Biotechnological Production of Vanillin from Natural Feedstocks and Development of New Procedures for the Recovery of the Product.

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This PhD research project was focused on innovative biotechnological production and recovery of vanillin and vanillin precursors. Experiments carried out using whole cells or crude enzyme preaparations demonstrated that: selective recovery of the product, using macroporous resins, enhances the biological conversion of ferulic acid to vanillin using resting cells of *Escherichia coli* FR13 strain; (b) thermal pre-treatment at 55°C of crude PVA acylase from *Streptomyces mobaraensis* DSM40847 improves the conversion of capsaicin to vanillylamine.

Produzione Biotecnologica di Vanillina da Matrici Naturali e Sviluppo di nuove tecniche di Recupero del Prodotto

Il progetto di tesi di dottorato riguarda l'innovativa produzione biotecnologica e il recupero di vanillina e di precursori naturali della vanillina. Gli esperimenti eseguiti con cellule e preparati enzimatici grezzi hanno dimostrato che: (a) il recupero selettivo del prodotto con una resina macroporosa, favorisce la bioconversione dell'acido ferulico in vanillina usando cellule resting del ceppo di *Escherichia coli* FR13; (b) un pre-trattamento termico a 55°C della PVA acilasi ottenuta dal ceppo di *Streptomyces mobaraensis* DSM40847 nel preparato enzimatico grezzo migliora la conversione di capsaicina in vanillilamina.

Key words: vanillin; enzymatic synthesis; microbial acylase; *Streptomyces mobaraensis*; *Escherichia coli*; product recovery; adsorbent resin.

1. Introduction

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. 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 (Figure 1). The aims of the thesis were: to evaluate different strategies for enhancing the production of the capsaicin acylase from *Streptomyces mobaraensis* DSM40847 strain (1), to identify optimal conditions for capsaicin hydrolysis by using the acylase from *Streptomyces mobaraensis* DSM40847 strain (2), to develop efficient procedures for the recovery of vanillin from diluted aqueous solutions (3), and to enhance vanillin production from the recombinant strain of *E. coli* FR13, starting from ferulic acid and by using XAD-4® resin (4).

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

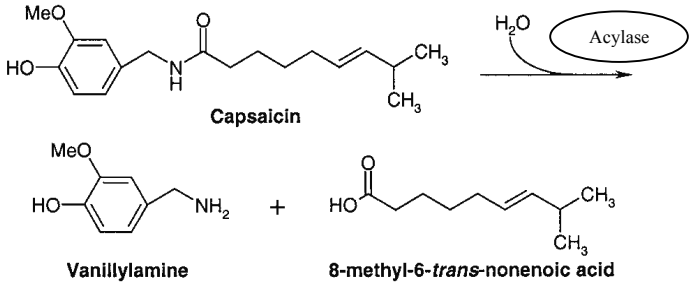

Figure 1 *Enzymatic hydrolysis of capsaicin*

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 et al., 1999; Shizmann et al., 1990; Kim and Kim, 2001), *S. mobaraensis* has been shown to produce a capsaicin-hydrolysing acylase, (*Sm*-PVA) that is secreted in the culture medium (Koreishi et al. 2006). In order to develop a cost-effective process for the biotechnological production of vanillin, we considered the use of crude enzyme and the performance of different extraction procedures on the recovery of vanillin from diluted aqueous solutions. 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. 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. We investigated the in situ product adsorption using adsorbent resin to improve the vanillin yield using *E. coli* FR13resting cells.

2. Materials and Methods

2.1 Microorganisms and culture conditions

Conversion of capsaicin to vanillylamine was carried out using *Sm*-PVA acilasi from *Streptomyces mobaraensis*. Cultivation of DSM40847 strain 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). For inocolum the microorganism 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 production of *Sm*-PVA, the strain was grown on media *M* or *MT* amended with glucose (5 g/L) or soluble starch (40g/L) as carbon source. Medium M contains: 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 MT contains: 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).
Bioconversion of ferulic acid to vanillin was carried out in phosphate salin buffer using *Escherichia coli* FR13. Cells were grown in LB medium (Composition per liter: tryptone 10 g; NaCl 5 g; yeast extract 5g) containing kanamicin (25 μg/ml). Conversion of ferulic acid to vanillin and hydrolysis of capsaicin were monitored by HPLC (see below).

2.2 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).

2.3 Preparation of *Sm*-PVA

Crude preparations from the liquid culture of *Streptomyces mobaraensis* DSM40847 strain were obtained by the precipitation of extracellular proteins with ammonium sulfate at 65% in Tris-HCl 50 mM, pH 7.80.

2.4 Vanillin recovery

XAD-4® macroporous adsorption resin with crosslinked styrene-divinylbenzene framework were used in this research. 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. The kinetic studies were carried out at 30°C, with resin 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. 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], with resin at 0,1 g/ml. 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. 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).

3. Results and discussion

3.1 Effect of medium composition on the production of *Sm*-PVA from *Streptomyces mobaraensis*

In preliminary experiments we evaluated the effect of different carbon and nitrogen sources (see Materials and Methods) on the production of *Sm*-PVA. Data reported in Figure 2 indicated that both profile and production level of acylase were affected from the source of nitrogen provided for the growth. The highest level of acylase (140 U/L) was obtained on medium M providing soluble starch as carbon source and polypeptone and meat extract as nitrogen sources.

3.2 Effect of temperature on the conversion of capsaicin to vanillylamine

The effect of the temperature on the hydrolysis of capsaicin using crude preparations of *Sm*-PVA acylase was investigated. Experiments were carried out in the range of temperature 37-60°C, and acylase activity was measured by HPLC monitoring the consumption of capsaicin during the time. The results, reported in Figure 3a, indicated that: PVA acylase from DSM40847 strain was active in a wide range of temperature and the highest enzymatic activity was measured at 55°C.

3.3 Effect of cobalt ions on the hydrolysis of capsaicin using *Sm*-PVA

Experiments carried out using PVA from *Streptomyces 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 crude preparations of *Sm*-PVA from strain DSM40847. Hydrolysis reactions were carried out at different temperatures, using ~7.5 mU of acylase activity (measured at 55°C) (see Materials and Methods). The results reported in Figure 3b indicated that the stimulatory effect of cobalt ions on the acylase activity was detected only at low temperature (37°C). In this condition the addiction of cobalt ions determined an increase from 1.2 ±0.1 U/L to 1.5±0.07 U/L. The same effect was not observed at higher temperatures using crude enzyme preparations of *Sm*-PVA acylase.

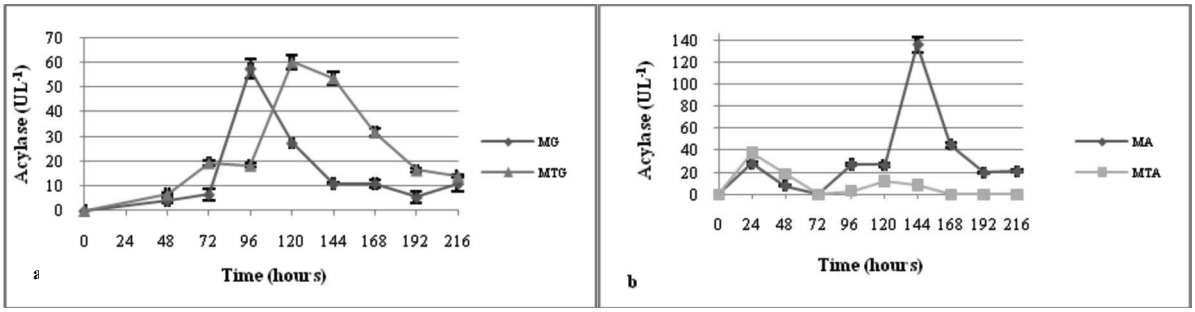

Figure2 *Acylase production of Streptomyces mobaraensis grown on medium M and MT containing soluble starch as carbon source (a); Acylase production of Streptomyces mobaraesins DSM40847 grown on medium M and MT containing glucose (5 g/L) as carbon source (b). Error bars indicate standard deviations.*

3.4 Stability of crude enzyme preparations of *Sm*-PVA

Stability of *Sm*-PVA was determined on samples stored for 7 and 14 days at three different temperatures: -20°C, 4°C and 20°C for. All enzymatic reactions were carried out at 55°C for 2 hours. The results reported in Figure 3c, indicated that crude preparations of *Sm*-PVA at 4°C and 20°C showed a rapid loss of activity (75% after 14 days). The highest residual activity (60% after 14 days) was obtained maintaining the enzymatic preparation at -20°C. Pre-incubation of crude enzyme stored at -20°C for at least 2 hours determined a 5-fold increase in the

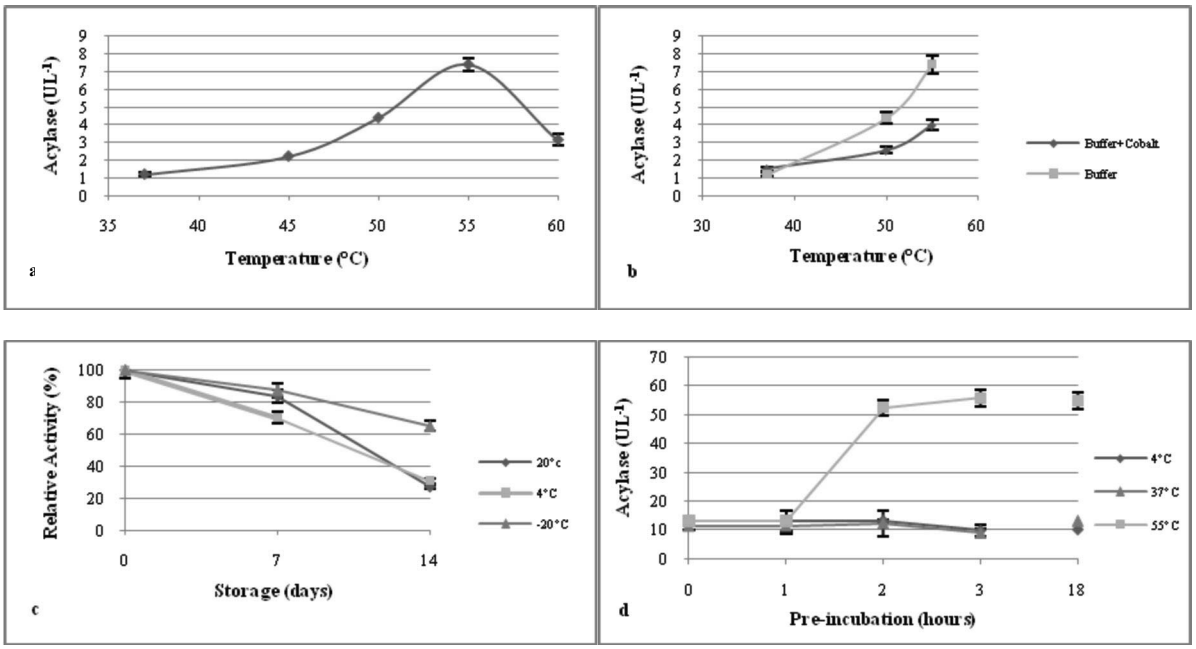

Figure 3 *Effect of temperature (a) and of cobalt ions (b) on acylase activity from S. mobaraensis; Acylase activity levels of crude enzymatic preparation pre-incubated at different temperature in the range of 0 – 18 hours (c); Relative activity (%) of crude enzyme stored for 14 days (d). Experiments were carried out using raw enzymatic preparation obtained from fermentation broths of Streptomyces mobaraensis strain cultivated on MA medium. Acylase activity was determined using capsaicin as substrate and measured by HPLC analysis. Error bars are standard deviations.*

level of acylase activity compared to untreated samples or to the samples incubated at 4°C or 37°C (Figure 2d).

3.5 Selective recovery of vanillin

Ferulic acid and vanillin can be recovered from liquid medium using macroporous resins, such as XAD-4® resin. 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. Results, reported in Figure 4a, 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. 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.

We also investigated the recovery of vanillin and ferulic acid in acqueous mixture by using liquid/liquid extraction with *n*-butyl acetate. The results reported in Figure 4b indicated that recovery vanillin percentage was 98% and that high recovery and high selectivity were obtained.

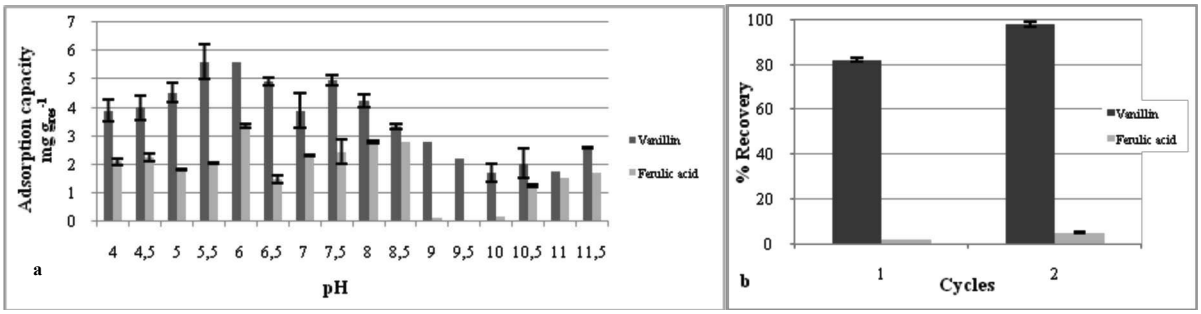

Figure 4 *Adsorption capacity of XAD-4® resin (a); Recovery of vanillin (%) by using liquid/liquid extraction with n-butyl acetate (b). Liquid/liquid experiments were carried out at 20°C. Vanillin concentration in acqueous phase was measured by HPLC analysis. Error bars are standard deviations.*

4. Conclusions

Streptomyces mobaraensis DSM40847 strain produces an acylase that can be used for the conversion of capsaicin to vanillylamine. The production of this enzyme is stimulated growing the microorganism medium containing soluble starch as carbon source and meat extract and polypeptone as source of organic nitrogen. A crude preparation of this acylase can be successfully used for the production of vanillylamine carrying out the reaction at 55°C and pre-treating the crude enzyme preparation at the same temperature for at least 2 hours. XAD-4® resin can be used for the selective recovery of vanillin working at alkaline pH. Under this condition the conversion of ferulic acid to vanillin using resting cells of *E.coli* FR13 strain can be also obtained. 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.

5. References

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