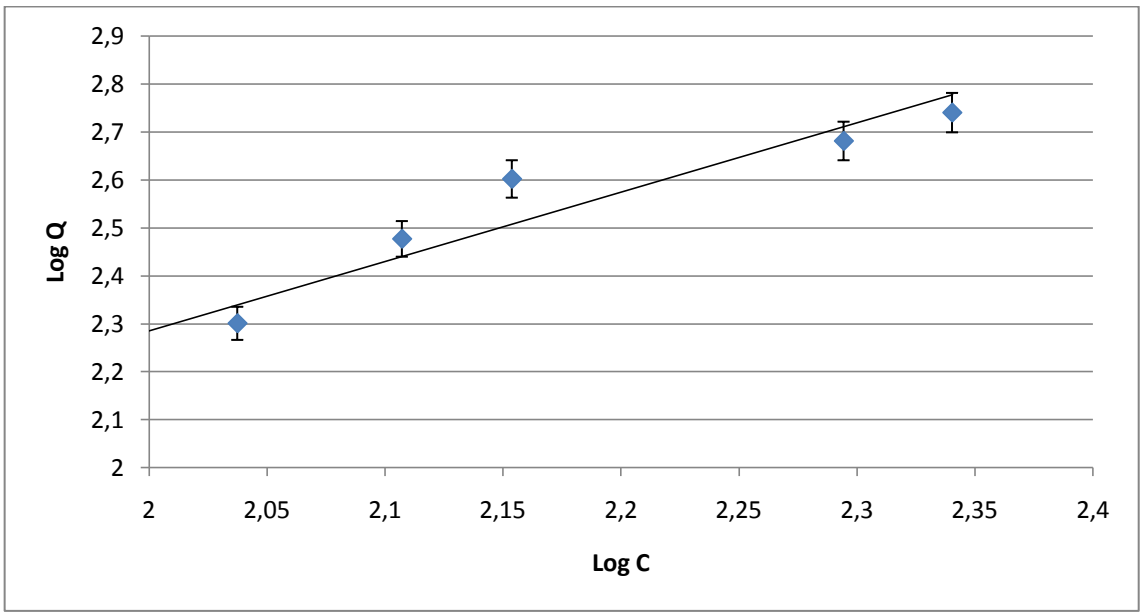


Fig. 53 Freundlich isotherms of resin at 30°C and pH 5.50. Adsorption experiments were carried out in solution of vanillin (0.55 mg/ml) in M9 buffer using different amount of resin. Adsorption time was 2 hours to reach the equilibrium. Quantification of vanillin and ferulic acid in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

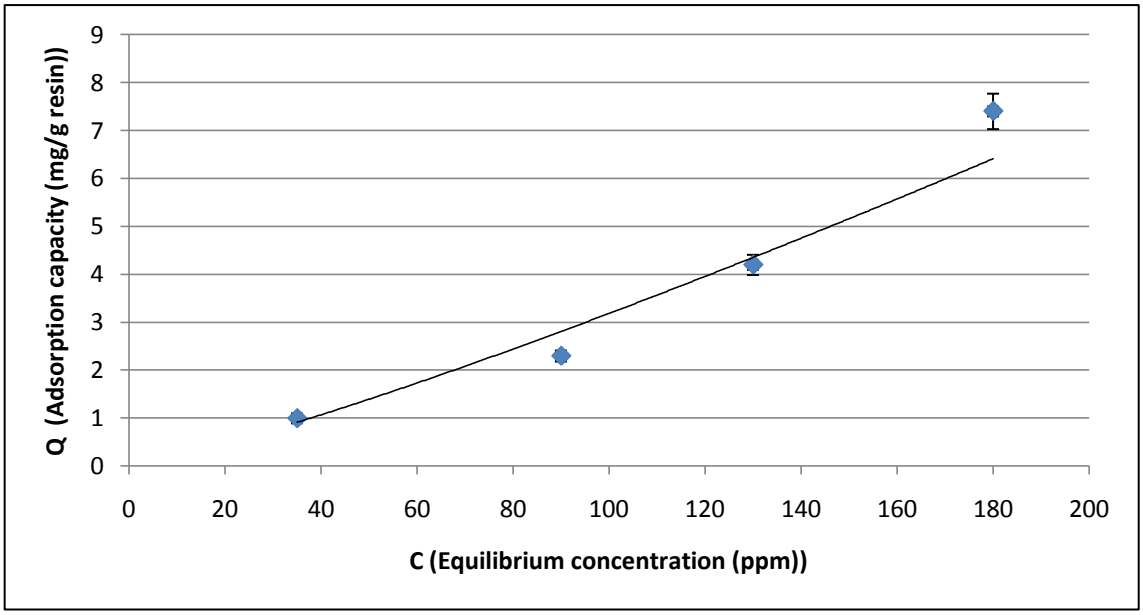


Fig.54 Equilibrium adsorption isotherm of resin at 30°C and pH 9.00. Adsorption experiments were carried out in solution of vanillin (0.55 mg/ml) in M9 buffer using different amount of resin. Adsorption time was 2 hours to reach the equilibrium. Adsorption capacity was expressed as mg of metabolites adsorbed per gram of resin. Quantification of vanillin and ferulic acid in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

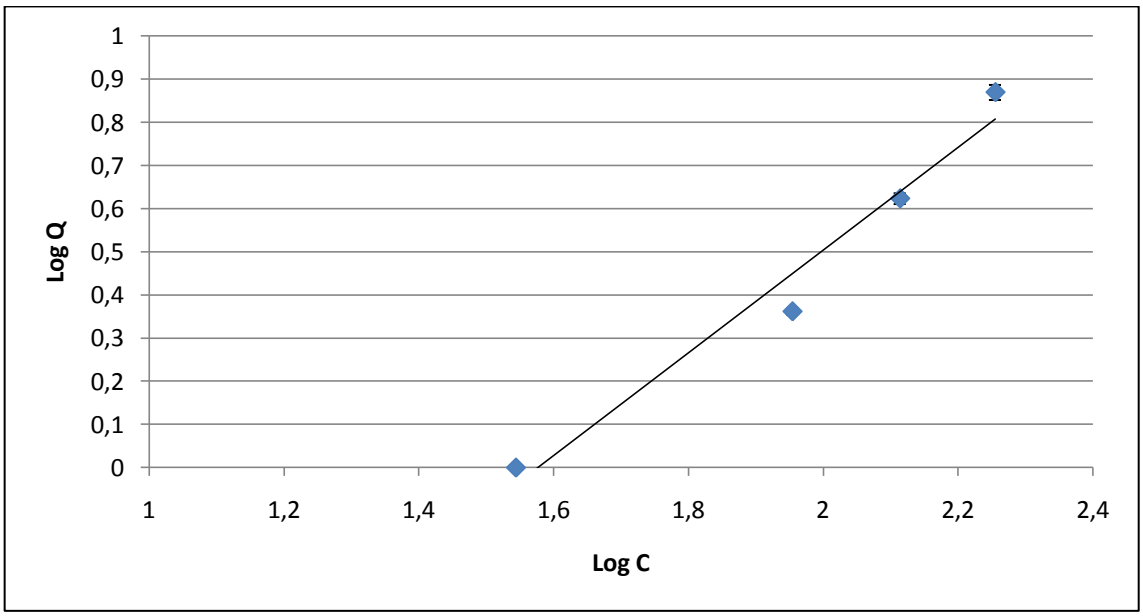


Fig. 55 Freundlich isotherms of resin at 30°C and pH 9.00. Adsorption experiments were carried out in solution of vanillin (0,55 mg/ml) in M9 buffer using different amount of resin. Adsorption time was 2 hours to reach the equilibrium. Quantification of vanillin and ferulic acid in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

Effect of temperature on the adsorption capacity of XAD-4® resin

To evaluate the effect of temperature on the adsorption capacity of XAD-4® resin, adsorption experiments were carried out in acqueous solutions of vanillin, in the range of 30 to 44°C. As shown in fig. 56, the equilibrium adsorption isotherms of XAD-4® resin are similar at different temperatures in the range considered. The increasing of temperature has not a relevant influence on vanillin adsorption on XAD-4® resin.

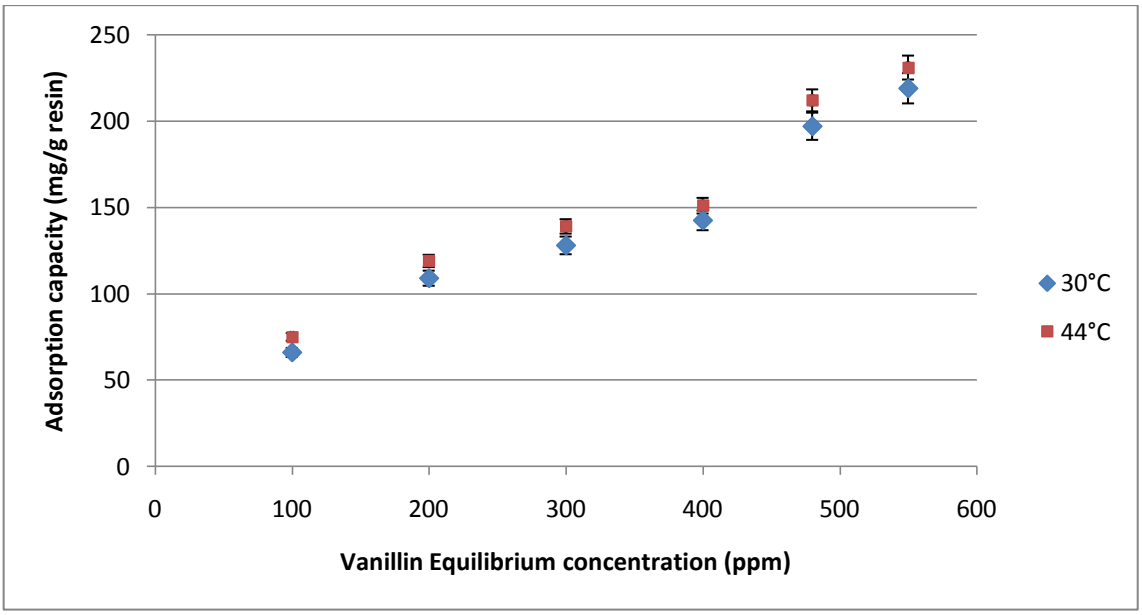


Fig.56 Equilibrium adsorption isotherms of resin at 30°C and 44°C (pH 5.50). Adsorption experiments were carried out in solution of vanillin in M9 buffer using different amount of resin. Adsorption time was 2 hours to reach the equilibrium. Adsorption capacity was expressed as mg of metabolites adsorbed per gram of resin. Quantification of vanillin and ferulic acid in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

Effect of pH on the adsorption capacity of active coal

Adsorption experiments using active coal, at 0.1 g/ml, in mixture of vanillin, (0.75 mg/ml), and ferulic acid, (0.5 mg/ml), were carried out at 30°C, and at different pH, in shaken flasks. The effect of pH on the adsorption capacity of active coal was not relevant. The results showed that the values of adsorption capacity of active coal at pH 7.50 and 5.00 were similar (fig.57).

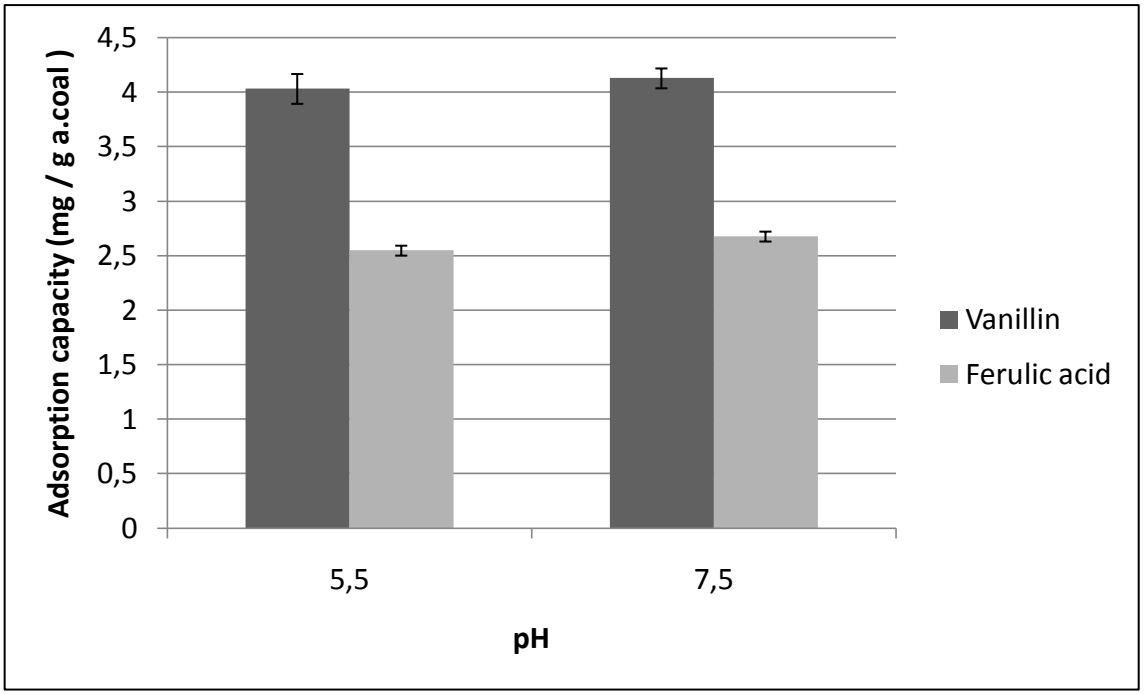


Fig. 57 Active coal adsorption capacity. Adsorption experiments were carried out in solution of vanillin (1, 7 mg/ml) in M9 buffer. The amount of active coal used was 0.15 g / ml. Adsorption time was 2 hours to reach the equilibrium. Adsorption capacity was expressed as mg of metabolites adsorbed per gram of active coal. Quantification of vanillin and ferulic acid in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

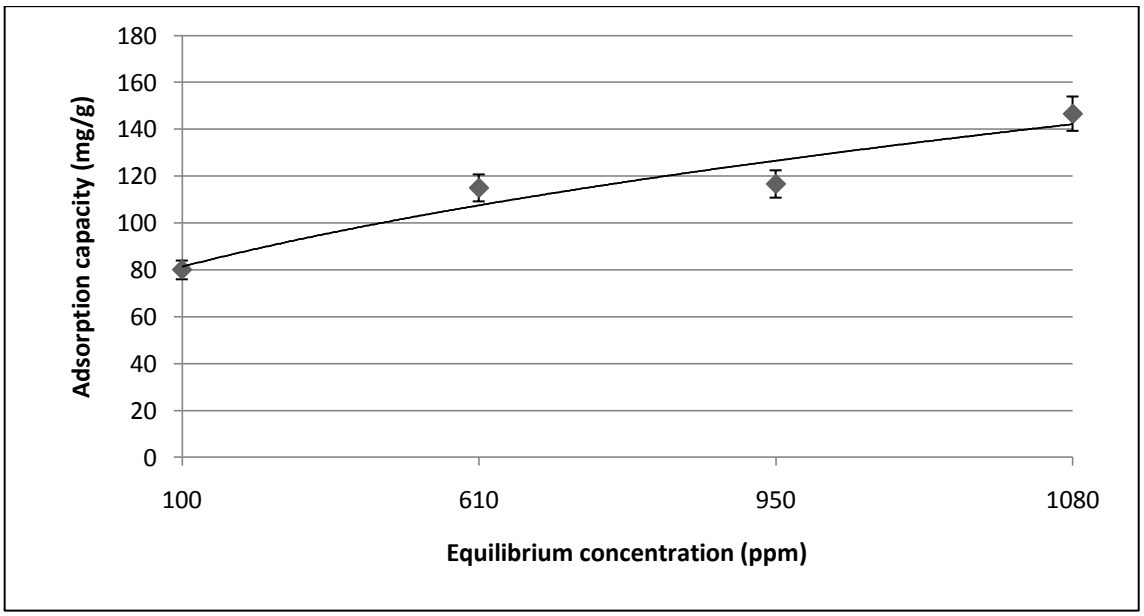


Fig.58 Equilibrium adsorption isotherm of active coal at 30°C and pH 7.50. Adsorption experiments were carried out in solution of vanillin (1,74 mg/ml) in M9 buffer using different amount of active coal. Adsorption time was 2 hours to reach the equilibrium. Adsorption capacity was expressed as mg of metabolites adsorbed per gram of active coal.

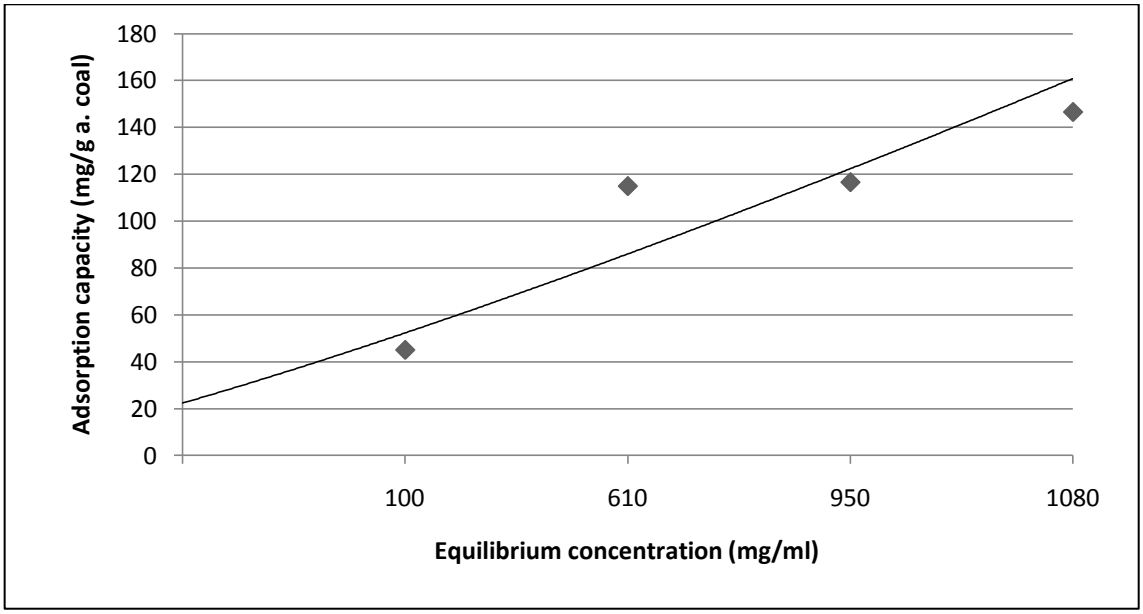


Fig.59 Equilibrium adsorption isotherm of active coal at 30°C and pH 5.00. Adsorption experiments were carried out in solution of vanillin (1.7 mg/ml) in M9 buffer using different amount of active coal. Adsorption time was 2 hours to reach the equilibrium. Adsorption capacity was expressed as mg of metabolites adsorbed per gram of active coal. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

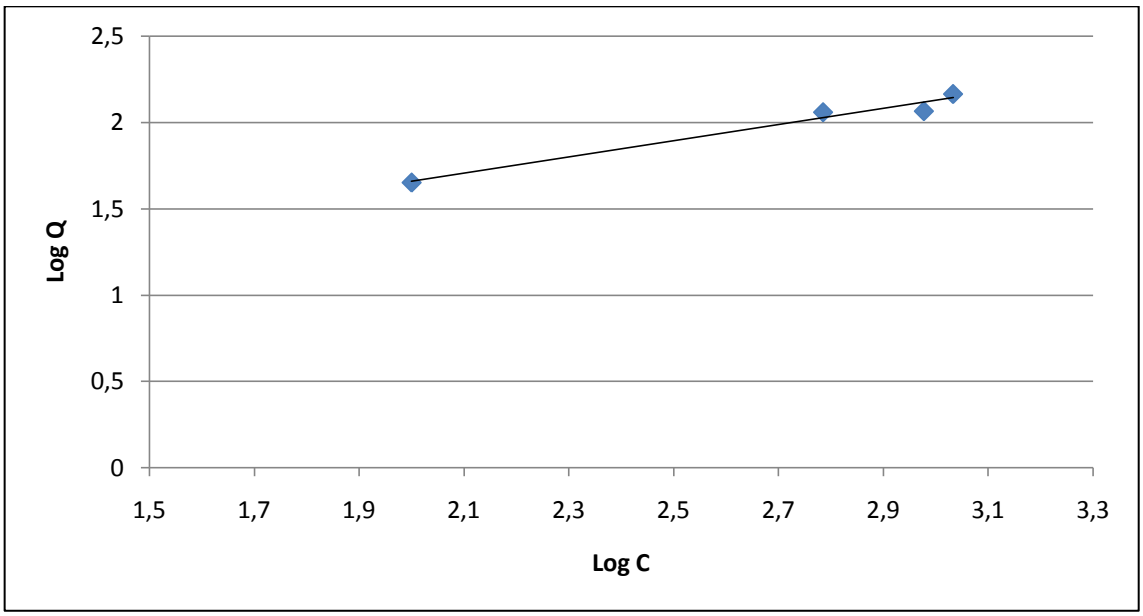


Fig.60 Freundlich isotherms of active coal at 30°C and pH 5.00. Adsorption experiments were carried out in solution of vanillin (1.7 mg/ml) in M9 buffer using different amount of active coal. Adsorption time was 2 hours to reach the equilibrium. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

Kinetic adsorption on XAD-4® resin

The kinetic studies were carried out at 30°C, with resin at 0.1 g/ml in solutions of vanillin in M9 buffer. The kinetic adsorption results of the resin are shown in fig.61. There was a sharp decrease of vanillin concentration in the solution during the first 15 minutes both at pH 5.50 and 7.50. In 2 hours of adsorption when the equilibrium was reached, the highest value of adsorption rate was obtained at pH 5.50.

Kinetic adsorption on active coal

The kinetic studies were carried out at 30°C, with resin at 0,1 g/ml in solutions of vanillin in M9 buffer. The results obtained using active coal are reported in fig. 62. The highest value of adsorption rate was obtained at pH 5.50 and during the first 15 minutes of adsorption a strong decrease in vanillin concentration was obtained both at pH 5.50 or 7.50.

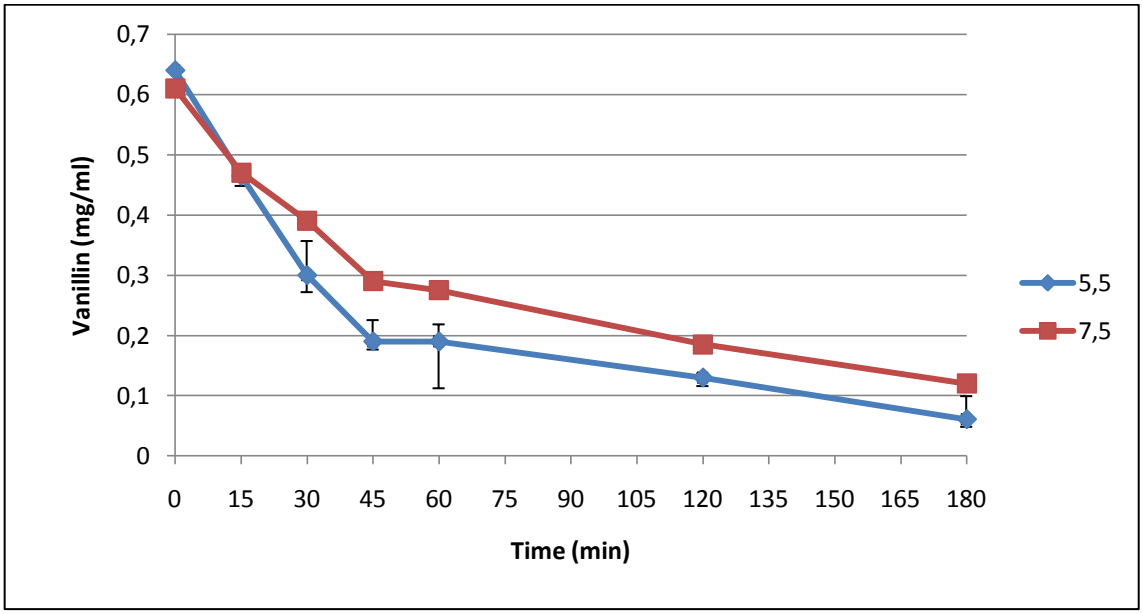


Fig. 61 Kinetic adsorption results of resin at 30°C. Experiments were carried out at pH 7.50 and 5.50 in solution of vanillin (0.6 mg/ml) in M9 buffer. The amount of resin used was 0.1 g /ml. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

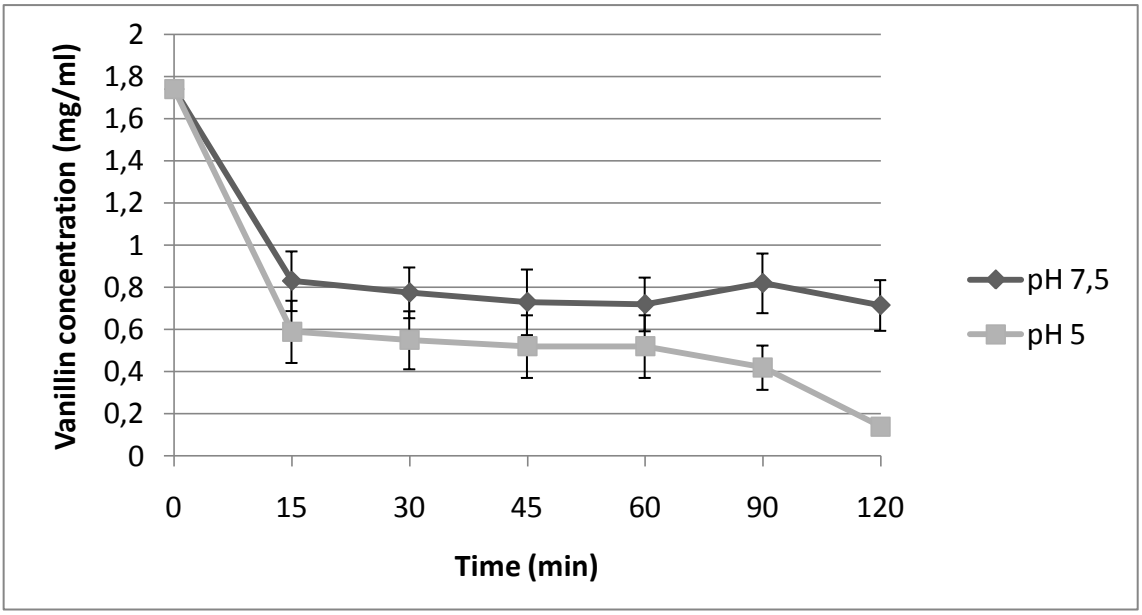


Fig. 62 Kinetic adsorption results of active coal at 30°C. Experiments were carried out at pH 7.50 and 5.50 in solution of vanillin (1.74 mg/ml) in M9 buffer. The amount of active coal used was 0.006 g /ml. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

Resin XAD-4® supplied better results than active coal, as shown in fig. 63. After 30 minutes of adsorption, adsorbed vanillin quantity on resin was 25% more than that on active coal.

The differences in the adsorption rate between resin and active coal were due to the surface areas and polarities.

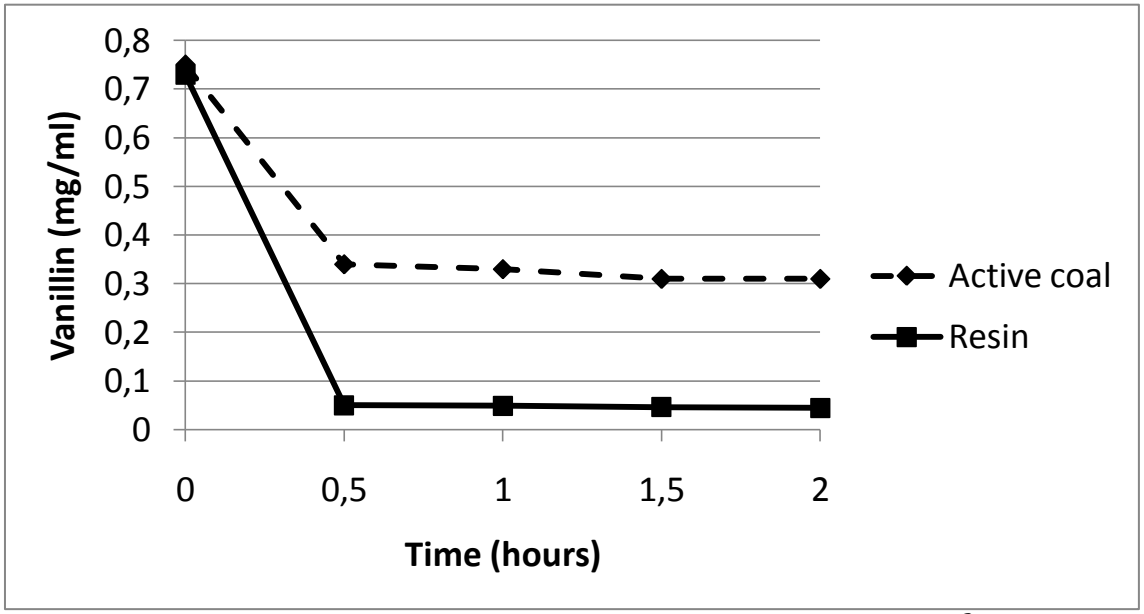


Figure 63 Vanillin concentration during adsorption on active coal and XAD-4® resin at pH 5.50 and 30°C. The amount of active coal or resin used was 0.1 g /ml. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

Column exhaustion studies

In order to evaluate the saturated adsorption capacity of vanillin on XAD-4® resin column exhaustion study was performed at room temperature, using vanillin solutions at pH 5.50 and 7.50. The breakthrough curve was obtained plotting the concentration of vanillin in outlet versus bed volumes of liquid passed into the column. Fixed bed adsorption, using data obtained on a small scale, can be analyzed by the mass transfer zone motion model in which the fixed bed is divided into three parts named equilibrium zone, mass transfer zone (MTZ) and unused zone. For a resin with good mass transfer characteristics, the MTZ is low. The following equation can be used to calculate MTZ from experimental data. (94, 100):

$$MTZ = L_0\,(\theta_s - \theta_b)/\theta_s$$

where L_0 is the total bed height, Θ_s is the time at breakthrough and Θ_b is the time at which breakpoint occurs. The results obtained showed that the MTZ of the resin was 2.42 cm. The area under the curve in fig. 64 represent the amount of unadsorbed vanillin during the adsorption process.

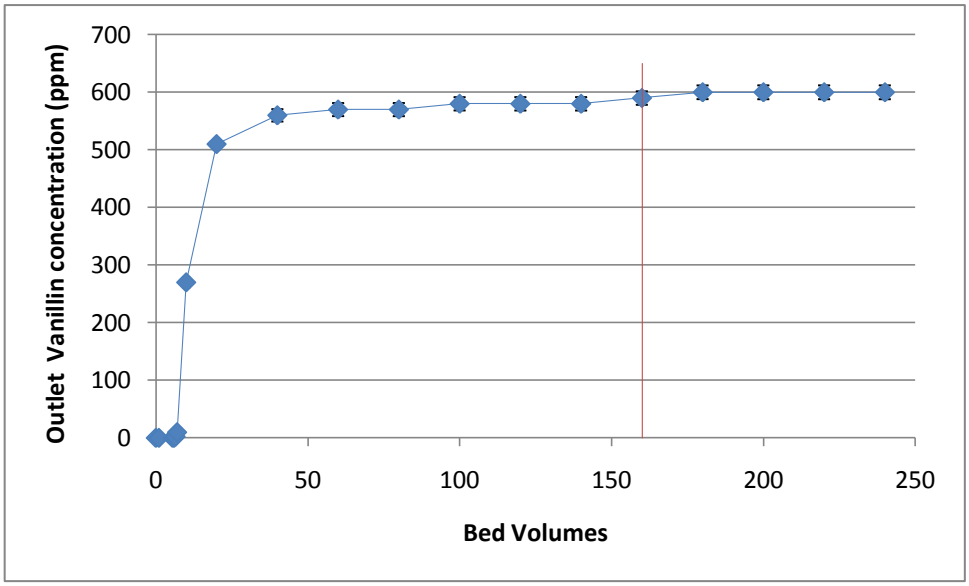


Fig. 64 The breakthrough curve for XAD-4® resin. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

The results showed that the saturated adsorption capacity of vanillin on XAD-4® resin was 420,61 mg/g (vanillin /resin).

Recovery of vanillin and regeneration of the resin

The economical feasibility of the adsorptive recovery process was determined from the easy regeneration of resin and from the recovery of vanillin. Solvent regeneration can be used for polymer adsorbent resins. The solvent can break the binding and van der Waals forces between the adsorbate and adsornet resin. In order to recover vanillin, usually used in food industry as a fragrant ingredient, toxic and non-accetable odor solvents cannot be used. Ethanol is non toxic and it is usually used in flavours field. It can be easily removed from vanillin by distillation because has a low solubility parameter of 12.7 and boiling point of 77–78°C. In this study the elution was carried out using different elution solutions such as ethanol at 96% in concentration, ethanol/water solution (1:1), water, M9 buffer at pH 7.00, Tris/HCl buffer at pH 7.00 and NaOH 1 M, in the range of temperature 30°C-70°C (fig. 68). Elution with ethanol at 30°C supplied the best results and the 96.00% of adsorbed vanillin can be eluted using only two bed volumes of ethanol (fig. 69).

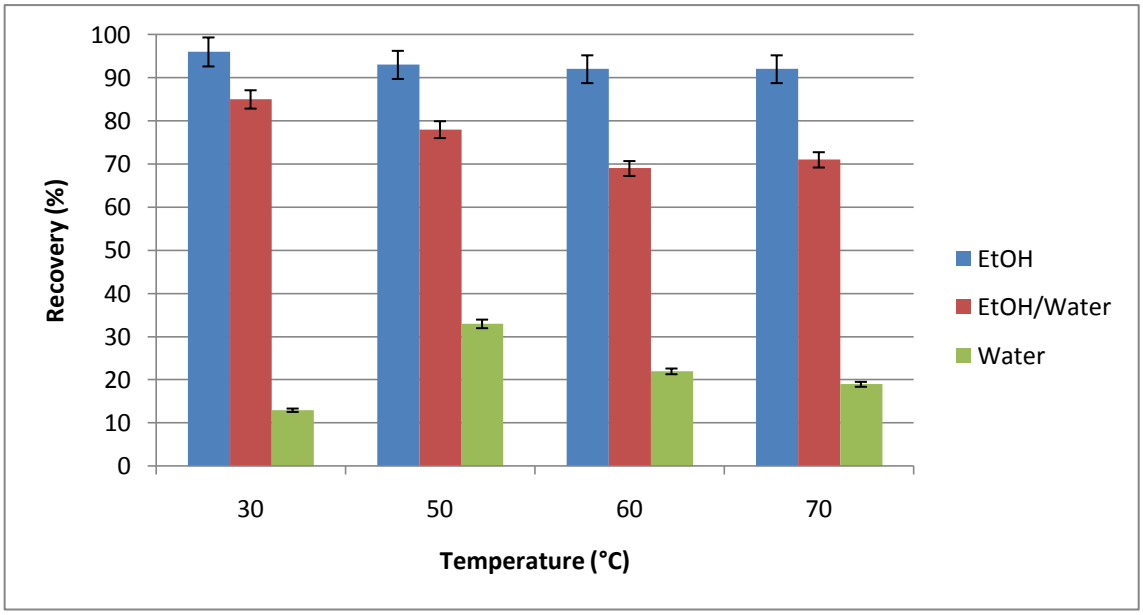


Fig.68 Recovery of vanillin from XAD-4® resin by elution after two-hours incubation. Experiments were carried out at 30°C. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

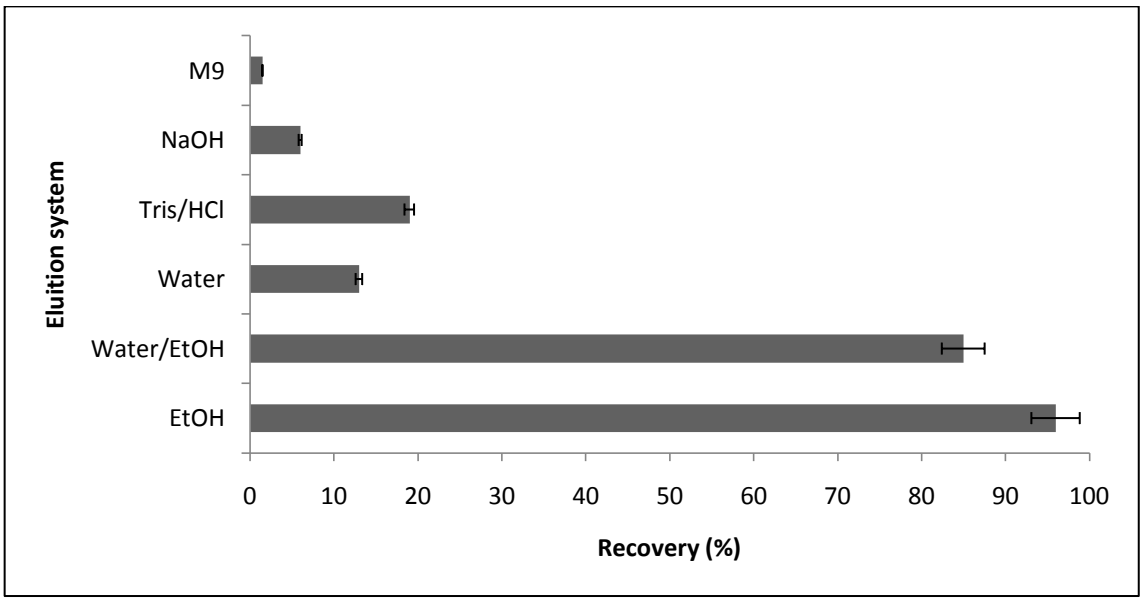


Fig.69 Comparison of elution system for the recovery of vanillin from XAD-4® resin after two-hours incubation. Experiments were carried out at 30°C. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

Liquid/liquid extraction by using n-butyl acetate

Liquid/liquid extraction experiments with *n*-butyl acetate were carried out with mixture of vanillin, (0.5 mg/ml) and ferulic acid (0.5 mg/ml), at 20°C with a total volume ratio of solution and organic solvent of ½ in a double extraction process. When *n*-butyl acetate was used, the adsorbed vanillin percentage was 98%, as shown in fig.70. Liquid/liquid extraction with *n*-butyl acetate allowed high recovery and high selectivity and supplied the best result in the recovery of vanillin from aqueous solutions.

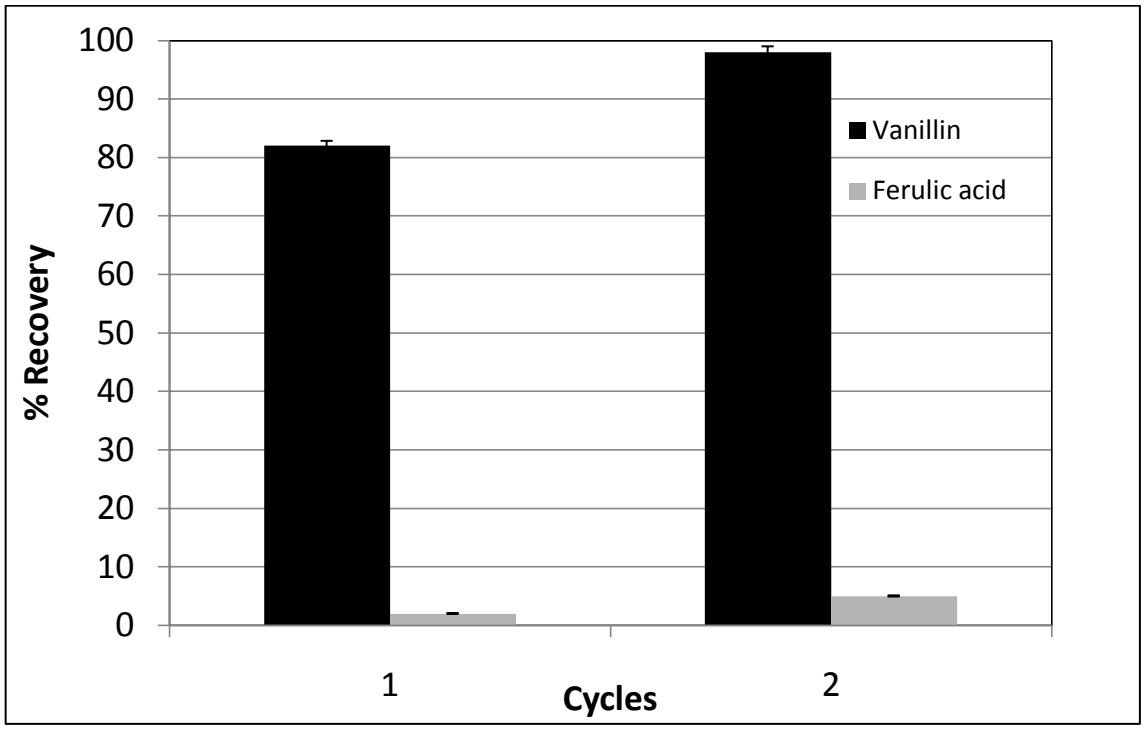


Figure 70 Recovery of vanillin (%) by using liquid/liquid extraction with n-butyl acetate.
Liquid/liquid experiments were carried out at 20°C. Quantification of vanillin in the liquid phase was determining by HPLC chromatography. Error bars are standard deviations.

CONCLUSIONS

Vanillin produced by biotechnological routes starting from natural feedstocks represents an example of sustainable production of natural flavours and a promising target of the modern food industry.

The use of crude enzyme preparation from *S.mobaraensis*, that efficiently hydrolyze capsaicin provides a valuable opportunity to develop a cost-effective process for enzymatic synthesis of natural vanillin precursors.

Experiments carried out using crude enzyme preparations of *Sm*-PVA demonstrated that:

- The highest level of acylase activity was obtained cultivating the microorganism in CSTR reactor on medium containing soluble starch, as carbon source, and meat extract and polypeptone, as source of organic nitrogen;
- The highest vanillylamine production using crude PVA acylase from Streptomyces mobaraensis DSM40847 strain was obtained carrying out the bioconversion at 55°C;
- A thermal pre-treatment at 55°C of crude PVA acylase from *Streptomyces mobaraensis* DSM40847 improves the conversion of capsaicin to vanillylamine;
- Crude enzyme preparations obtained cultivating the microorganism under controlled (CSTR) or uncontrolled (shaken flask) conditions respond in different way to a thermal treatment at 55°C.

At present, ferulic acid bioconversion by fungi and actinomycetes shows the highest vanillin molar yield; however, recently it has been demonstrated that the employing of unicellular microorganisms from selected/tailored bacteria for the vanillin production can be a good option. Experiments carried out using resting cells of *E.coli* engineering strains demonstrated that:

- The use of agarose gels plugs allow a modulated release of ferulic acid into the bioconversion buffer and permits to reduce the toxicity effect of the substrate;
- Selective recovery of the product, using macroporous resins is possible in an off-line process.

Recovery of vanillin from bioconversion buffers is a fundamental step for the whole biotechnological process. Moreover vanillin and ferulic acid can be toxic for the cells and this can cause low vanillin molar yield.

Recovery experiments were carried out using different extraction techniques and supplied the following results:

- XAD-4® Resin had the best capacity to adsorb vanillin and showed no selectivity in moderate acidic condition. The highest selectivity was obtained at alkaline pH.

- Liquid/Liquid extraction with *n*-butyl acetate of acqueous solutions containing mixture of ferulic acid and vanillin allowed high recovery of vanillin and high selectivity.

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