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Enzymic release of cellobiose from sugar beet pulp, and its use to favour vanillin production in *Pycnoporus cinnabarinus* from vanillic acid

E. Bonnin[a,*], H. Grangé[a], L. Lesage-Meessen[b,1], M. Asther[b], J.-F. Thibault[a]

[a]*Unité de Recherches sur les Polysaccharides, leurs Organisations et Interactions, INRA, BP 71627, 44316 Nantes Cedex 03, France*
[b]*Unité de Biotechnologie des Champignons Filamenteux, INRA, CP 925, Faculté des Sciences de Luminy, Centre d'Enseignement Supérieur en Biotechnologie, 163 avenue de Luminy, 13288 Marseille Cedex 09, France*

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Abstract

This study was undertaken in order to optimise the release of cellobiose from sugar beet pulp by enzymic treatment, and to evaluate the effect of this cellobiose in activating the vanillin production by filamentous fungi. Cellulose-rich residues were obtained from sugar beet pulp by a pectinase treatment, and they were then hydrolysed by a commercial cellulase. Various pretreatments (drying, grinding) and storage were applied, in order to modify the physical state of the cellulose-rich residues, such as degree of crystallinity of cellulose. After cellulase treatment, 51–64% of the initial cellulose was degraded into cellobiose and glucose, depending on the pretreatment. Whatever the pretreatment applied, cellobiose was maximally produced after 2–4 h of hydrolysis. Thereafter, cellobiose was degraded by the β-glucosidase present in the commercial cellulase, but this activity could be inhibited by the addition of D-glucono-δ-lactone. Only in some cases, could the extent of degradation of cellulose be related to the physical state of cellulose-rich residues.

The best conditions were used to produce large quantities of cellobiose in order to test its influence in the bioconversion of vanillic acid into vanillin by the filamentous fungus, *Pycnoporus cinnabarinus*. Addition of cellobiose to a 3-day-old culture yielded a 3.3-fold increase in vanillin production when compared to a control culture devoid of cellobiose.

Keywords: Cellobiose; Enzymic degradation; Sugar beet pulp; Vanillin; Biotransformation; *Pycnoporus cinnabarinus*

1. Introduction

Sugar beet pulp, an abundant by-product of the sugar refining industry, is mainly used in cattle feeding. However, its composition suggests some alternative utilisation with a higher added-value. Sugar beet pulp contains high levels of galacturonic acid (≈200 mg g^{-1}) and arabinose (≈200 mg g^{-1}), and also rhamnose (≈200 mg g^{-1}) and ferulic acid (≈8 mg g^{-1}; Micard, Renard, & Thibault, 1996). All these monomers may be released by enzymic saccharification and used for some specific purposes. For example, galacturonic acid can be transformed into surface-active agents by esterification with various fatty acids (Petit, Ralainirina, Favre, & De Baynast, 1993) or into L-ascorbic acid by enzymic conversion (Kulbe et al., 1997). Arabinose has anti-Parkinson properties after derivatisation to 5-deoxy-L-arabinose, and is also a precursor of L-fructose and L-glucose which can be used as sweeteners (Vogel, 1991). Rhamnose can be chemically transformed into aroma such as "furaneol" used in caramel, roasted and fruit flavour applications (Wong, Mazenod & Whiteside, 1983). Ferulic acid is known for its anti-oxidant properties (Graf, 1992).

This paper is part of our work dealing with the use of ferulic acid as a precursor of vanillin. In order to produce "natural" vanillin, in agreement with the European legislation (EC Directives 88/838), living cells or their components, including enzymes, have to be used. Numerous commercial enzymes were tested for their ability to release ferulic acid from sugar beet pulp with the highest possible yield, and a pectinase produced by *Aspergillus niger* was selected (Micard et al., 1996). A bioconversion process using filamentous fungi was developed to transform ferulic acid into vanillin (Lesage-Meessen et al., 1996). First, *A. niger* I-1472 transformed ferulic acid to vanillic acid, and in the second step vanillic acid was reduced to vanillin by *Pycnoporus cinnabarinus*. In the second step, vanillin production is limited by oxidative decarboxylation of vanillic acid leading to methoxyhydroquinone. We have

* Corresponding author. Tel.: + 33-2-40-67-50-00; fax: + 33-2-40-67-50-66.

E-mail address: bonnin@nantes.inra.fr (E. Bonnin)

[1] Tel.: + 33-4-91-82-86-00; fax: + 33-4-91-82-86-01.

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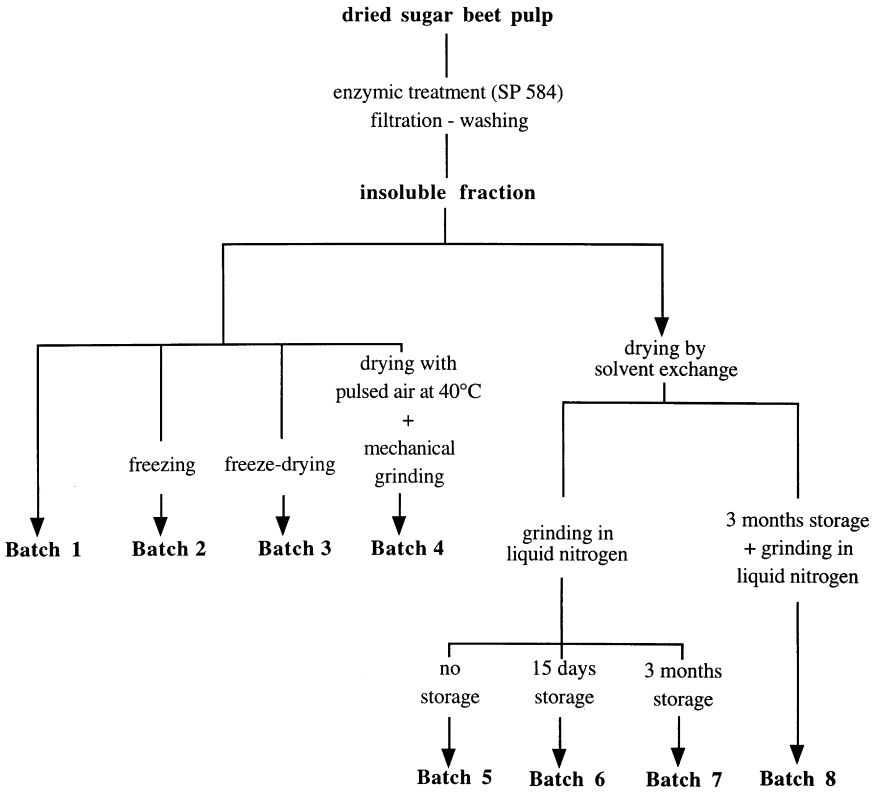

Fig. 1. Mode of preparation of the different cellulose-rich residues (batches 1–8), according to their drying method and storage.

previously shown that the yield of vanillin may be significantly increased by adding commercial cellobiose in the culture medium of *P. cinnabarinus*. Indeed, cellobiose channelled the vanillic acid metabolism via the reductive pathway, leading to vanillin (Lesage-Meessen, Haon, Delattre, Thibault, Colonna Ceccaldi, & Asther, 1997). As enzymic degradation of the pectic fraction from sugar beet pulp released a cellulose-rich residue containing about 550 mg glucose per gram residue (Micard et al., 1996), this residue may be used for cellobiose production. Therefore, the degradation of the cellulose-rich residue was studied.

In the present work, the release of cellobiose from the cellulose-rich residue by a commercial cellulase, in order to further scale up the process, and the use of cellobiose in vanillin-producing cultures were investigated. As the enzymic hydrolysis of cellulose depends on both the levels of enzymic activity and substrate reactivity, these two parameters have to be considered. The unfavourable parameters for the hydrolysis into cellobiose were the presence of β-glucosidases in the cellulolytic preparation, the retroinhibition of the enzymes by cellobiose and the physical state of cellulose in the residue. Thus, the conditions were optimised using well-characterised substrates, and various pretreatments were applied to the cellulosic residue in order to modify its physical state. In a second step, the best conditions of cellobiose production were used, and cellobiose-rich fraction obtained from sugar beet pulp was incorporated to *P. cinnabarinus* MUCL39533 cultures in order to test its effect on vanillin production.

2. Materials and methods

2.1. Enzymes

The pectinolytic mixture SP 584 (*Aspergillus niger*) and the cellulolytic mixture Celluclast 1.5L (*Trichoderma reesei*), obtained from Novo Nordisk A/S (Bagsvaerd, Denmark), were liquid preparations.

2.2. Enzymic assays

Cellulase activities in Celluclast 1.5L were determined towards microcrystalline cellulose (Avicel, particle size ≈ 50 μm, Fluka Chemie AG, Buchs, Switzerland) and carboxymethylcellulose (CMC, low viscosity, degree of polymerisation ≈ 400, Sigma Chemicals, St. Louis, MO, USA). Substrates were prepared at 1% in 0.05 mol l^{-1} acetate buffer, and activities were measured from the increase in reducing ends (Nelson, 1944). Glucose was used as a standard.

Glucosidase and cellobiohydrolase activities were measured with *para*-nitrophenyl β-D-glucoside (*p*Np-Glc, Sigma, N7006) and *para*-nitrophenyl β-D-cellobioside (*p*NP-Cel, Sigma, N5759) as substrates (4 mmol in 0.05 mol l^{-1} acetate buffer) (Rouau and Odier, 1986). Glycosidase activities were calculated by monitoring the release of *p*-nitrophenol at 400 nm ($\epsilon = 18350\ M^{-1}\ cm^{-1}$).

These activities were tested as a function of pH in the

range of 4–6.5, and temperature in the range of 25–80°C. Thermal stability was established by measuring the residual activities after 15 min pretreatment at 25–65°C.

The enzymic activities were expressed in nanokatals (nkat), one nkat produces one nmol of product per second. In the case of evaluation of pH or temperature effects, they were expressed as a percentage of the maximal activity.

2.3. Cellulose-rich materials

Sugar beet pulp (Générale Sucriére, France) was ground in a Forplex hammer mill equipped with a 2 mm grid. The fraction with particle size (0.08 mm (yield: 940 mg g^{-1}) was degraded by SP 584 (20 ml enzymic preparation in 3 l water containing 100 g pulp, for 120 h at 40°C; Micard et al., 1996). After filtration on a G3 sintered filter and washing with water, the residue was divided into eight aliquots (Fig. 1). Batches 1 and 2 were not dried, and batch 2 was freezed after washing. Batch 3 was freeze-dried. Batch 4 was dried for 5 d at 40°C, then ground (Analysenmühle A.10, Ika Labortechnik, Staufen, Germany). Batches 5–8 were dried first by solvent exchange (ethanol 95%, acetone) and then by incubation overnight at 40°C under vacuum. Batches 5–7 were ground in liquid nitrogen (freezermill, Spex 6700, Edison, NJ, USA). Batch 5 was not stored while the others were stored at room temperature for 15 d (batch 6) or 3 months (batch 7). Batch 8 was stored for 3 months and ground after storage under the same conditions. Batches 1–5 were used for cellulose degradation as soon as they were obtained.

2.4. Analytical methods

Dry matter was determined by drying the sample at 120°C for 2 h. Data were expressed on a moisture-free basis. Ashes were weighed after overnight incineration of the samples at 550°C and for 1 h at 900°C.

The neutral sugar composition was determined after 1 h of pre-hydrolysis in 72% sulphuric acid (Seaman, Moore, Mitchell, & Millet, 1954) and 6 h of hydrolysis in 1 mol l^{-1} sulphuric acid. Alditol acetates of monosaccharides were analysed by gas–liquid chromatography (Blakeney, Harris, Henry, & Stone, 1983) on a DI 200 chromatograph (Delsi Nermag Instruments, Argenteuil, France) equipped with a BP225 fused silica capillary column (Scientific Glass Engineering Sarl, Villeneuve-St Georges, France) and a flame ionisation detector, with *myo*-inositol as an internal standard. Uronic acid content was colorimetrically determined by the automated *m*-hydroxydiphenyl method (Thibault, 1979) using galacturonic acid as a standard. Proteins in the enzymic preparations were determined according to the Bradford procedure (Bradford, 1976), using bovine serum albumin as a standard.

Swelling was calculated by measuring the volume of 100 mg of dry sample in 10 ml of deionised water soaked overnight at 25°C (Kuniak and Marchessault, 1972).

In order to determine the cellulose crystallinity, diffraction diagrams were recorded using an Inel X-ray generator operating at 40 kV and 30 mA, and a curve position sensitive detector (Inel CPS 120). CuKα_1 radiation (0.15405 nm) was selected using a quartz monochromator. The samples (25 mg dry matter) were sealed between two sheets of aluminium foil to prevent any significant change in the water content during the measurement. A standard of amorphous cellulose was prepared from defatted cotton by solubilisation in 13 mol l^{-1} sulphuric acid and precipitation in water, and a standard of crystalline cellulose (cellulose I) was obtained by hydrolysis of cotton cellulose in 2 mol l^{-1} boiling hydrochloric acid. The crystalline cellulose content in the sample was determined by linear regression from the results obtained for the two standards (Wakelin, Virgin, & Crystal, 1959).

2.5. Enzymic degradation by Celluclast 1.5L

100 mg dry matter of cellulosic residue were suspended in 10 ml of 0.05 mol l^{-1} acetate buffer pH 4.5 and hydrolysed with 1 mg enzymic proteins from Celluclast 1.5L. Samples were removed at intervals over an 8 h incubation. The release of glucose and cellobiose was followed by HPLC on a column Aminex HPX-87P (300 × 7.8 mm, Bio-Rad S.A., Ivry sur Seine, France) eluted at 85°C with degassed water at a flow rate of 0.6 ml min^{-1} with a programmable pump (Waters, Massachusetts, USA). Elution was monitored by a differential refractometer (Erma, Tokyo, Japan). Glucose and cellobiose solutions of known concentrations were used as external standards.

2.6. Preparation of large quantities of cellobiose-rich fraction

Cellobiose-rich fraction was obtained from 200 g sugar beet pulp degraded by SP 584, as previously described (see Section 2.3). The degradation yielded 66.12 g of dry matter residue, which was recovered by filtration and then treated without drying by a volume of Celluclast 1.5L corresponding to 660 mg proteins. After cellulolytic treatment, the supernatant was recovered by filtration, analysed by HPLC and freeze-dried.

2.7. Production of vanillin by Pycnoporus cinnabarinus MUCL39533

Pycnoporus cinnabarinus MUCL39533 was obtained from the Mycothèque de l'Université Catholique de Louvain (Louvain-La-Neuve, Belgique). The fungus was grown in a basal medium containing maltose (20 g l^{-1}) as the carbon source, diammonium tartrate (1.842 g l^{-1}) as the nitrogen source, yeast extract (0.5 g l^{-1}), KH_2PO_4 (0.2 g l^{-1}), $CaCl_2$ (0.0132 g l^{-1}) and $MgSO_4$ (0.5 g l^{-1}). Cultures were inoculated and incubated as previously described (Falconnier et al., 1994). After 3 d of incubation, 2.5 g l^{-1} cellobiose, obtained from a commercial source (Sigma Chemical Co, St Louis, USA) or from enzymic

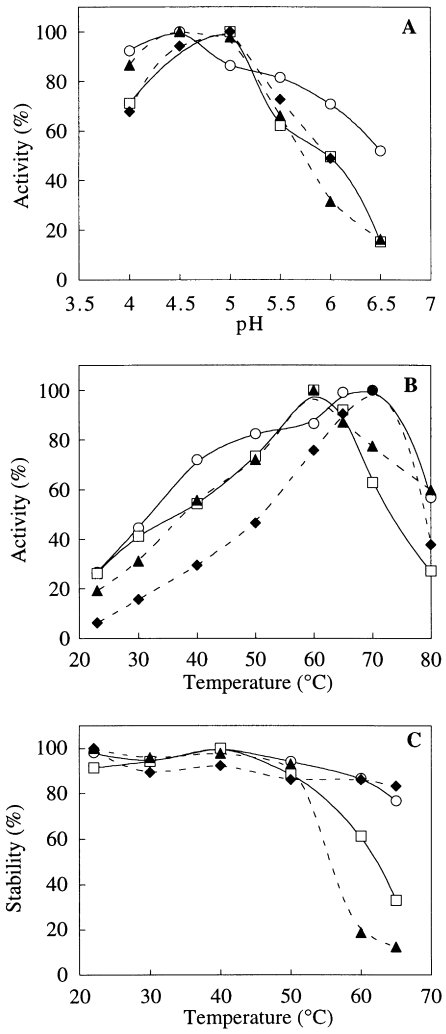

Fig. 2. Activities towards model substrates in Celluclast 1.5L: cellulase activity measured towards CMC (○), Avicel (□), cellobiohydrolase activity measured toward *p*NPCel (▲), β-glucosidase activity measured toward *p*NPGlc (◆). (A) Activities as a function of pH in the range 4–6.5; (B) activities as a function of temperature in range 25–80°C; and (C) stabilities as a function of temperature in the range of 25–65°C.

hydrolysis of sugar beet pulp, were added to the culture medium, followed by 0.6 g l^{-1} sodium vanillate (Fluka, St Quentin Fallavier, France) as vanillin precursor. From days 3–5, the culture medium was supplemented daily with 0.6 g l^{-1} sodium vanillate. Flasks without any cellobiose supplementation served as controls. Phenolic compounds derived from vanillic acid metabolism were quantified by HPLC as described by Falconnier et al. (1994). Each experiment was run in duplicate and repeated at least twice. The standard deviation of the analyses was less than 5%.

3. Results

Celluclast 1.5L was previously found to be the most efficient commercial preparation to produce cellobiose (Micard, Renard, & Thibault, 1997). However, it contained β-glucosidases that hydrolysed cellobiose into glucose. Therefore, the influence of some physicochemical parameters on the activities were studied in order to find conditions, leading to a limited glucosidase activity and favouring production of cellobiose.

3.1. Enzymic activities in Celluclast 1.5L

Activities in Celluclast 1.5L were measured towards model substrates as a function of pH and temperature (Fig. 2). *p*NP-Glc was specific for β-glucosidases as *p*NP-Cel can be hydrolysed by both glucosidases and cellobiohydrolases.

The pH and temperature curves indicated that depolymerases and glycosidases present in Celluclast 1.5L worked optimally at pH 4.5–5 (Fig. 2(A)) and 60–70°C (Fig. 2(B)). Activity towards Avicel and *p*NP-Cel decreased by 40 and 80%, respectively, after 15 min pre-incubation at 60°C, whereas CMC-hydrolysing enzymes and β-glucosidases were stable up to 65°C (Fig. 2(C)). Thus, it was not possible to decrease selectively the β-glucosidase activity by thermal pretreatment of the preparation.

Table 1
Characterisation of sugar beet pulp, cellulose-rich residues obtained from different pretreatments, and Celluclast-resistant fraction (ndb: not detectable, ndd: not determined)

	Sugar beet pulp	Cellulosic residues							Resistant fraction (from batch 3)
		Batches 1–2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	
Dry matter (mg/g)	908.6	975.0	975.0	926.0	947.0	967.0	981.0	975.0	975.0
Composition (mg/g)									
Neutral sugars	476.7	520.3	520.3	515.3	537.0	552.8	513.3	560.6	359.0
Rha	23.1	9.5	9.5	11.1	5.4	10.4	5.9	6.0	7.0
Ara	181.4	29.5	29.5	32.3	17.3	16.6	14.7	16.4	16.0
Xyl	14.6	31.4	31.4	33.0	34.5	34.6	32.4	35.4	35.0
Man	12.4	23.5	23.5	24.3	24.1	26.8	23.4	25.3	20.0
Gal	45.3	13.8	13.8	15.0	10.9	11.3	10.0	10.4	11.0
Glc	199.9	412.6	412.6	399.6	444.7	453.1	426.9	467.1	270.0
Uronic acids	184.0	31.0	31.0	36.0	23.0	25.0	25.0	25.0	25.0
Swelling (ml/g)	11.0	ndd	14.0	3.0	7.8	7.5	8.0	8.5	ndd
Crystalline cellulose (% total cellulose)	ndb	ndd	50.9	72.3	65.1	68.4	62.8	53.3	ndd

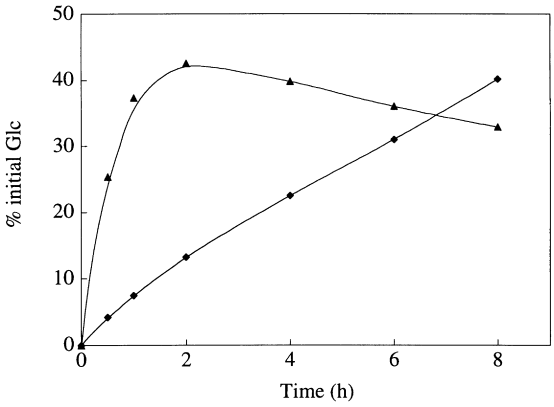

Fig. 3. Time course of release of cellobiose (▲) and glucose (◆) during enzymic hydrolysis by Celluclast 1.5L of freeze-dried cellulose-rich residue from sugar beet pulp (batch 3).

From these results, the following conditions were selected for the enzymic degradation of cellulosic residues by Celluclast 1.5L—pH 4.5 in 50 mmol l^{-1} acetate buffer and 40°C. Under these conditions, activities in Celluclast 1.5L towards CMC, Avicel, *p*NP-Glc and *p*NP-Cel were 3900, 805, 535 and 218 nkat ml^{-1}, respectively.

3.2. Characterisation of sugar beet pulp and cellulosic residues

Chemical composition and physical characterisation of sugar beet pulp and cellulose-rich residues are summarised in Table 1. The main sugars in beet pulp were glucose, arabinose and uronic acid. Glucose (≈200 mg g^{-1}) originated from cellulose, whereas arabinose (≈180 mg g^{-1}) and uronic acids, as galacturonic acid (≈184 mg g^{-1}), originated from pectins. This composition was in agreement with the previously published data (Micard et al., 1996). Degradation of pectins in sugar beet pulp by SP 584 yielded about 307 mg g^{-1} insoluble fraction. Eight different cellulose-rich residues (batches 1–8) were obtained from this insoluble fraction, by various methods of drying (Fig. 1). The water content varied from 19 mg g^{-1} after drying by solvent exchange (batch 7) to 74 mg g^{-1} after pulsed air drying (batch 4). Neutral sugar and uronic acid analyses in the cellulosic residues showed two different groups—(i) the first group (batches 1–4), where the residues contained about 30 mg g^{-1} arabinose and uronic acids; and (ii) the second group (batches 5–8), where the residues had lower amounts of arabinose and uronic acids (about 15 and 25 mg g^{-1}, respectively). From these results, it may be concluded that ethanol and acetone used for drying (batches 5–8) led to further washing of the pectic monomers. The commercial pectinase SP 584, used for the enzymic saccharification of the pectic fraction of sugar beet pulp, released a residue containing 67.5% of the initial glucose content, confirming that it was efficient for pectin degradation and did not extensively degrade cellulose (Micard et al., 1997).

The physical state of cellulosic residues was evaluated by X-ray diffraction and swelling (Table 1). Crystallinity of cellulose was measured as they were prepared, but was not determined in batches 1 and 2, because of their high water content. Percentage of crystalline cellulose was markedly influenced by drying method as it reached 50.9% after freeze-drying (batch 3), and 72.3% after drying for several days with pulsed air (batch 4). Crystalline cellulose in the residues dried by solvent exchange represented about 65% of total cellulose (batches 5–7) but it seemed to be lowered by grinding at the end of the storage (batch 8). Swelling also differed from 3 ml g^{-1} in batch 4 to 14 ml g^{-1} in batch 3, indicating that the higher the crystalline cellulose content, the lower the swelling. A high swelling may allow a faster diffusion of the enzymes in the material.

Therefore, different cellulosic substrates with similar compositions but different physical states were obtained.

3.3. Degradation of cellulosic residues into cellobiose

The different cellulosic residues were degraded by Celluclast 1.5L, and the release of cellobiose and glucose was measured. The results were compared with those obtained with standard celluloses degraded under the same conditions.

The time course of enzymic degradation of cellulosic residues was similar for all batches. Batch 3 is shown as an example (Fig. 3). The maximum concentration of cellobiose was reached after 2 h, except for batches 5 and 8 for which the maximum concentration was reached after 4 h (data not shown). Further, cellobiose concentration decreased due to its hydrolysis to glucose by β-glucosidases. The release of glucose was linear for the first hour and slowed down after that. After 2 h of hydrolysis by

Table 2
Residual cellulose, glucose and maximum contents in cellobiose after hydrolysis of cellulose-rich residues or standard celluloses by Celluclast 1.5L

	Batches of cellulose-rich residues								Standard celluloses	
	1	2	3	4	5	6	7	8	CMC	Avicel
Time of hydrolysis (h)	2	2	2	2	4	2	2	4	4	4
Residual cellulose[a]	42.4	44.7	44.3	49.0	36.0	37.2	43.4	38.3	43.0	82.6
Cellobiose[a]	43.5	42.3	42.5	38.2	42.8	45.1	41.7	35.2	35.0	9.1
Glucose[a]	14.1	13.0	13.2	12.8	21.2	17.7	14.9	26.5	22.0	8.3

[a] Expressed as a percentage of glucose initially present in cellulose-rich residues from sugar beet pulp or in standard celluloses.

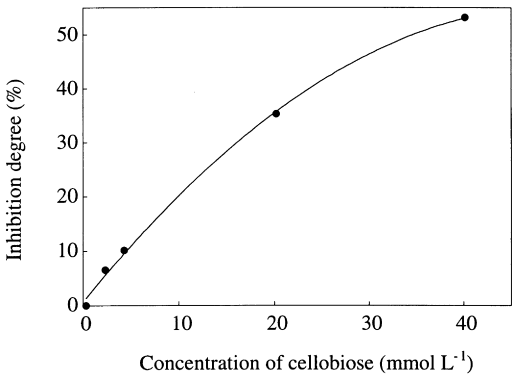

Fig. 4. Inhibition of cellobiohydrolase activities in Celluclast 1.5L measured on *p*NPCel with increasing concentrations of cellobiose up to 40 mmol l^{-1}.

Celluclast 1.5L, glucose content in the cellulase-resistant fraction was lowered but still represented 270 mg g^{-1} (Table 1).

The extent of cellulose degradation and the maximum concentration of cellobiose and glucose varied according to the residues. Table 2 shows that the maximum cellobiose production varied from 35.2% of the initial glucose content for batch 8 to 45.1% for batch 6. During the same time, glucose release varied from 12.8% for batch 4 to 24.5% for batch 6. The residue containing the highest level of crystalline cellulose (batch 4) was the least degraded as the cellulose content in its resistant fraction represented 49% of initial cellulose. When the residues were dried by solvent exchange and stored for a longer time (batches 5–7), the percentage of residual cellulose increased, although the crystallinity of cellulose was not significantly increased. Moreover, the cellulose was less crystalline and more extensively degraded if the residue was ground at the end of the storage (batch 8). Comparison of the results obtained with batches 1 and 5 indicated that drying by solvent exchange enhanced the degradability of the cellulosic material, though it also increased the content in crystalline cellulose. On the contrary, more glucose was produced after drying by solvent exchange. The comparison of batches 1 and 3 showed that freeze-drying had no effect on the extent of degradation.

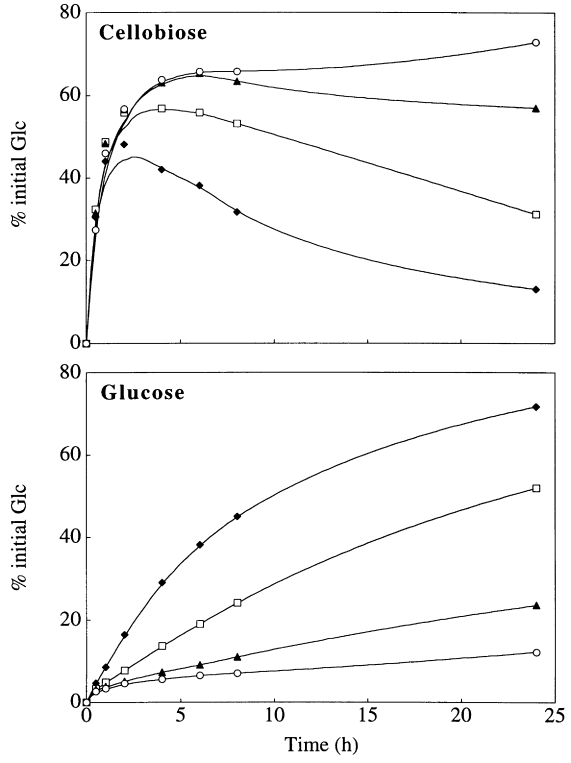

Fig. 5. Time course of release of cellobiose and glucose during enzymic hydrolysis of batch 3 by Celluclast 1.5L carried out in the presence of increasing concentrations in D-glucono-δ-lactone: Control without D-glucono-δ-lactone (◆); D-glucono-δ-lactone = 1 mmol l^{-1} (□); D-glucono-δ-lactone = 5 mmol l^{-1} (▲); D-glucono-δ-lactone = 20 mmol l^{-1} (□).

The ratio cellobiose/glucose was different according to the residues, although all incubations were carried out under the same conditions. This led to the conclusion that it was also influenced by the physical state of the substrate. Actually, the residue obtained from batch 4 and containing the highest percentage of crystalline cellulose (72.3%) released the lowest amount of glucose (12.8%). The opposite was not observed as batch 3 had the lowest percentage of crystalline cellulose (50.9%) and released the same amount of glucose as batch 4 (13.2%), while batch 8 had also a low percentage of crystalline cellulose (53.3%) but released much larger amount of glucose (26.5%). Batches 1, 2 and 3 were the most interesting for cellobiose production as they released the highest level of cellobiose and the lowest level of glucose.

CMC and Avicel were degraded by Celluclast 1.5L under the same conditions as the cellulosic residues (Table 2). The concentration in cellobiose obtained after 4 h of hydrolysis represented 35.0 and 9.1% of initial amount of glucose for CMC and Avicel, respectively. Avicel was very poorly degraded and this could be related to its crystalline nature. CMC degradation was lower and slower than that obtained from the cellulose-rich residues from sugar beet pulp, although in this case cellulose was totally amorphous. Likewise, the residual cellulose in the resistant fraction obtained from CMC was of the same order as that observed for the cellulose-rich residues. These results may be due to the presence of carboxymethyl groups, which led the polymer to be soluble but which may also decrease the affinity of enzymes.

3.4. Inhibition in Celluclast 1.5L

In order to evaluate the retroinhibition of Celluclast 1.5L activities by cellobiose, cellobiohydrolase activity was measured towards *p*NPCel in the presence of increasing concentrations of cellobiose up to 40 mmol l^{-1}. The

Table 3
Phenolic compounds (mg l^{-1}) in 6-day-old cultures of *P. cinnabarinus* MUCL39533 daily supplemented from day 3 to day 5 with 600 mg l^{-1} vanillate

	Control[a]	Commercial cellobiose[b]	Cellobiose from SBP[c]
Vanillin	222	694	725
Vanillyl alcohol	0	40	186
Methoxyhydroquinone	764	351	268

[a] Without cellobiose.
[b] With 2.5 g l^{-1} commercial cellobiose supplemented on day 3.
[c] With cellobiose-enriched fraction from sugar beet pulp supplemented on day 3.

inhibition degree was calculated as follows:

$$\text{Inhibition degree} = \left[1 - \frac{\text{Initial rate without cellobiose}}{\text{Initial rate with cellobiose}}\right] \times 100$$

Fig. 4 shows a polynomial curve of order 2 whose equation is given by $y = 0.639 + 2.172x - 0.022x^2$ ($r^2 = 0.998$). By knowing the cellobiose concentration released during the hydrolysis of cellulosic residues by Celluclast 1.5L, this equation allowed us to calculate the degree of retroinhibition due to cellobiose. The highest concentration of cellobiose in the reaction mixture was 5.9 mmol ml^{-1}, obtained with batch 6. According to this equation, a 12.8% inhibition of cellobiohydrolases was calculated, which was considered to hinder the cellulose hydrolysis very weakly.

The use of D-glucono-δ-lactone, known to inhibit glucosidases, was tested. First, activities in Celluclast 1.5L were measured towards model substrates in the presence of increasing concentrations of D-glucono-δ-lactone up to 20 mmol l^{-1}. With 1 mmol l^{-1} inhibitor, β-glucosidase activity was 23.9 nkat ml^{-1}, implying a loss of 95.5% activity whereas activities towards CMC, Avicel or *p*-NPCel were not affected. Thus, a cellulose-rich residue (batch 3) was hydrolysed by Celluclast 1.5L in the presence of the same concentrations of D-glucono-δ-lactone. As shown in Fig. 5, the release of glucose decreased with increasing concentration of inhibitor, whereas cellobiose appearance increased. It reached 56.3, 65.3 and 65.7% of initial glucose in the presence of 1, 5 and 20 mmol l^{-1} glucono-δ-lactone, respectively, to be compared with 48.2% initial glucose reached without inhibitor. Thus, the use of D-glucono-δ-lactone appeared to be advantageous for the production of cellobiose from cellulose-rich residues obtained from sugar beet pulp.

3.5. Vanillin production in the presence of cellobiose

Larger quantity of cellobiose-rich fraction was prepared from 200 g of sugar beet pulp in order to test its influence on the biotransformation of vanillic acid into vanillin by *P. cinnabarinus* MUCL39533. HPLC analysis of this fraction revealed 10.7 g cellobiose, 7.6 g glucose, and 2.6 g of cellodextrins with a degree of polymerisation larger than 2. Compared to the initial weight of sugar beet pulp, the successive degradation by pectinase and cellulase yielded 5.3, 3.8 and 1.3% of cellobiose, glucose and cellodextrins, respectively.

Cellobiose-rich fraction from sugar beet pulp was used in *P. cinnabarinus* MUCL39533 culture media. The concentrations of phenolic compounds were measured and compared with those obtained in the presence of commercial cellobiose. At the optimum of metabolite production (on day 6), the biotransformation of vanillic acid by *P. cinnabarinus* in the presence or absence of cellobiose is shown in Table 3. In the control cultures, methoxyhydroquinone accumulated, reaching 764 mg l^{-1} with a molar yield of 64.5%, while only 222 mg l^{-1} of vanillin and no vanillyl alcohol was produced. When commercial cellobiose was added to 3-day-old cultures, a 2.2-fold decrease in the methoxyhydroquinone level was recorded, while the vanillin production increased 3.1-fold. Under these conditions, small quantities of vanillyl alcohol were detected. When the cellobiose-rich fraction from sugar beet pulp was added to 3-day-old cultures of *P. cinnabarinus*, a 2.9-fold decrease in the methoxyhydroquinone level was recorded, while the vanillin production increased 3.3-fold, reaching a molar yield of 55.6%, and the level of vanillyl alcohol reached 186 mg l^{-1}. In all these assays, 80–85% of total vanillic acid added in the culture media were consumed.

4. Discussion

The enzymic release of ferulic acid from sugar beet pulp left a cellulose-rich residue, used as cellobiose source. When cellobiose was used in *P. cinnabarinus* culture medium, it largely channelled the vanillic acid metabolism to vanillin and vanillyl alcohol, and decreased the methoxyhydroquinone appearance. It was previously reported that *P. cinnabarinus* degraded vanillic acid via either a reductive pathway leading to vanillin and vanillyl alcohol or an oxidative decarboxylation pathway leading to methoxyhydroquinone (Falconnier et al., 1994; Lesage-Meessen et al., 1996). Adding cellobiose by-passed the methoxyhydroquinone formation, thus favouring the production of vanillin (Lesage-Meessen et al., 1997). Cellobiose may act as a supplier of energy source required for the expression of

the reductive pathway or as an inducer of cellobiose: quinone oxidoreductase, which inhibits vanillic acid decarboxylation. However, the use of an efficient resin could limit the formation of vanillic alcohol (Stentelaire et al., 1998). Under these conditions, vanillin production could correspond to the total reduced products, i.e. 734 mg l^{-1} in the case of commercial cellobiose and 911 mg l^{-1} in the case of cellobiose from sugar beet pulp. The differences observed in the production of phenolic compounds in the presence of commercial cellobiose or cellobiose-rich fraction from sugar beet pulp could be related with other sugars contained in the last one and originating from cellulose degradation. Particularly, glucose seemed to be favourable to the vanillin synthesis.

Celluclast 1.5L was produced by *Trichoderma reesei*, containing several cellulose-degrading enzymes. In this work, it was used to produce cellobiose from sugar beet pulp. It is known that endo-β-D-glucanases and cellobiohydrolases act synergistically to completely degrade amorphous or crystalline cellulose (Wood, 1985). Celluclast 1.5L also contained β-glucosidases, which degraded cellobiose as soon as it appeared. All these enzymes are part of the same cellulase system (Walker and Wilson, 1991) and consequently work optimally under the same conditions of pH and temperature. It is probably for this reason that it was not possible to discriminate cellobiohydrolases and β-glucosidases. Under the conditions chosen for the use of Celluclast 1.5L, cellobiohydrolases exhibited their highest activity. Nevertheless, this activity was lower than β-glucosidases.

Cellulose hydrolysis by *T. reesei* cellulases produced cellobiose and glucose. Both are known to inhibit the degradation of cellulose (Walker and Wilson, 1991), low concentration of cellobiose desorbing the Cellulose-Binding Domain of hydrolases from the cellulose (Takada et al., 1996). However, Micard et al. (1997) showed that removing the hydrolysis products, i.e. glucose and cellobiose, did not enhance the degradation of cellulose-rich residues from sugar beet pulp. This had to be related to the low concentration of cellobiose and the low retroinhibition degree shown by this to be related to the low concentration of cellobiose and the low retroinhibition degree shown by this work. On the contrary, the use of D-glucono-δ-lactone efficiently inhibited the hydrolysis of cellobiose by inhibiting the glucosidase activity. However, its use in the context of the European legislation concerning "natural" flavours is questionable.

Moreover, our results demonstrated that enzymic hydrolysis depended on the physical state of cellulose in the cellulose-rich residues. Storage was shown to be a critical step in the degradability of cellulosic materials. It could induce an aggregation of fine particles and therefore a lower specific area. Grinding increased the specific area of the residues and favoured subsequent cellulose hydrolysis.

These results confirmed the industrial interest of sugar beet pulp in the production of vanillin. In the context of "natural" vanillin, enzymically-hydrolysed sugar beet pulp could provide on the one hand ferulic acid, as the aroma precursor able to be biotransformed into vanillic acid by *A. niger*, and on the other hand cellobiose as an activator of the metabolic pathway from vanillic acid to vanillin in *P. cinnabarinus*.

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