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To cite this article: Amanda Inés Mejía, Betty Lucy Lopez & Michael Hess (2016) Bioconversion of poly(vinyl alcohol) to vanillin in a *Phanerochaete chrysosporum* culture medium, Materials Research Innovations, 7:3, 144-148, DOI: [10.1007/s10019-003-0239-1](https://doi.org/10.1007/s10019-003-0239-1)

To link to this article: <https://doi.org/10.1007/s10019-003-0239-1>



Published online: 13 Oct 2016.



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Received: 25 September 2002 / Revised: 30 January 2003 / Accepted: 25 September 2002 / Published online: 10 April 2003
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Abstract The bioconversion of *Phanerochaete chrysosporium* cultures in aqueous poly(vinyl alcohol) – PVAL – solutions was studied. A 50% weight loss was observed within 20 days, caused by a ligninperoxidase – LiP – enzyme which is produced during the secondary metabolism of *Phanerochaete chrysosporium*. The changes of the chemical structure of PVAL were studied by UV- and IR-spectroscopy. The presence of vanillin – the major metabolic reaction product, benzaldehyde, and veratryl-alcohol was determined chromatographically (HPLC) in the extra cellular medium after different reaction times. A possible mechanism of the biological degradation is outlined.

Keywords Biodegradation · Poly(vinyl alcohol) · Vanillin · *Phanerochaete chrysosporium*

Introduction

Vanillin and related flavour compounds are of great economical importance because of the demand and their price. Low-expansive biotechnological processes therefore are of great interest. The demand for vanillin, for example exceeds its natural production. Only 0.2% of the flavour's world market is presently covered by natural resources; the major part is synthetic material [1]. The tendency to use the products from natural sources

increases because it is found that they frequently are healthier so that vanillin, for example, can be applied in higher concentration. The drawback however, is the high price. Biotechnologically produced vanillin is considered as “natural.”

Vanillin is a bioactive compound which is easily recognized because of its scent. Its biotechnological production makes it available independent from agricultural problems like the climate, contamination by fertilizers, pesticides or plagues. Also, market restrictions or socio-political uncertainties can be ruled out.

The highest concentration of natural vanillin (1–3%) is found in matured grains of the Vanilla orchid, an easily satisfied parasitic tropical plant which does not need much more than clean air and water to survive [2]. Vanillin can be isolated from the mature grains of the plant as a brown alcoholic solution and has multiple applications in this crude form. However, the amount of material produced is strongly depending on the climatic conditions and the price is high, so early attempts for a synthetic production were investigated. The first large scale production of synthetic vanillin was started from sulphated lignin waste waters in 1936 by the Salvo Corporation in the USA [2].

Vanillin is also an intermediate product in the metabolic oxidation of lignin by the fungus *Phanerochaete chrysosporium* in the course of the biodegradation of lignin, lignin model compounds and aromatic pollutants [3]. Other intermediate products during biodegradation are substituted quinones, hydroquinones, and benzoic acid. Open ring fragments and benzaldehyde are later formed by intracellular metabolic processes [4, 5].

Pseudomonas putida and *Streptomyces setonii* are also able to produce vanillin from eugenol or ferulic acid substrates [5] in quantities of 5 g/L. Several strains of *Nocardia* are also able to produce vanillin from vanillic acid [6]. Also gen-technical methods have been developed [7]. Recent investigations [8] report about a biotechnological potential of producing vanillin by enzymatic hydrolysis of red pepper and capsaicin [8]. Among several phenolic components vanillin was identified after

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22 days under controlled conditions from cultures of the fungus *Pycnoporous cinnabarinus* (DMS-1184). Literature does not reveal any results referring to the production of vanillin from PVAL using the enzymatic system of the fungus *Phanerochaete chrysosporum*. PVAL is a polymer which is widely used in pharmaceutical, cosmetic, and chemical applications [9]. The production of PVAL was 500,000 t in 1995 world wide [10]. In previous publications [11, 12], we have studied the biodegradation of PVAL with enzymatic extracts of the *Phanerochaete chrysosporium* fungus, in which the lignin peroxidase activity (LiP) was detected. The results of Gel Permeation Chromatography (GPC) there showed that given the conditions of that study, in 15 days the average molar mass of the polymer decreases about 80%. Benzaldehyde and vanillin were the main degradation products. In the present study, we now want to quantify the amounts of vanillin produced during the degradation using the same biodegradation conditions as applied in ref. 11 and 12.

Materials and methods

The PVAL used in this study had been produced by the BASF Chemical Company, distributed in Colombia by Generic Chemical Products S.A. (gmp), and had an average molar mass of 160,000 g/mol.

The degree of hydrolysis (HD) of the polymer, the volatiles, sodium acetate and ash content were determined according to JIS K6726-1977 [13]. All values were determined twice. The inserted PVAL had a degree of hydrolysis of 98.99% (mol), 0.568% w/w ash content, and 0.0567% (mol) Na-acetate in the dry product.

Phanerochaete chrysosporum BKM-F-1767 (ATCC 24725) was cultivated in YMPG medium containing 1% w/v yeast extract, 1% w/v malt extract, 0.2% w/v bacteriological peptone, 1% w/v glucose, 0.1% w/v asparagine, 0.2% w/v KH_2PO_4 , 0.1% w/v MgSO_4 , 1% w/v thiamine hydrochloride, and 2% w/v agar. It was sterilized and incubated for 5 days at 37 °C. After that treatment it was kept at 4 °C [14, 15].

The expression fluid where the enzyme ligninperoxidase – LiP – is produced contained 0.1 g/mL% PVAL, 0.1% w/v glucose, 0.02% w/v ammonium tartrate, 0.05% w/v MgSO_4 , 0.01% w/v 5 $\text{CaCl}_2 \cdot 12 \text{H}_2\text{O}$, 0.05% w/v between 80, 0.1% w/v thiamine, 2.5 m/mol veratryl alcohol and 70 ml/L of trace element solutions (1 L of this trace element solution contained 1.5 g nitroacetic acid, 0.3 g $\text{MgSO}_4 \cdot 5 \text{H}_2\text{O}$, 0.1 g NaCl, 0.1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 12 \text{H}_2\text{O}$, 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$, 0.01 g HBO_3 , $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ [16]). The pH was adjusted to 4.5 with a sodium tartrate buffer. The ionic strength was about 0.2 mol.

The expression fluid was filtered through 0.45 μm membranes. Twelve aliquots of 175 ml of this solution were poured into 500 ml Erlenmeyer covered with cotton roundes, under sterile conditions. In nine of them freshly replied *Phanerochaete chrysosporium* spores (not older than 3 weeks) were inoculated until a final concentration of 18×10^4 spores/mL was reached [16, 17] and the lasted three without spores are used as control samples. Orbital shaking speed was set at 150 rpm at 25 °C.

Three of the PVAL samples were maintained during 10 days, three during 20 days, and finally three during 30 days. Three control samples were maintained during 30 days. For the analysis, the total amount of each Erlenmeyer flask was filtered using 0.45 μm membranes.

LiP-activity

The activity of LiP was determined according to Kirk et al. [18] using a Varian Cary 50 Bio uv-vis spectrometer.

Extraction of the metabolites

Each sample was adjusted to pH 2 with HCl 3 M and extracted with three aliquots of 20 mL of fresh mixtures of acetonitrile/methylenechloride (90:10). The combined extracts were concentrated at 55 °C with a Rotavapor™. The organic phase (with a strong vanillin smell) was kept at –20 °C until further analysis. After thawing the sample a thin white layer of PVAL had precipitated. The precipitate was filtered and dried for further analysis.

Concentration of residual PVAL

The concentrations of residual PVAL was determined according to the method of Alzate et al. [19], which is based on, the method described by Finch [20]. The calibration curve of PVAL was composed in the culture medium.

Residual PVAL was separated from the culture solution after defined reaction times by freeze-precipitation at –20 °C, separated by centrifugation and dried to constant weight at 70 °C. Solutions containing 0.1% g/mL were analysed as described below.

IR-Spectroscopy

A Perkin-Elmer Spectrum one FTIR spectrometer was used. A PVAL film was produced by evaporating the aqueous solution of the polymer in a SeZn-ATR cell. These solid-state spectra cannot be used for an exact quantitative analysis, qualitative and relative information, however, can be drawn from them.

GC-MS analysis

The combined extracts of each sample as described above was subjected to a GC-MS analysis (Varian) on OV-5 polar columns (30 m length), temperature ramp 70–220 °C with a heating rate of 10 K/min. The components were identified with the corresponding standards.

HPLC-analysis

A Waters liquid chromatography equipped with a 996 UV diode array and C-18 columns (4.6 mm \times 250 mm, particle size 5 μm , mobile phase: 53% ortho phosphoric acid, 0.2% H_2O , 47% acetonitrile; flow rate: 1 mL/min; injected volume 20 μL) was used for quantitative determination of vanillin, veratryl alcohol and benzaldehyde after establishing the corresponding calibrations with the pure components.

Results and discussion

The time dependence of the LiP-activity and the PVAL concentration is given in Fig. 1a and the vanillin production is given in Fig. 1b. The activity of the LiP and the vanillin production increases with the time after 10 days. The fungus secretes LiP enzyme into extra cellular environment under nutrient limited conditions-specific glucose and nitrogen- [11, 12, 21]. The presence of the LiP causes the degradation of the PVAL to produce

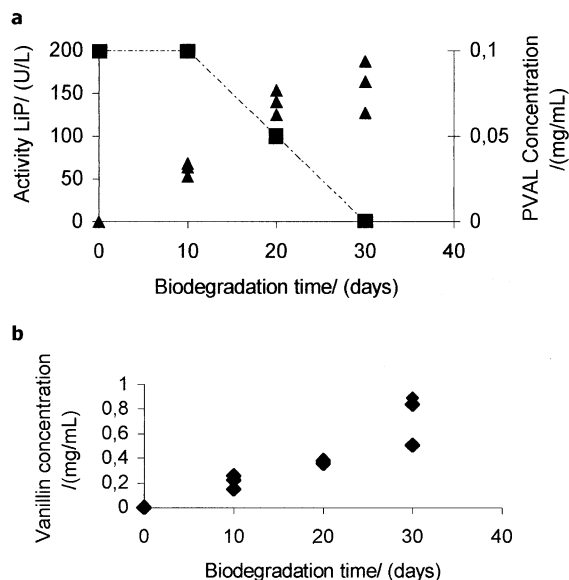


Fig. 1 a) time dependence of LiP-activity (triangles) and polymer concentration (squares). The unit “U” used for description of biochemical enzyme activity is defined by $U \equiv \mu\text{mol}/\text{min}$, b) time dependence of the vanillin concentration during the enzymatic degradation of PVAL

vanillin as one of the main degradation product. There is no activity and it is no vanillin production in the PVAL control samples.

Although the molar mass of the polymers were not explicitly determined in this work, previous work under the same biodegradation conditions showed that the average molar mass of the polymer determined by GPC decreased after 10 days [11, 12]. It seems that during the initial period of the degradation (<10 days) a decrease of the polymer chain length is negligible and the predominant reactions are functionalization reactions of a still rather intact main chain (see spectroscopic results). After 30 days, no precipitate could be isolated, indicating that the degradation of the PVAL chains had reached a state where only oligomeric material is left which soluble in the culture medium even at low temperatures.

The infrared spectra of the PVAL after different incubation times are given in Fig. 2.

The peak at the wave number 3314 cm^{-1} shows the unchanged OH-stretching vibration of the PVAL in the control sample and degraded polymer after 10 days respectively 20 days, as indicated. The splitting of the 3314 cm^{-1} signal into two peaks indicates the presence of hydroperoxide groups produced during degradation. At 2936 cm^{-1} is correlated with the CH-stretching vibration and becomes markedly weaker with increasing incubation time – in particular for times >20 days. The signal at 2974 cm^{-1} merging around 20 days of reaction time is attributed to the appearance of $=\text{C}-\text{H}$ stretching vibrations [22, 23]. The intensity of vibrations of carbonyl groups – 1710 cm^{-1} – is only small and found for samples with a high degree of hydrolysis [9]. However, it increases in intensity for samples incubated >20 days.

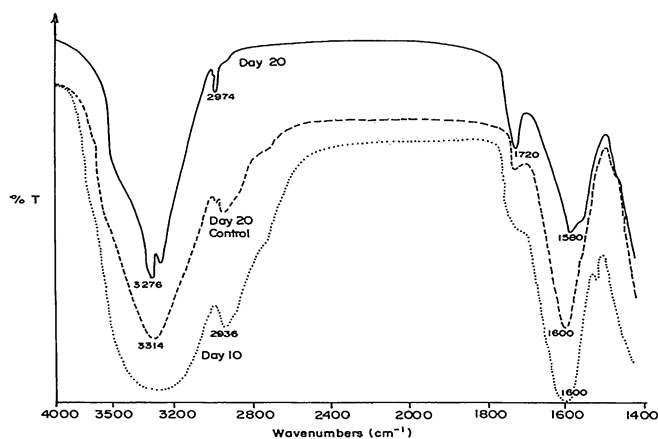


Fig. 2 Time dependence of IR-spectrum of PVAL during the course of biodegradation (precipitated as a film), for details see text

The $\text{C}=\text{C}$, indicated by an absorption at 1600 cm^{-1} [22] increases with increasing incubation time. For incubation times >20 days this band shifts to 1580 cm^{-1} indicating a possible conjugation with the carbonyl function of an aldehyde or a ketone [22].

Considering the decreasing mass of the precipitating PVAL being due to decreasing chain length of the polymers, the biotransformation of PVAL seems to be initiated by a longer period of functionalization reactions before the chain degradation mechanism – probably dominated by intra-chenar cyclization reactions – takes over after about twenty days.

Since the chain length of PVAL does not change considerably during the first 10 days, and no vanillin production was found in the absence of PVAL, a possible explanation is that degradation starts at the chain ends or is due to completely soluble oligomeric material present in the polymer which is not detected by the precipitation analysis used in this work.

GC-MS analyses show the presence of vanillin, veratryl alcohol and benzaldehyde. Benzaldehyde was only found in some samples with variable concentration. This effect is apparently caused by the sensibility of this reaction product to oxidation and other side reactions.

The initial concentration of veratryl alcohol added to the culture medium was $2.5\text{ mmol}/\text{L}$ and we found $3.0\text{ mmol}/\text{L}$ after 10 days, because this compound is formed by the ligninolytic machinery of *Phanerochaete chrysosporium* [11, 23]. After 20 days and 30 days, the concentration of veratryl alcohol decreased to $2.1\text{ mmol}/\text{L}$ and $1 \times 10^{-2}\text{ mmol}/\text{L}$ respectively; no change in the concentration of the veratryl alcohol was observed in the control. This substance acts as a mediator in the production of LiP.

The linear single strand PVAL can contain small rings formed by etherification side reactions as shown in the reaction scheme (Table 1). The enzyme LiP can cause deterioration of PVAL during the first (<10 days) and the second stage (>10 days) of the biodegradation process. During the first stage, the enzyme eliminates residual

veratryl alcohol, vanillin, and the reproducibility of the benzaldehyde production, the analysis of the radical reactions with ESR and conformational analysis of the polymer should give deeper insight into the reactions.

Acknowledgements We thank the Universidad de Antioquia for the resources assigned to this project.

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