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Design and Scale-up of a Bioprocess for the Production of Natural Vanillin from Agricultural By-Products

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Final Report Executive Summary

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Consortium: The contract was co-ordinated by the Pernod-Ricard Research Centre, Creteil (France), in partnership with Institute of Food Research, Norwich (UK), Laboratoire de Biochimie et Technologie des Glucides, INRA Nantes (France), Laboratoire de Biotechnologie des Champignons Filamenteux, INRA Marseille (France), Centre for Systems Engineering and Applied Mechanics, Louvain La Neuve (Belgium), agro-Industries recherche Et Developpment, Pomacle (France), Department of Applied Chemistry and Microbiology, University of Helsinki (Finland), Amersham-Pharmacia Biotech Europe GmbH, Freiburg (Germany).

EXECUTIVE SUMMARY

Introduction

The objectives of the project were focused on the design and scale-up of an industrial biotechnological process for the transformation of agricultural by-products into high added value natural vanillin. This non-food valorisation, as flavours are considered as additives but not as food, have been realised on the basis on the experience and results obtained at the laboratory level in the previous "Agriculture and Agro-Industry including Fisheries" RTD programme AIRI-CT92-0026 "New processes for the biological transformation of agricultural residues for the production of high added value flavours".

This project addresses all problems associated with the transfer of basic research to industrial scale and is divided into four phases:

(a) Preparation of fermentation feedstock by fractionation of agricultural plant cell walls. The scale-up of the methodologies of enzymatic/biological fractionation and separation of agricultural by-products (wheat and maize bran, sugar-beet pulp) investigated in order to prepare, in cheap and environmental friendly conditions, high amounts of fermentation feedstock for vanillin overproduction : (i) ferulic acid-rich fractions or ferulic acid in a pure form which will be afterwards converted directly into vanillin, (ii) co-products which are extremely important for the economy of the process, xylose (as carbon source) and cellobiose (as activator of the vanillin pathway).

(b) Modifications of fungal pathways for vanillin overproduction. Identification and amplification of essential cellular mechanisms involved in

biotransformation of ferulic acid to vanillin have been studied in *Aspergillus niger* and *Pycnoporus cinnabarinus* in order to improve levels of vanillin productivity.

(c) Industrial fermentation process scale-up. Studies of bioreactor design suitable for filamentous fungi (such as comparison of pneumatic versus mechanically-agitated bioreactor, control of fungal pelleting, characterisation of fermentation conditions, determination of optimal feeding strategies), continuous vanillin extraction system, new monitoring techniques and advanced control methodologies have been developed for the optimisation of an industrial unit of natural vanillin production.

(d) Authentication and labelling of the natural vanillin. The aim of this study is to characterise the biotechnological vanillin extracts from different agricultural sources by isotopic mass spectrometry and nuclear magnetic resonance, in order to authenticate this new product to obtain the "natural" label.

Results

Phase A: preparation of fermentation feedstock by fractionation of plant cell walls

Task A .1: Studies on A. niger

Sub-task A.1.1: Cloninig and amplification of the FAE gene of Aspergillus niger

The *fae*A gene was over-expressed (copy number = 25) in a protease-deficient *A. niger* mutant (from Jaap Visser's group, in Wageningen, The Netherlands) and in Norwich was shown to be identical to FAE-III by Molecular mass, pI, pH and temperature optima, N-terminal sequence and activity on methyl ferulate (same K_M and V_{max}). The yield of purified FAE-III from recombinant culture supernatants was 50 mg/L (compared to 1-2 mg/L for wild type).

The cloning and characterisation of the gene coding for CinnAE was also undertaken in collaboration with Jaap Visser. N-terminal and two internal sequences from highly purified I for CinnAE were obtained and used to generate primers for polymerase chain reactions amplification of specific sequences. Six new sequences were obtained for CinnAE.

Sub-task A 1 2 : Nature of the enzyme secreted by A. niger and the complementation

Large scale purification from wheat bran led us to obtain highly pure 8-0-4' diFA and chemical synthesis allowed us to produce enough of some of the main diferulates in order to calculate the correct extinction coefficients and response factors for each compound. We have determined the diferulate composition of wheat bran after sequential alkaline hydrolysis. The three main dimers detected were identified and quantified as 5,5' diFA, 8-0-4' diFA and 8,5' BeFu diFA representing approximately 21% of the total phenolic compounds. 8,5' BeFu diFA and 8-0-4' diFA were predominant. The antioxidant properties of the 5-5-, 8- 0-4- and 8-5-Benzofuran diferulic acids were assessed. The results showed that dimerisation affects the antioxidant capacity of ferulic acid but the effect depends on the nature of the linkage between the monomers. Increasing number of free phenolic hydroxyl groups and higher degree of conjugation seem to be associated with better antioxidant capacity. In the lipid phase, all the ferulic acid dehydrodimers examined were more effective inhibitors of lipid peroxidation than ferulic acid.

Ferulic Acid Esterase (FAEIII) activity on diethyl diferulates: The best efficiency is observed for the 5-5 diester followed by the 8-5 Benzofuran diester. The 8-0-4 diester is not a good substrate for FAEIII. The monoesters are all poorer substrates, in particular the 8-5 monoester for which we were not able to detect any activity. even after several hours of incubation with a high concentration of pure enzyme.

Search for new enzymes able to release 8-5 Benzofuran diferulic acid from the monoester: FAEA from *Aspergillus niger* is highly specific for hydrolysis of dehydrodiferulates but does not always form the free diferulic acid suggesting an significant role for this esterase in the degradation of plant cell walls through cleavage of at least one ester bond from the diferulate cross-links which exist between wall polymers. The activity of an *Aspergillus oryzae* tannase was also tested on diesters. The results show that tannase has some esterase activity on the diferuloylated substrates and that this activity is much lower than the activity of FAEIE on the same substrates. However, tannase is able to hydrolyse the second ester bond of the 8-5-Benzofuran monoester -to release free acid.

Nature of the enzymes secreted by A. niger I-1472 and P. cinnabarinus MUCL 39533: This task was focused on the characterisation of cell-'wall-degrading enzymes synthesised by the fungi selected for the bioconversion process. The production of polysaccharide-degrading enzymes by *P. cinnabarinus* grown on sugar beet pulp began a little later compared to *A. niger* since only xylanase was found in the 3 day-old culture supernatant. All activities stayed lower than in *A. niger* supernatants. In the control cultures carried out on maltose, all the activities produced were near zero, demonstrating that most of the enzymatic activities were induced by sugar beet pulp. Concentrations of *A. niger* supernatants allowed to display ferulate esterase. In contrast, no ferulate esterase activity was found in *P. cinnabarinus* supernatants, even after concentration.

In order to enhance the synthesis of ferulate esterases, *A. niger* was grown on various carbon sources, i.e. sugar beet pulp, cereal bran and ferulic acid-enriched fractions originating from these by-products. Polysaccharide-degrading enzymatic activities produced by *A. niger* I-1472 grown on cereal bran, after autoclaving pre-treatment or not, were lower than those obtained when the fungus was grown on sugar beet pulp, except for xylanases. However, the highest esterase activity was produced when the fungus was grown on autoclaved maize bran, which contained also the highest amount of esterified ferulic acid.

Selection of the best raw material for direct biotransformation of ferulic acid to vanillic acid by A. niger: On day 3 of incubation, which corresponded to the maximal day of hydrolase activities of the fungus, raw materials or their fractions rich in feruloylated oligosaccharides were added to the culture medium as source of ferulic acid. The different combination were all tested and the best result in terms of vanillic acid production was obtained when sugar-beet pulp was used as carbon source inducer and concentrated autoclaved maize bran as ferulic acid source. More than 2.2 g.1-1 vanillic acid after 7 days of incubation were produced with a molar yield of 77.4%. These conditions were applied to mechanically-agitated bioreactor (2 1) and allowed to produce 800 mg/l vanillic acid.

Combination of A. niger 1-1472 and P. cinnabarinus MUCL 39533 potentialities to produce vanillin from autoclaved maize bran: With respect to the economy of the process, two new strategies were defined using cheap autoclaved maize bran instead of expensive free ferulic acid as precursor.

The first strategy consists to directly produce vanillin by adding *A. niger* 1- 1472 filtered culture broth containing exocellular polysaccharide-degrading enzymes and ferulate esterases, in 3-day-old cultures of *P. cinnabarinus* MUCL 39533 and

feeding, these cultures with spray-dried autoclaved maize bran as ferulic acid source. The second strategy consists to produce vanillic acid with *A. niger* I- 1 472 cultures feeding with autoclaved spray-dried maize bran as ferulic acid source, to remove vanillic acid from *A. niger* I-1472 cultures and to use it as vanillin precursor in 3-day-old cultures of *P. cinnabarinus* MUCL39533.

Optimisation of enzyme production by A. niger I-1472 for ferulic acid de-esterification: Considering the p-NP arabinofuranosidase activity, a low aeration (0,3 vvm) was shown to be more adapted than a high aeration (0.8 vvm). Besides with respect to this activity, *A. niger* cultures were successfully scaled-up. On the opposite, considering 0-glucosidase activity, without control on dissolved oxygen in the bioreactor, enzyme activity was near 0. Quite no ferulic acid was freed and little vanillin was produced thereafter. When 50 % dissolved 02 is maintained in the fermentor, the activity is closer to flask results and allowed to use these enzymes in *P. cinnabarinis* cultures.

Freeze-drying was not suitable for concentrating *A. niger* culture broth. Consequently, the enzyme concentration was mainly realised by ultrafiltration on a suitable membrane Assays were successfully conducted in flasks and in 12 L bioreactor. In flasks, the ultrafiltration allowed to concentrate 8.1 times the volume of the enzymes solution and 7.4 times the activity of pNP arabinofuranosidase. In the case of *A. niger* 12L bioreactor culture broth, the filtration on the press-filter led to a loss of 60% of pNP arabinofuranosidase and 60% of the protein content. Moreover, the ultrafiltration was successful and allowed to concentrate both the volume of the enzyme solution and the activity of pNP arabinofuranosidase. Using a 1O kD cut-off membrane of 0.5 m^2, the best results considering beta-glucosidase activity were obtained after saturation of the membrane with casein. Enzymes have been concentrated 10 times for a volumetric concentration of 15. The recovery yield was higher than without saturation of the membrane i.e. 67% instead of 23%.

Task A 2 : Design and development of the enzymatic and microbial process of plant cell wall hydrolysis

Sub-task A 2 1 .- Process for the production of natural ferulic acid

Release of ferulic acid by heat or/and enzymatic treatment

From wheat bran: Ferulic acid was obtained after an overnight treatment with a *Trichoderma* xylanase (Novozvm 431L, Novo Nordisk) and a second overnight incubation of the concentrated soluble material with a purified esterase. FAE-III from *Aspergillus niger*. The yield obtained was 82% of the total ferulic acid released by alkaline hydrolysis of the same material (17.7 g).

Study of the extraction of linked-ferulic acid from maize bran: Autoclaving treatments solubilised arabinoxylans as well as ferulic acid and in a very efficient way from maize bran. Treating the samples for 1 min at 190°C or at 160°C for lh gave an optimal yield of 63% in weight of solubilised material. In both cases small oligomers are produced and ferulic acid is still esterified to sugars. At 140°C and 120°C, the time duration for a maximum level of linked ferulic acid are respectively 3 and 6 hours. Autoclaving for 3 hours at 140°C is the most suitable treatment as it can be used at pilot plant scale.

The heat treatment was also tested for the extraction of linked-ferulic acid from beet pulp and wheat bran. Due to its high ferulic acid content (up to 2%) maize bran is the most interesting raw material. The quantity of ferulic acid found in the liquid fraction after treatment is about equivalent to the quantity of ferulic acid in the raw material, showing an important release of linked-ferulic acid by this treatment. Extrusion-cooking treatments didn't allow any release of ferulic acid or

improvement of enzymatic degradation of maize bran and was therefore abandoned.

Release of free ferulic acid by commercial enzymes: Comparative tests were carried out with pre-selected commercially available enzymes and different operating conditions. Direct enzymatic hydrolysis of the maize bran was not efficient with the 3 tested enzymes. Viscozyme seems to give better results than Spezym and OC 140, but in all cases the quantity of ferulic acid is very low (less than 200 ppm). Moreover the ferulic acid released is linked (more than 90%). Enzymatic hydrolysis of the liquid fraction produced by heat treatment (140°C - 3h) releases more than 80% of free ferulic acid from the juice. The amount of free ferulic acid increases with the enzyme concentration. pH regulation has no significant influence on the process. This process gives more than I g of ferulic acid per 100 g of maize bran.

Release of ferulic acid by enzymes produced by A. niger: The enzymes produced by *A. niger* (analysed in Sub-task A. 1.2) were tested with model substrates in the crude enzymes and commercial mixtures (Novozym 342, SP 584). *A. niger* I-1472 exhibited all the activities detected in the commercial mixtures at the same time, the most important being, in decreasing order, arabinofuranosidase, xylanase. arabinanase, polygalacturonase and rhamnogalacturonase. Ferulate esterases were about 10-fold higher than in Novozym 342.

Using either SP 584 or crude enzymes from *A. niger* on sugar beet pulp yielded the same amount of residue and about the same sugar composition. Thus, SP 584 released 78.9% of ferulic acid, including 49.4% of free ferulic acid, whereas crude enzymes from *A. niger* released 70.2% of ferulic acid including 38.7% of free ferulic acid. Therefore. *A. niger* was not so efficient than SP 584 in the release of ferulic acid from sugar beet pulp. On autoclaved maize bran, release of glucose or galactose was lower with *A. niger* than with Novozym 342. In contrast Novozym 342 liberated only 32.8% of free ferulic acid, while *A. niger* released almost 100% of free ferulic acid, demonstrating the efficiency of ferulate esterases synthesised by *A. niger*.

Sub-task A 2.4 .- Enzymatic production of cellobiose

Characterisation and enzymatic degradation of cellulose-rich residues: During the previous project, Celluclast 1.5L was selected among thirteen cellulose degrading preparations for its ability to release neutral sugars from a cellulosic residue. But it- contained P-glucosidase activity that hydrolysed cellobiose. Therefore physicochemical conditions favourable for the release of cellobiose but unfavourable for its degradation were studied, Large amounts of cellulose-rich residues from sugar beet pulp were characterised for sugar composition and percentages of crystalline cellulose (determined by Xray diffraction). and swelling capacities.

Depending on the method of drying and storage, a maximum value of cellobiose was obtained after 2 or 4 h hydrolysis. Further, the cellobiose concentration decreased due to its hydrolysis in glucose. The release of glucose was linear for the first four hours and slowed down after.

The dry matter of the different juices was very low (less than 0.2%). Therefore different concentrations of dry matter were tested and the influence on the enzymatic hydrolysis by Celluclast 1.5L was measured. The results show that the ratio cellobiose/glucose decreases when increasing the dry matter concentration. Nevertheless at 0.25% DM the very low concentration of cellobiose (up to 0.5 g/1) is making the process economically profitable. The production yield of cellobiose is around 50% of the cellulose content of the solid fraction. This process was developed at large scale.

Task A 3 : Industrial fractionation process scale-up of plant cell walls

Sub-task A 3 1 Scale-up of the plant cell wall degradation

Required amounts of cellobiose were provided to the other partners following the previously established method with Celluclast 1.5L. - Required amounts of free ferulic acid in syrup form were produced from maize bran following the process established in Sub-task A.2.1 with commercial enzymes. - Required amounts of linked-ferulic acid in powder form were produced from maize bran following the process established in Sub-task A.2. 1.

Sub-task A 3 2 ., Purification of the precursor

The ferulic acid can be purified by the use of powdered activated carbon. The free ferulic acid produced in the previous task was therefore purified and dried, The complete process after purification gives more than 5g of ferulic acid per 500 of maize bran.

Sub-task A 3 3 .- Production of specific carbon sources

The process for cellobiose production at large scale is optimised. Trials are currently performed. Cellobiose was provided to the partner PI and P3 for bioconversion trials using natural cellobiose. Production of other carbon sources (like xylose) was not purchased as they were no more useful in the process.

Phase B: Modification of Fungal Pathways for Vanillin Overproduction

Task B I : Control of vanillic acid formation from ferulic acid in Aspergilllis niger and Pycnoporus cinnabarinus

Sub-task B I I .- Identification of ferulic acid peroxisomal beta-oxidation

The invoivement of P-oxidation for the metabolism of the phenyl propenoic acid, ferulic acid, to vanillic acid by *A. niger* I-1472 was tested at the level of enzyme activities (hydroxyacyl CoA dehydrogenase, especially). Despite the various tested conditions, only very low activities (picokatal/mg protein) were detected, unusable results to confirm the involvement of P-oxidation in the biotransformation of ferulic acid to vanillic acid.

A new strategy was proposed which consists to isolate and characterise the metabolic intermediates (as thio-esters of CoenzymeA) between ferulic acid and vanillic acid. But this method was also unsuccessful. Two hypothetical metabolic intermediates involved in the P-oxidation of ferulic acid to vanillic acid were then synthesised and tested for their bioconversion by *P. cinnabarinus.* Almost no vanillic acid was detected in the flasks but anyway the hypothesis of a P-oxidation seems to be confirmed as some vanillin and methoxyhydroquinone were produced. But only in vitro experiments can confirmed this hypothesis.

Task B 2 : Control of vanillin formation from vanillic acid in Pycnoporus cinnabarinus

Sub-task B 2 1 : Purification, characterisation and sequencing of vanillate reductase

The use of NADH oxidation as test allowed to obtain enzyme activity associated to the presence of vanillic acid, but extraction of samples followed by HPLC analysis didn't lead to the presence of vanillin. Three hypotheses were developed, (i) the activity of vanillate reductase was so low that vanillin produced was not

detectable, (ii) vanillin produced was immediately transformed to another product and (iii) NADH oxidation would not be involved into the reductive way conducting to vanillin from vanillic acid.

In vivo, the bioconversion of vanillic acid to vanillin was always very efficient. In this context, a new strategy of analysis was developed using radioactive methods to detect very low amounts of products released in the reactions. But in vitro, the results were very variable even with labelled vanillic acid. Finally, we succeeded to measure VAR activity in vitro using an enzyme extract obtained from slightly ground mycelium under very protective conditions and incubated with a mixture of labelled and unlabelled vanillic acid in the presence of cofactors. An oxygen-deficient atmosphere didn't allow to improve vanillin production in these conditions. Labelled ferulic acid was also used in vitro in order to produce vanillin and intermediates, but it resulted in no significant improvement of the activity test.

Now, we can define vanillate reductase as an intracellular aromatic acid reductase, stable and induced by one of its main substrate, vanillic acid. A simple calculation was realised considering a consumption of 600 mg/l vanillic acid per day in vivo VAR activity was estimated to 4. 2 picomoles/s/ml or 0.24 U (micromoles/min/1), which is very low. From these results. further experiments were conducted in order to isolate the enzymes responsible of ferulic acid transformation to vanillin by *P. cinnabarinis*, but no real prepurification was obtained.

Task B 3 : By-pass of unwanted aromatic products

Sub-task B. 3. 1. Control of vanillyl alcohol formation from vanillin - *See C 2 2.*

Sub-task B 3 2: Control of methoxyhydroquinone formation from vanillic acid

As for vanillate reductase, no enzyme activity test was sensitive enough to be used for vanillate hydroxylase measurements except methods based on 14 C-labelled vanillic acid. The reaction requires cofactors such as FAD and NADPH. The method appeared to be suitable but tedious, requiring the run of HPLC for every sample. The assay is not well suitable for enzyme kinetic work. The peaks from the chromatograms were identified by comparison with their UV absorbency using unlabelled vanillic acid, MHQ, vanillin, vanillyl alcohol, p-benzoquinone (p-BQ), hydroquinone (HQ), ferulic acid and 1,2,4- trihydroxybenzene (THB) as standards in the same column and elution gradient. All of these results are to be confirmed by LC-MS analysis.

Purification of vanillate hydroxylase: After analysis of protein pool on SDS-PAGE gel, a rather dark band is visible. This band appears to be rather near the 67 kDa mw standard band. However, it is necessary to continue purification further because there are many other protein bands which also exist in the same pooled fraction. Further purification of VH was continued with improved techniques using hydrophobic interaction chromatography (HIC) columns in FPLC apparatus. Then the fractions from many separate FPLC runs were pooled and these were determined for VH activity. A very low activity was detected. The results indicated that the VH probably was somewhat larger than 67 kDa might. However, this fraction still contained other protein bands, so this might also be another protein.

Phase C: Industrial Fermentation Process Scale-Up

Task C I : Effect of bioreactor design and fermentation condition on process performance

Sub-task C 1 1.- Analysis of the effect of fermentation conditions on fungal pelleting and sub task C 1 2 : Characterisation of fermentation conditions

Production of vanillin from pure synthetic vanillic acid by Pycnoporus cinnabarinus. Optimisation in bioreactor: Combining the effect of doubling the concentration of the inoculum and an earlier addition of vanillic acid (day 2) led to an earlier production of vanillin from vanillic acid with a high productivity but limited the total vanillin production. By increasing the aeration (90 l/h) during the vegetative phase of the fermentation resulted in higher amounts of total metabolites from vanillic acid and would allow to improve vanillin production. In these assays, vanillin production reached 1.2 $g.1^{-1}$ in 7 days.

Applying Y-AD-2 resin in cultures allowed to trap specifically and efficiently vanillin. The scale up of vanillin production in 101 bioreactor was also encouraging. The best production of vanillin from vanillic acid in 1.81 bioreactor was obtained when XAD-2 resin was added in the culture medium. An optimum of 1.6 $g.1^{-1}$ vanillin on day 7 was recorded, corresponding to a molar yield of around 80 % and a productivity of 225 mg/l/day.

Production of vanillin from pure synthetic ferulic acid by Pycnoporus cinnabarinus: As very little was known on the large-scale fermentation of Basidiomycetes and especially *Pycnoporus cinnabarinus*, we had to optimise the fermentation condition from scratch. There is a strong influence of the fermentor design on the growth of the fungus and the bioconversion, agitation devices giving high shear-stress (Rushton turbines which are the most common design for large fermentors) being very detrimental to the fungus. The best designs were shown to be a bench-top fermentor with marine propeller or an air-lift fermentor.

Optimisation of bioconversion conditions using high cell density cultures: The still low productivity of this process is certainly due mainly to the very low biomass obtained in these conditions, 1 to 2 g/l as dry matter. A selection of carbon and nitrogen sources able to produce more biomass has been done in flask then verified in ferrnentor. The global production can reach up to 15 g/l of biomass using phospholipids or lecithin and a cocktail of vitamins and amino-acids. Cultures were also realised in the presence or not of a buffer, 26 mM DiMethyl Succinate (DMS) maintaining the pH at 5.5. The presence of the buffer did not enhance the vanillin production whatever the carbon source is. The best result was obtained on non- buffered medium containing glucose and Nat 89 as carbon sources giving 671 $mg.1^{-1}$ vanillin after 13 days. In order to confirm the good results obtained in 250-ml flask using glucose and Nat 89 as carbon sources, a preliminary assay in 1.8 1 bioreactor was realised using constant conditions of aeration, 30 1/h. After 15 days of fermentation, 804 $mg.l^{-1}$ vanillin (with a molar yield of 50 % and a productivity of 54 $mg.1^{-1}.d^{-1}$ was produced.

The use of an easily assimilable sugar alone (like glucose or fructose) lead to very low production of vanillin. Therefore we try to obtain this biomass in a "competent" state using a change in the composition of the medium before the bioconversion phase. Best results were obtained with fructose as first carbon source and maltose during the bioconversion. 500 mg/l vanillin was obtained in 10 days and about 82% of ferulic acid was bioconverted into identified phenolic compounds. But a lot of by-products were still produced. especially methoxyhvdroquinon and vanillyl alcoholwith a molar yield of vanillin is of 34%. Using a medium containing 2 carbon sources., 470 mg/l vanillin was obtained in 13 days with 10 g/l glucose (totally consumed during the growth phase) and 30 g/l maltose. By feeding with sugars during the bioconversion we were able to reach 600 mg/l vanillin at day 19.

Cultivation in an air-lift bioreactor allowed to produce vanillin but also a lot of

methoxyhydroquinone which production is favoured by the oxidative conditions. The growth is also increased when the biomass is immobilised. Aim of the immobilisation is to allow a change of medium and to ease the continuous extraction of vanillin. Different components of the medium were tested for their influence on the immobilised biomass and on the vanillin production. Despite many attempts vanillin production was not improved. Compared to the free culture control in maltose, vanillin production is lower and slower when the fungus is immobilised and grown in maltose medium, i.e. 195 in 15 days instead of 278 mg/l in 7 days in free cultures. By adding phospholipids in the medium two-fold more biomass (14.1 g/1) was obtained than without Nat 89 addition, but vanillin concentration only reached 140 mg/I.

Conditions of aeration, pH and temperature: With 100 l/h aeration (0,9 vvm) quite no vanillin was obtained after 10 days. The fungus consumed all the ferulic acid but didn't produce vanillin. It probably degraded the precursor.

In contrast 20 1/h (0. 19 vvm) aeration resulted in the production of 300 mg/l vanillin after 9 days, i.e. about 47 mg/l/d. A lot of methoxyhydroquinone was still produced but the acceleration only occurred after 7 days of fermentation. As *P. cinnabarinus* has a strong aerobic metabolism, 20 1/h aeration was too low for optimal conditions of bioconversion, In further experiments aeration was lowered to 30 1/h (0.28 vvm) so oxygen was non-limiting, When *P. cinnabarinus* is grown at 35°C during the 3 first days, the biomass is not higher than the control at 3°C. Vanillin production is then performed at 30°C. The switch of temperature from 35°C to 30°C does not improved vanillin production compare to the control. pH regulation at 4 or 5 does not improve vanillin production compare to free pH evolution.

Addition of XAD-2 resin: On the basis of the encouraging result obtained in the high density cultures of *P. cinnabarinus* MUCL39533 grown with glucose and Nat 89 as carbon sources, new cultures were realised in which XAD-2 resin was applied on days 4, 5, 6, 7 and 8 of fermentation. The best result was obtained when XAD-2 resin was applied on day 7. Most of the vanillin was trapped by resin, avoiding its transformation to vanillyl alcohol. Consequently more than 900 $mg.l^{-1}$ vanillin (with a molar yield of 36 %) were recovered after 14 days of fermentation.

Bioconversion with natural cellobiose: Pectic juice (raw fraction enriched in cellobiose obtained during ferulic acid production process) cannot be used as this in *P. cinnabarinus* cultures. On the contrary, vanillin production is close to the control with pure cellobiose when using enriched fraction provided by INRA, i.e. 401 and 427 mg/l. MHQ production is also reduced in the same range, showing that inhibition by cellobiose is efficient.

Bioconversion with ferulic acid of natural origin: Vanillin production with free ferulic acid from sugar beet pulp and maize bran provided by ARD was compared to results obtained with synthetic ferulic acid. Maize fraction enriched in esterified ferulic acid (autoclaved maize bran) was also tested. In flask vanillin production was the highest with free ferulic acid from maize bran and, in contrast, almost no vanillin was obtained from autoclaved maize bran. Bioconversion was therefore scaled Lip in 2- litre fermentor with free ferulic acid from maize bran. 459 mg/l vanillin were obtained in I 1 days with ferulic acid from maize bran. against 424 mg/l in the control fermentor with synthetic pure ferulic acid. Also the molar yield in vanillin was higher. i.e. 40 instead of 30

Combination of A. niger I-1472 and P. cinnabarinus MUCL 39533 and their potential to produce vanillin from autoclaved maize bran: P. cinnabarinus MUCL 39533 was not able to produce vanillin directly from autoclaved maize bran, as it does not produced esterases. Therefore esterases will be provided by *A. niger* I-

1472 and two strategies were defined (See Sub-task A.2.1.).

First strategy

- *Addition mode of the enzymes.* First, vanillin production with single addition of enzymes at day 3 was compared to double addition at day 3 and day 7. Vanillin production (503 mg/1) is not improved by adding twice the same quantity of enzymes. It is even a little less than with a single addition (559 mg/1): a part is certainly due to the dilution.
- *Daily addition of autoclaved maize bran* The molar yield in vanillin is the same whatever the daily addition of autoclaved maize bran is (expressed in equivalent quantity of ferulic acid). However the vanillin production reached only 202 mg/l after 6 days with 300 mg/l/day of ferulic acid, while 415 and 550 mg/l were obtained in 10 days, respectively with 400 and 500 mg/l/day. Larger quantities of by-products were also produced, especially vanillyl alcohol.
- *Origin and concentration of enzymes* As enzyme production by *A. niger* was quite different in flask and fermentor, vanillin productions with the help of enzymes from both origins were compared. In order to reduce the volume of enzymes added to the culture, *A. niger* culture filtrates were concentrated by different methods (freeze-drying or ultrafiltration on 10 kD membrane see Sub-task 2.1). The concentrated enzymes were then used for vanillin production in *P. cinnabarinus* cultures. Only 404 mg/l of vanillin were obtained using enzymes produced in fermentor, against 665 mg/l using enzymes produced in flask. Despite ferulic acid desesterification (high amounts of free ferulic acid were measured in the medium), all the experiments with concentration exhibit lower vanillin production than with non-concentrated enzymes.
- *Use of XAD-2 resin* As more vanillyl alcohol was produced in cultures with autoclaved maize bran, XAD- 2 resin was tested directly in the medium for removing vanillin. This resin was already selected by INRA Marseille for its specificity. The XAD-2 resin was added at day 7. The addition of resin reduced the quantity of vanillyl alcohol by removing the vanillin from the medium. After elution, 802 mg/l of vanillin were measured, against 687 mg/l in the control culture without resin. The yield in vanillin was also higher by using XAD-2, i.e. 41 % instead of 32

Second strategy

- *Influence of the quantity of autoclaved spray-dried maize bran added* Two modes of ferulic acid addition were tested. The first consisted of the sequential addition of autoclaved spray-dried maize bran solution equivalent to 600 mg/L/day total ferulic acid. The second depended on a single addition (in one time on day three culture) of autoclaved spray- dried maize bran solution equivalent to 1.5 and 2.5 g/l total ferulic acid. Sequential addition of ferulic acid source (equivalent to 600 mg/L total ferulic acid each time) was defined as the best condition for vanillic acid production by *A. niger* I- 1472. All the other experiments have been done in these conditions.
- *Influence of carbon source : assays with phospholipids* In order to favour biomass production, *A. niger* I-1472 was grown with 30 g/L glucose, 5g/L of phospholipids and ten-fold more yeast extract. In every case, ferulic acid source (autoclaved spray-dried maize bran) was sequentially added as previously indicated. As expected, on day 3 of cultivation, the biomass of *A. niger* 1-1472 was increased until 11.8 g/L with the phospholipid medium compared to 9 g/L with maltose medium. With the medium containing glucose, phospholipids and high level of yeast extract, the maximal vanillic acid production was delayed on day 9 and reached 1.64 g/L with a productivity of 180 mg/l /day instead of 270 mg/L/day in the reference

conditions. These conditions didn't favour ferulic acid biotransformation to vanillic acid by *A. niger* I-1472. Too high level of carbon sources and yeast extract certainly limit sugar beet pulp degradation and consequently enzyme production to release ferulic acid.

- *Influence of the concentration of Tween 80 in the culture medium* To facilitate vanillic acid recovery, it was necessary to limit foam formation. Consequently, three Tween 80 concentrations were tested : 0.5 g/L (reference conditions) , 0.1 g/L and 0.05 g/L. Ferulic acid source was sequentially added exactly as previously indicated. The maximal amounts of vanillic acid synthesised on day 6.5 of cultivation were similar for the three concentrations of Tween 80. Consequently, to limit foam formation in cultures (especially in bioreactors), 0.05 g/l Tween 80 was chosen for the further experiments.
- *Vanillic acid production in bioreactor* Vanillic acid production in 2-litre fermentor was successful, i.e. 2,65 g/L on day I') of cultivation with a molar yield of 96 % and a productivity of 204 mg/L/day. However, the time of vanillic acid production could be reduced to 8 days with 1.55 g/L vanillic acid corresponding to molar yield of 86% and a productivity of 207 mg/L/day. One assay of *A. niger* I-1472 culture in 12L bioreactor was conducted in a 12L mechanically stirred-tank bioreactor, at 30'C with an agitation rate of 120 rpm and with an aeration of O.3 vvm. The maximal production of vanillic acid was 886 mg/L on day 8 of cultivation with a molar yield of 22.5% and a productivity of 111 mg/L/day.
- *Recovery of vanillic acid from the broth culture of A. niger 1-1472* In order to recover vanillic acid, the cultures of *A. niger* I-1472 were stopped when a significant amount of vanillic acid (more than 1.5 g/L) was produced and a minimum quantity, of ferulic acid (less than 200 mg/L) remained in the medium. Two methods were tested for vanillic acid recovery: - filtration and concentration of the culture medium under low pressure at 50°C (Rotavapor) up to 6-10 g/L vanillic acid. As the sterilisation is not possible. The shelf life stockage of the vanillic acid solution can not exceed 2 days at 4°C. - adsorption on a specific resin for vanillic acid, such as SP207, elution bv ethanol and concentration by evaporation. This method allowed a recovery of 93% of the vanillic acid that icient but introduced can be sterilised by filtration on a 0.45 microm membrane. This method is efficient as a supplementary step in the process of natural vanillin production.
- *Vanillin production by P. cinnabarinus MUCL 39533 from vanillic acid came from raw materials transformed by A. niger 1-1472* Significant productions in vanillin from vanillic acid came from *A. niger* cultures could be observed by *P. cinnabarinus* MUCL 39533 with a maximum when a sequential addition of 300 mg/L/day was performed from day 3 to day 7: 580 mg/L vanillin with molar yield of 53/6 ere produced and 916 mg/L vanillic acid were consumed after 7 days of cultivation. Ferulic acid present in vanillic acid solution from *A. niger* accumulated during the cultivation because it was not consumed by *P. cinnabarinus.* However, by adding 600 mg/L of vanillic acid (came from *A. niger* I-1472 cultures) led to no production of vanillin. Actually vanillic acid was not consumed.

Comparing different media, the glucose/phospholipids medium did not led to an increase of the production of vanillin. The use of XAD2 resin in the culture medium allowed to improve the biotransforination of vanillic acid to vanillin. Nevertheless, Y-AD2 resin was more efficient with commercial vanillic acid than with *A. niger* vanillic acid. In the case of commercial vanillic acid, the amount of vanillin synthesised was multiplied by 1.6-1.8 in the presence of XAD2 resin. In the case of *A. niger* vanillic acid, the amount of vanillin was multiplied by 1.3-1.5. Moreover, at the end of cultivation (J7), *A. niger* vanillic acid remained in the culture medium especially in the presence of XAD2 resin although commercial vanillic acid has been totally consumed.

Task C 2 : Design and development of continuous vanillin extraction system

Sub-task C 2 1 .- Separation of the fermentation medium from the fungal biomass

Separation devices like sintered glass filter of metallic grids have been tested. It is possible to pre-filter the medium in the fermentor using a sintered glass filter at the bottom of the fermentor and to remove all the medium by this way. During all the experiments presented in task C. I., 2 different filtration systems were compared both using tangential filtration. One was more efficient as it offers a larger surface for filtration with an agitation chamber to prevent clogging. This device was used on-line with a programmator and a collector for sampling. It was also used off-line in order to test the column for vanillin extraction (See sub- task C.2.2.).

Sub-task C 2 2 .- Selection of specific adsorbents for continuous vanillin extraction The idea at the beginning of the project was to use the Streamline™ technology, i. e. an Expanded Bed Adsorption Technology from Amersham - Pharmacia. based on a fluidised bed of resins of different sizes and densities to keep it stable, thus allowing the loading with "rough" media. Different gels having different coatings (none, phenyl-, hydroxypropyl, Dextran-) have been tested in packed bed columns with synthetic compounds (vanillic acid and vanillin). Other matrix have been tested in parallel like LH 20, LH 3O, LH 60, Source RPC, Source RPC ')O...

The Streamline technology was finally not chosen because of the excessive charge of the fermentation medium and also because the available resins don't allow an on/off adsorption selectivity for such-close compounds. Starting from actual fermentation broth, another technique has been developed : First the compounds are separated on a LH 20 colurrm, then the outlet of this column corresponding to the peak of vanillin is derived to a trapping column of Source RPC using a automatic switch based on the UV adsorption. When fully loaded the last column can be eluted with ethanol.

Using two separation columns alternatively, it is thus possible to proceed to a continuous detoxification of the fermentation medium.

The two step purification of vanillin at pilot scale was performed on clarified fermentation broth of *P. cinnabarinus* MUCL 59533 after 8 days of growth with synthetic ferulic acid as precursor. We were able to separate a fraction containing pure vanillin. As the solvent for run LH20 was pure water, all flow through fractions could be recycled to the fermenter via a sterile inline filter in a continuous process.

Samples of *P. cinnabarinus* culture medium, containing vanillin from natural free ferulic acid, were directly sampled from the fermentor through a filter. Vanillin was extracted off-line on LH-20 column with a good yield and a high purity. We try to recycle the medium but there was no incidence on the vanillin production except the dilution. Other experiments were made with culture filtrates containing vanillin from autoclaved maize bran with the same success of vanillin recovery and purification.

Task C 3: Mathematical modelling

Sub-Task C 3 1 : Model developments In the literature, fungal models have been generally developed for *Penicillium* sp. or *Aspergillus* sp., and no modelling work has been done for *Pycnoporus* sp. A protocol for data transfer has been established by UCL which should minimise the risk of errors in the use of data and

allow an automatic treatment of data. Computer programs written in MATLAB allow data displaying (DATAREAD.M), interpolation and smoothing (MAKESPLINE.M). Another program (SCHEMREAC.M) has been developed to test a mass balance model corresponding to a given reaction scheme and to study its validation.

The data provided by INRA and Pemod on growth and biotransformation of *Pycnoporus cinnabarinus* growing in 2 1 bioreactors have been carefully studied. In a first step, the more relevant metabolic pathways for the dynamics of the biotransformation have been hypothesised from bibliographic study, data analysis and discussion with Pernod and INRA. From these reactions, a mass balance model has been derived. This model represents the dynamic evolution of biomass and of the main extracellular compounds that are consumed or excreted in the medium : carbon and nitrogen sources, phenolic compounds and dissolved gases. For the variables whose model prediction was unsatisfactory. the reaction network was corrected : a reaction for death of the mycelium was added. and maltose was taken into account in the reaction of biotransformation. It turns out that 6 reactions are necessary to describe growth and biotransformation. These reactions involve 6 yield coefficients and transfer coefficients (liquid/gas transfer coefficients and Henry' s constants).

A validation study has been lead by comparing the model predictions with the data. Yield coefficients have been determined by least square regressions. The high level of noise in the data for gases did not allow the validation of the dynamics of the dissolved gases (and their role in the reactions). Moreover, some trouble in the mass balance of phenolic compounds (more phenolic compounds were measured than was added for some experiment) explain why the parameters associated with phenolics will have to be re-estimated.

Two types of models have been constructed to model the reaction rates. First a classical approach has been carried out. In this approach the data smoothed and interpolated by spline functions have lead us to formulate assumptions on the expression of the kinetics for the different reactions considered. The parameters of the kinetics have then been calibrated using some of the experiments. The data for oxygen could not allow us to validate and calibrate this part of the model, therefore oxygen profile have been taken as inputs of the model. Finally, the effect of pH on the dynamics of CO_2 has been studied. It turns out that during the first phase of biomass growth bicarbonate is transferred to CO_2. The correction that should be made to dynamics Of CO_2 has been studied. They requires only pH measurements and the liquid/gas transfer coefficients.

The alternative approach to modelling of the reaction rates by neural networks is used too. The influence of concentrations of different substances was studied and tested. Different network configurations, set of inputs and other parameters (shapes of the Gaussian curves, number of hidden neurones, requested error levels etc.) have been considered and the most appropriate with respect to the data have been chosen.

Various computer programs have been written to represent the data in the appropriate format (DTREAD.M), for network training (TRAINING.M), for calibration (CALIB.M) and validation (VALID.M) in Matlab v.5.0.

A sensitivity study has been led in order to determine the parameters that are the most important for the model. From this study, the inhibiting role of oxygen in the biotransformation of vanillic acid into vanillin has been confirmed. The kinetic modelling for the bioconversion of ferulic acid into vanillic acid has also been improved. From the experiments analysis it turns out that the role of activating or inhibiting role of oxygen was not clear. Indeed, we propose now a model independent of the oxygen for this first biotransformation step. The variability

observed during the experiments (it can be seen that for a large number of experiments a "stress phase" appears during which the biotransformation is stopped) gave rise to a model for the production of vanillic acid from ferulic acid that must be considered with care.

Sub-Task C 3 2 .- Structural analysis and simulation studies

Agreement between model predictions and experimental results have been successful tested with experiments that have not been used for model calibration. First qualitative conditions for optimal biotransformation have been derived from the model.

Despite the problems of oxygen measurements and of phenolic compounds balance the general agreement between the model predictions and the data is very good. It appears that a very low level of oxygen is required for biotransformation (at least for the biotransformation of vanillic acid into vanillin). An optimal feeding strategy will probably consist (as it will be determined in sub-task C. 5. 1) to let a very high oxygen concentration in the medium during the growth phase, and then to decrease this concentration to zero.

All fermentation measurements were well described using the proposal model except for the by-products (MHQ and vanillyl alcohol). Some biases may be due to the difficulty encountered for the HPLC measurements to separate properly the phenolic compounds, which causes often problems in the mass balance of the phenolics. The conservation equation of the mass balance through the model have been written, these equations are then the main basis to derive the software sensor (see Sub-task C.4.3), A mathematical analysis of the model and an extensive simulation study have been led to determine the set of control objectives that are achievable. This study have been carried out for each of the two steps: biomass growth and biotransformation. These properties have then been used for the determination of the optimal strategies to maximise the vanillin production (see Sub-task C.5. 1).

Task C 4 : Development of tools and facilities for on-line process monitoring

Sub-Task C 4 1 : On-line gaseous measurement

For practical reasons of too low air-flow in the 2-litre fermentors and limitation of the. detector system with low variation of oxygen and C02, it was not possible to have on-line gaseous measurements but only off-line.

Sub-Task C 4 2 : On-line analysis of the fermentation medium

By sampling more frequently (like every 2 hours), we saw that it seems interesting to follow more precisely the evolution of the different phenolic concentrations during a whole night or during a week-end. With this aim, a new HPLC device was installed with a sampler coupled to the filtration system. The system is totally operational for on-line medium analysis.

Sub-Task C 4 3 .- Design of model-based software sensors

The structural properties of the mass balance model have allowed to derive software sensors that can determine biomass and phenolic compounds concentration from gases, nitrogen and carbon in the medium. These software sensors have been validated at the same time as the reaction scheme, and therefore it is also submitted to the same troubles as the mass balance model (gaseous measurements, phenolic compounds balances). These software sensors

require the measurements of the concentrations in the influent, some measurements in the medium (ammonium and sugars concentration), and measurements of dissolved and gaseous CO_2 and O_2. They also need the values of 6 parameters. It is noteworthy that this software sensor does not separate vanillin from vanillic alcohol : it estimates the sum of these two phenolic compounds.

The software sensor for biomass estimation and for vanillin have been tested with experimental data. The result are very promising if we remember that they should be applied when the fungal death is low, which is not the case in the considered experiments. Another estimator has been developed in order to filter the on-line data of the phenolic compounds (see Sub-task C.4.2) and to estimate the reaction rates. It has been extensively tested on simulations perturbed by additive white noise. This estimator is used for the adaptive controller (see Sub-task C.5.2).

Task C 5 : Development of an automated computer control system for process optimisation

Sub-task C 5 1 .- Optimal reactor feeding and vanillin extraction strategies

The software used for HPLC analysis is able to control the filtration system as well as the precursor feeding pump. Currently the interface for sending the signal to the pumps (depending on ferulic acid and vanillin concentrations) has to be created. The model has been examined in order to determine the optimal conditions for the biotransformation. First, a strategy has been determine aiming at maximising at each time instant the specific vanillin production rate. This strategy leads to maintain in the medium a vanillic acid concentration proportional to the square root of the present vanillin. A second strategy consist in maintaining setpoints for the vanillic acid and the vanillin concentrations. A control law based on the precursor addition control or on the vanillin extraction rate can achieve these setpoints. Finally we use the first one, which is given by an analytic equation. Another problem has been solved: the determination of the optimal time instant to' switch from growth to production. This has been achieved by extensive simulations of the vanillin obtained for a given time of precursor addition. The optimal strategy proposed previously has been modified to take into account the modifications in the model. A suboptimal feeding strategy has then - been proposed, this solution is very close to the (intractable) actual optimal one. Criterion closer to the industrial requirements than only the total amount of recovered vanillin have been considered: the yield of the biotransformation, the value added to the precursor and the expected benefit. It turns out that the strategy consisting in maintaining in the medium a vanillic acid concentration proportional to the square root of the present vanillin is also a (sub)optimal solution when the value added is considered as criterion to optimise. Finally the three tested strategies (corresponding to the optimisation of various criteria) gave very close results. So we use the control action which is given by an analytic equation. Then extensive simulations when the optimal controller is applied allowed to determine for each of these criterion an optimal time to switch from the biomass production phase to the biotransformation phase. This optimum depends on the carrying capacity of the medium (i.e. of the maximum achievable biomass), Finally we determined the optimal duration of the biotransformation. Qualitative conclusions were drawn, in order to keep the "philosophy" of the optimisation approach even if the process is evolving. These study was lead also in the case of the one step process (ferulic acid is used as precursor of vanillin). In that case the optimal control is simpler: it consists in regulating the ferulic acid concentration to a set point level.

Sub-task C 5 2 .- Design of model-based control algorithms

In order-to apply the optimal strategy defined in Sub-task C.5.1, a controller has been designed. A linearizing controller appears to be a good candidate to maintain the system close to the optimal surface. Nevertheless it requires the knowledge of the vanillin production rate. We have then developed an algorithm to estimate from the available phenolic compounds concentrations the reactions rates and to filter the measurements so that they can be used by the controller. The so called adaptive controller has been extensively tested with simulations perturbed by white noise. The result of this controller are very promising. The controller seems to be also efficient when the parameters are not known with an uncertainty margin. Nevertheless, if the phenolic compounds are not known with a short enough sampling period, then the performance of the controller are degraded.

Sub-task C.5.3. .- Implementation and validation on a laboratory pilot bioreactor

Since partners PI and P5 were not ready for an online implementation of the controller, this task was not performed. The actual efficiency of the controller was thus only tested on extensive simulations (see sub-Task C.5.2). In fact, some experiments were made but they were not useful for the validation (the use of autoclaved maize bran has led to too many changes in the medium).

Task C 6: Industrial fermentation scale-up studies

Sub-Task C 6 1 : Scale-up to a preindustrial pilot plant scale

Pre-studies have been made in order to evaluate the number of experiment necessary for an optimisation of the process up to industrial scale. Some experiments were performed in flasks at Recordati (Partner P5). With vanillic acid, high vanillin concentration was obtained (I g/1) at day 8 using low aeration, while no more than 580 mg/l were reached in high aeration conditions. In the same aeration conditions, 100 mg/l and 580 mg/l of vanillin were obtained from ferulic acid in respectively high and low aeration. These results confirmed the previous results obtained by Partner P 1 and P3. A study on the vaniiiin stability in the conditions used for bioconversion has shown that vanillin is not very stable and is quickly consumed. Some experiments were performed in flasks at Recordati (Partner P5).

Experiments were made in 20 litre bioreactor by Partner PI using autoclaved maize bran as precursor.

- *with non-concentrated enzymes* The quantity of vanillin is lower in 20-litre fermentor than in 2-litre fermentors. 253 mg/l vanillin was produced with a molar yield of 35 %, against about 670 mg/l in 2-litre fermentor with 34 % vield. The maximum is also only obtained after 15 days, against 12 days in 2-litre bioreactor and 10 days in flask.
- *with concentrated enzymes* Enzymes were successfully concentrated using a 10 kD cut-off membrane of 0.5 m best results were obtained after saturation of the membrane with casein. Enzymes have been concentrated 10 times for a volumetric concentration of 15. The recovery -yield was 67%. The concentrated medium was used in a 20-litre fermentor for vanillin production. ,In 20-litre fermentor, 600 ml of *A. niger* culture (after concentration and filtration) were added into 16 litres of *P. cinnabarinus* culture.

Enzymes were active, as free ferulic acid was measured in the medium. But only 163 mg/l of vanillin were produced after 10 days. There was perhaps a too low aeration for the bioconversion phase.

Sub-task C 6.2 Scale-up to industrial plant scale

Phase D: Authentication and labelling

Task D I : Preparation of commercial biotechnological vanillin extract of natural origin

15 litres of the fermentation medium from the Chemap fermentor using autoclaved maize bran as precursor were filtered. Small amounts (- 1 50 mg) of vanillin were purified and crystallised.

Task D 2 : Vanillin authentication

Organoleptic study was done on liquid extracts containing natural biotechnological vanillin from maize bran, among vanillin of various origin, ethylvanillin and a vanilla extract of Bourbon type. This last one was completely different from all the other, with a rich and complex aromatic profile. From all other samples, biotechnological vanillin exhibited a strong vanilla flavour and taste, like ethylvanillin, but with a woody phenolic secondary note. Isotopic ratio $^{13}C/^{12}C$ was measured on the crystallised vanillin and the - 1 8.3 ‰ PDB obtained value is close to the ratio obtained with pure vanillin from vanilla bean.

Task D 3: Vanillin labelling

A study on vanilla market was developed and presented by Partner PI in order to look after the place that our biotechnological vanillin can take in this market. Up to now the production price is too close to the expected sale price to expect an industrial exploitation of the process, taking into account also the price of the purification and crystallisation and the margins of the companies involved in the process (production of the precursor, fermentation, purification and sale). The only way to overcome that would be to increase the final yield by a factor of at least 5 to 10. This closeness is mainly due to the strong decrease of the price of vanilla beans from Madagascar during the completion of the project (from 70 Euros to 20 Euros /kg).

Discussion and conclusions

Purely enzymatic treatment (wheat bran, beet pulp) or a combination of mild physical pre- treatment followed by enzymatic processing (maize bran) allowed us to obtain preparation of ferulic acid useful as precursor for the vanillin bioconversion, and carbon source for the fungus (cellobiose). Maize bran is particularly important because of his high content in ferulic acid (up to 3%), but other sources are also useful because they are easier to treat. The optimisation of ferulic acid extraction from raw material has been realised by combining heat treatment and enzymatic hydrolysis. Kilograms of fractions enriched in ferulic acid were produced.

Two strains, *A. niger* I-1472 and *P. cinnabarinus* MUCL 39533, were evaluated for their production of some polysaccharide-degrading-enzymes and esterases. The results showed the high potentialities of the enzymes produced by *A. niger* I- 1 472 to release free ferulic acid even directly from a raw material or a simple derivative. This free acid then could be biotransformed to vanillic acid in the same culture or into vanillin by *P. cinnabarinus* MUCL 39533 cultures. In this aim, two strategies will be further studied to produce biotechnological vanillin:

- First strategy: combination of *A. niger* 1-1472 and *P. cinnabarinus* MUCL39533 potentialities
-
    - enzyme production by *A. niger* I- 1 472 with raw materials as carbon sources.

- addition of *A. niger* 1-1472 exocellular enzymes in *P. cinnabarinus* cultures with autoclaved feruloylated fractions as source of ferulic acid to produce vanillin.
- Second strategy : vanillic acid production by *A. niger* I- 1 472 and use of vanillic acid as precursor of vanillin in *P. cinnabarinus* MUCL 39533 cultures.

The isolation and characterisation of the enzymatic activities involved in the biotransformation (VAR and P-oxidation) or in the formation of side-products (VAH) were not fully successful. Many strategies were tested but it seems that these enzymes are different in *P. cinnabarinus* than in other previously studied *Basidiomycete*. For the first time, we succeeded to define a test of enzyme activity for the bioconversion *in vitro* of ferulic acid to vanillin by *P. cinnabarinus*, but it was not possible to isolate the enzymes responsible of this activity.

Concerning the bioconversion step, the process using two successive fermentation steps with pure precursors transformed by two different fungi (*A. niger* for the conversion of free ferulic acid to vanillic acid and *Pycnoporus cinnabarinus* for the bioconversion to vanillin) is no more studied because of its complexity, despite a better yield and a higher final concentration of vanillin. All the work in fermentor was then focused on the direct transformation of raw materials to vanillin by the two fungus or of free ferulic acid by *Pycnoporus cinnabarinus*..

We have been successful trying to obtain higher biomass to improve the productivity of the process. Different medium conditions were tested for bioconversion of pure synthetic ferulic acid in vanillin by *P. cinnabarinus*. Results were similar when using 2 different carbon sources (Nat 89, fructose or glucose with maltose) as when exchanging the medium between growth and bioconversion. Because of industrial feasibility the process with a single fermentation medium seems to be more realistic and will be further studied. The best result was obtained with addition of an adsorbent allowing to produce 900 m /1 vanillin in 14 davs.

Immobilisation experiments in air-lift bioreactor allowed to increase biomass in some media especially with phospholipids. Medium is clear and medium exchange or recycling are easier than with a free fungus. However we weren't able to obtained as much vanillin as with the free culture.

With free natural ferulic acid obtained from maize bran and sugar beet pulp, vanillin production was as good as with pure synthetic ferulic acid or even higher in 2 litre bioreactor. The two strategies defined for the transformation of autoclaved maize bran lead both to high productions of vanillin. About 600 mg/l of vanillin was produced within 12-13 days in 2-litre fermentors.

The use of mathematical modelling showed the influence of physical parameters on the different steps of the bioconversion, allowing us to optimise some fermentation parameters like oxygen concentration.

Separation of the final product from fermentation medium has been proved feasible using a simple extractor coupled to an external circulation loop, decreasing the production of by-products which are detrimental to the final yield.

The organoleptic study of biotechnological vanillin from maize bran exhibited a strong vanilla flavour and taste, with a woody phenolic secondary note. This flavour is typical from so called woody products (like whiskies for example). It is certainly due to some residual by- products such as ferulic acid or acetovanillone. Isotopic ratio $^{13}C/^{12}C$ measured on the crystallised vanillin produced from maize bran shows a typical value close to the ratio obtained with pure vanillin from vanilla bean.

Due to a very strong decrease of the price of vanilla beans since 1995, the process is not economically competitive. Only increase of the yield and/or the price of vanilla beans could give way to an industrial exploitation.
