
Grass cell wall degradation by fungal cellulases and hemicellulases

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Hallesmeersch, I. and Vandamme, E.J. (2003). Grass cell wall degradation by fungal cellulases and hemicellulases. *Fungal Diversity* 13: 13-27.

There has been an intensified interest in the microbial and enzymatic conversion of renewable raw materials into useful products, such as feed, chemicals and energy. Enzymes involved in these conversions have potential commercial applications in quite different fields. An interesting ascomycete able to convert up to 30% and 55% of grass cell wall components after 7 and 21 days respectively, was isolated during a screening programme for grass cell wall degrading microorganisms. This fungus may serve as an interesting source of novel xylanases and cellulases, since other cellulosic and hemicellulosic substrates, such as microcrystalline cellulose, cotton, paper and xylan were also converted. For example, unprinted newspaper (20g/L) was completely solubilised within 4-5 days at 30°C. According to the type of cellulosic or hemicellulosic substrate, used as carbon source in the growth medium, enzyme synthesis profiles differed considerably, not only did the level of enzyme activity differ, also the type of enzyme produced differed. The pH and temperature profiles of the fungal enzyme activities present in the crude supernatant were determined. Most enzymes functioned optimally at 50-60°C and at neutral pH. Thermal stability of the enzymes was compared at 30°C and 60°C. Addition of glycerol (30% w/v) stabilised some of the enzymes from thermal inactivation. The conversion of different substrates by the crude supernatant was followed by HPLC-analysis of the released mono- and oligosaccharides. The highest degree of conversion was observed with substrates such as unprinted newspaper (72%), xylan (61%), carboxymethyl cellulose (60%), Whatmann paper (53%) and cotton (44%). Microcrystalline cellulose and grass were hydrolysed to a lesser extent. Further characterisation of these enzymes is now underway, as well as the taxonomic identification of the ascomycete.

Key words: cellulase, grass cell wall, hemicellulase, screening

Introduction

There has been considerable interest in microbial and enzymatic conversion of cellulosic and hemicellulosic substrates, such as waste plant cell wall material. Its successful utilization as renewable raw material depends on the development of economically feasible technologies for the hydrolysis into

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low molecular weight products and for the conversion of these hydrolysis products into commercial products such as liquid fuel, food materials, and chemicals (Risna and Suhirman, 2002). Enzymes involved in these conversions can find commercial applications in quite different fields.

In this context, a screening of grass (*Lolium perenne*) cell wall degrading microorganisms was performed. Grass was chosen as plant material, since it produces large amounts of waste, which is difficult to degrade through composting.

Materials and methods

Plant material

Harvested grass (*Lolium perenne*) was stored by freezing; prior to use, it was freeze-dried and ground (1-2mm).

Screening procedure

Samples from various origins were suspended, in sterile physiological solution, diluted and plated on a medium containing 20g/l grass, 2.5g/l (NH₄)₂SO₄ and 20g/l agar (Oxoid) at pH 7 (SGrM). After 4 days of incubation at 30°C, single colonies were picked up and inoculated on a general storage medium (GSM) containing 20g/l glucose, 10g/l yeast extract (Oxoid), 10g/l tryptone (Oxoid), 10g/l K₂HPO₄ and 20g/l agar (Oxoid), at pH 7.

Grass conversion

Strains grown on SGrM during 4 days at 30°C were flooded with physiological solution (10ml); 1% (v/v) of this cell suspension was inoculated in liquid medium (LGrM) containing 5g/l buffer extracted grass, 2.5g/l (NH₄)₂SO₄, 1g/l K₂HPO₄, 1g/l yeast extract (Oxoid), 1g/l MgSO₄.7H₂O, 8 10⁻⁵g/l CuSO₄.2H₂O, 0.02g/l FeSO₄.7H₂O, 0.01g/l MnSO₄.6H₂O and 4 10⁻⁴g/l ZnSO₄.7H₂O at pH 7. Incubation took place at 200 rpm and 30°C over 7 days. The residual amount of grass and microbial biomass were collected by centrifugation and filtration (0.22µm) and washed twice with phosphate buffer (0.1M, pH 7). After removal of microbial cells, the amount of residual grass was gravimetrically determined (Akin, 1987). Blanks were included during the whole test.

Several methods were compared to remove microbial cells from the residual amount of grass:

- Chitinase (Sigma) for the removal of fungal cells: 5U/g grass was added and incubated at 25°C (Akin, 1987).
- Acidified pepsin (Sigma) to remove fungal and bacterial cells: 5ml/g grass of 0.2% pepsin in 0.08M HCl was added and incubated at 25°C (Akin, 1987).
- A chemical method, using 1M NaOH at 100°C and ethanol at 60°C to remove adherent microorganisms from grass (Lee *et al.*, 2000).

Protein

The total protein content was determined, using the total protein test kit (Sigma).

Growth and enzyme synthesis on various cellulosic and hemicellulosic substrates

Strain P13 was grown on GSM for 4 days at 30°C, subsequently a cell suspension was made, using 10ml of sterile physiological solution; inoculation was performed with 1% (v/v) of the cell suspension in liquid growth medium, containing 5g/l cellulosic or hemicellulosic substrate, 2.5g/l (NH₄)₂SO₄, 1g/l K₂HPO₄, 1g/l yeast extract (Oxoid), 1g/l MgSO₄.7H₂O, 8 10⁻⁵g/l CuSO₄.2H₂O, 0.02g/l FeSO₄.7H₂O, 0.01g/l MnSO₄.6H₂O and 4 10⁻⁴g/l ZnSO₄.7H₂O at pH 7. Grass, glucose, cellobiose, carboxymethyl cellulose (CMC), microcrystalline cellulose, shredded, unprinted newspaper, birchwood xylan, Whatman paper, pectin and cotton were used as carbon sources. Incubation took place at 200 rpm and 30°C during 7 days. At regular times, the total protein content, pH and various enzyme activities were measured.

Enzyme activities

All enzyme activities were measured by colorimetric methods. Carboxymethyl cellulose (CMC), birchwood xylan and galactomannan (locust bean gum) were used as substrates to measure respectively endo-β-1,4-glucanase (endocellulase), β-1,4-xylanase and β-1,4-mannanase. In each case, 0.3ml culture supernatant was incubated together with 0.3ml substrate solution (pH 7) at 30°C during 15minutes; after adding 0.9ml DNS-reagent, samples were boiled during 10minutes. D-glucose, D-xylose and D-mannose were used as standards. Enzyme activities were expressed as U/ml, with 1 Unit defined as 1μmol of reducing sugar equivalent released per minute. The absorbance was measured at 575 nm. β-1,4-Cellobiohydrolase, β-1,4-glucosidase and β-1,4-

xylosidase were determined using p-nitrophenyl (pnp)-glucosides (Sigma) as substrates; 0.25ml culture supernatant was incubated with 0.25ml pnp-substrate at 30°C during 30 minutes, afterwards, the absorbance was measured at 400nm. The enzyme activities were expressed as U/ml, with 1 Unit defined as 1µmol p-nitrophenol released per minute.

Temperature and pH-optimum

Determination of the optimal temperature and pH was performed by measuring the enzyme activity at different temperatures or at different pH-values. In these tests, culture supernatant of strain P13 grown on grass was used.

Stability of enzyme activities

The thermal stability was determined by incubating the culture supernatant at 25°C and 60°C; at regular times, the residual level of enzyme activities were measured. From these data, $T_{1/2}$ was calculated. In these tests, culture supernatant of strain P13 grown on grass was used.

The activation or inhibition effect of several ions and EDTA on the various enzymes was tested. Cu^{2+} , Mn^{2+} , Mg^{2+} , Cl^- , Ca^{2+} , Fe^{2+} , Zn^{2+} and EDTA were tested at 1mM, by adding CuSO_4 , MnSO_4 , MgSO_4 , MgCl_2 , FeSO_4 and CuSO_4 to the culture supernatant of strain P13 grown on grass. Incubation took place at 4°C for 24hours.

Hydrolysis of grass cell wall polysaccharides

Fifty ml of supernatant of a culture grown on grass was freeze-dried and redissolved in 25ml phosphate buffer (pH 7.0.1M), containing 5g/l substrate. The substrates used in this test were: cellobiose, carboxymethyl cellulose (CMC), microcrystalline cellulose, Whatman paper, unprinted and shredded newspaper, birchwood xylan and grass. Samples were taken at regular times, from which the amount of sugars released from the substrates was determined by HPLC-analysis (Dionex). A Dionex Carbopac PA-100 column (4 * 250 mm) with precolumn was used; the elution rate was 1ml/minute and detection of the sugars was performed with a pulsed amperometric detector. Two programs were used, one was developed for the detection of D-glucose, D-galactose, D-arabinose, L-rhamnose, D-fructose, D-xylose, sucrose and galacturonic acid; the second was used for the detection of D-glucose and cellooligosaccharides up to cellopentaose.

Results and discussion

Screening

Various samples such as compost, soil and decayed wood, were screened for grass degrading microorganisms. Many strains were able to grow on SGrM indicating the poor selectivity of this original screening medium. Growth was apparently possible on soluble sugars and proteins, present in the grass cells.

An extraction method was optimized to remove these readily utilizable nutrients. To minimize the effect on the cell wall structure, a phosphate buffer (1M, pH7) was used instead of solvents. The effect of the extraction period, the number of extractions and the need for washing of the residue was tested.

Further screening was performed with grass, extracted twice for 1hour with phosphate buffer (10g/L) and washed twice with distilled water. When comparing the number of strains isolated from the same sample, on both the original and the improved screening medium, it was observed that a buffer extraction of the grass resulted in an increased selectivity of the screening medium.

In total, 89 strains able to metabolize the extracted grass material were isolated, 44 were fungi and 45 bacteria.

All lignocellulosic materials are formed predominantly of three compounds. Cellulose is the major constituent and consists of linear β -1,4-linked D-glucose residues, it is responsible for strength and flexibility. Hemicellulose consists of more heterogenous polysaccharides, the major hemicellulose polymer is xylan, which consists of a β -1,4-linked D-xylose backbone and can be substituted by different side groups. Lignin is a polyaromatic heteropolymer conferring decay and hardness (De Vries and Visser, 2001; Sin *et al.*, 2002). The ability to digest cellulosic substrates is widely distributed among many genera in the domain *Bacteria* and in the fungal groups within the domain *Eucarya* (Abdel-Raheem and Shearer, 2002). Fungal cellulose utilization is distributed across the entire kingdom, from the primitive protist-like *Chytridomycetes* to the *Basidiomycetes*. Most cellulolytic fungi are, however, found in the ascomycetes, basidiomycetes and their anamorphs (Abdel-Raheem and Shearer, 2002; Lyund *et al.*, 2002).

Grass conversion

All isolated strains were evaluated for their grass converting capabilities. Determination of microbial growth and grass degradation could not be

performed by conventional methods, because of the insoluble and complex nature of the substrate. Akin (1987) describes a gravimetric method for the assessment of fungal biomass associated with plant material, whereby all microbial cells had to be removed from the grass residue after growth. In this research several enzymatic, physical and chemical methods were evaluated as to their efficiency to remove selectively microbial cells adhering to plant material. From the differences in dry weight before and after growth and after the removal of microbial cells, the microbial biomass formation and grass degradation could be calculated.

Based upon literature data, the use of chitinase, acidified pepsin and NaOH-ethanol seemed promising and were compared for the removal of fungal cells, while acidified pepsin and NaOH-ethanol were compared for the removal of bacterial cells (Akin, 1987; Sijtsma and Tan, 1996; Lee *et al.*, 2000). The results are represented in Table 1.

Table 1. The efficiency of different methods for the removal of microbial biomass from grass residues.

Microbial group	Treatment	Incubation time	% Grass Conversion	% Microbial Growth	% Effect on the Control	
Fungi	Chitinase	6	8.15 ± 1.21	6.25 ± 0.6	7.07	
		24	19.20 ± 0.66	14.09 ± 0.87	8.31	
		48	16.30 ± 1.16	12.38 ± 0.24	7.27	
	Acidified-pepsin	6	14.83 ± 0.96	10.06 ± 0.96	11.12	
		24	18.45 ± 1.25	8.15 ± 0.66	10.23	
		48	16.91 ± 0.51	9.11 ± 1.06	9.22	
	NaOH-ethanol			18.65 ± 2.39	8.04 ± 6.96	14.28
		Acidified-pepsin	6	6.15 ± 0.45	2.66 ± 0.45	11.12
			24	6.43 ± 0.36	3.11 ± 0.75	10.23
48	6.98 ± 1.01		2.96 ± 0.32	9.22		
Bacteria	NaOH-ethanol		7.15 ± 3.03	3.18 ± 1.89	14.28	

Chitinase and pepsin applied for a minimum of 24 hours seemed useful in causing lysis of fungal and bacterial cells. The use of NaOH-ethanol affected the grass cells and was less accurate.

It was also established whether spectrophotometrical measurements of the released chlorophyll in the growth medium during growth on grass material could be related to the biomass formed and grass converted. This was proven impossible, probably because several microbial strains produced pigments, which interfered with the measurements. Also the subsequent extraction of

chlorophyll from the grass residue could not be related to the amount of grass converted.

All isolated strains were tested as to their grass converting capabilities. The chitinase-treatment was used to remove fungal cells, while acidified-pepsin was used to remove bacterial cells (Figs. 1 and 2).

With the fungal strains, up to 30% conversion was observed after 7 days of incubation; 13 of the 44 fungi were able to convert more than 20% of the grass. With the bacterial strains, the highest grass degradation found was 8% after 7 days. These tests confirm that fungi are the most promising group of microorganisms, producing extracellular enzymes able to convert grass cell wall polymers. However, in nature lignocellulosic substrate utilization is thought to be carried out by multiple microbial species. Removal of certain plant cell wall polymers by one species or group improves the accessibility of a second group (Lyund *et al.*, 2002).

Three decay types are generally recognized in terrestrial fungi. Soft rot, is the enzyme decay of cellulose and hemicellulose, which is accompanied by little or no lignin degradation. This kind of decay is characteristic for many ascomycete and anamorphic genera. Other types of decay are white rot and brown rot, found only among basidiomycetes and a few higher ascomycete genera. During white rot decay, lignin is preferably degraded, while during brown rot decay, polysaccharides are degraded first and the lignin matrix remains nearly undigested but not unchanged (Jennings and Lysek, 1996; Sin *et al.*, 2002).

One of the most promising fungi from our screening, referred to as strain P13, was selected for further characterization; it converted up to 30% and 50% of the grass cell wall material after 7 days and 21 days respectively (Fig. 3).

Filamentous fungi, such as strain P13, are particularly interesting producers of cellulases and xylanases from an industrial point of view, due to the fact that they excrete these enzymes into the culture medium, thus eliminating the need for cell disruption. Furthermore, enzyme levels from fungal cultures are typically much higher than from yeasts or bacteria (Haltrich *et al.*, 1996).

Characterization of the fungal enzymes

Growth profile and enzyme synthesis

The optimal growth temperature of the ascomycetous fungus P13, was found to be 30°C; less growth was noticed at 25°C, while no growth occurred at

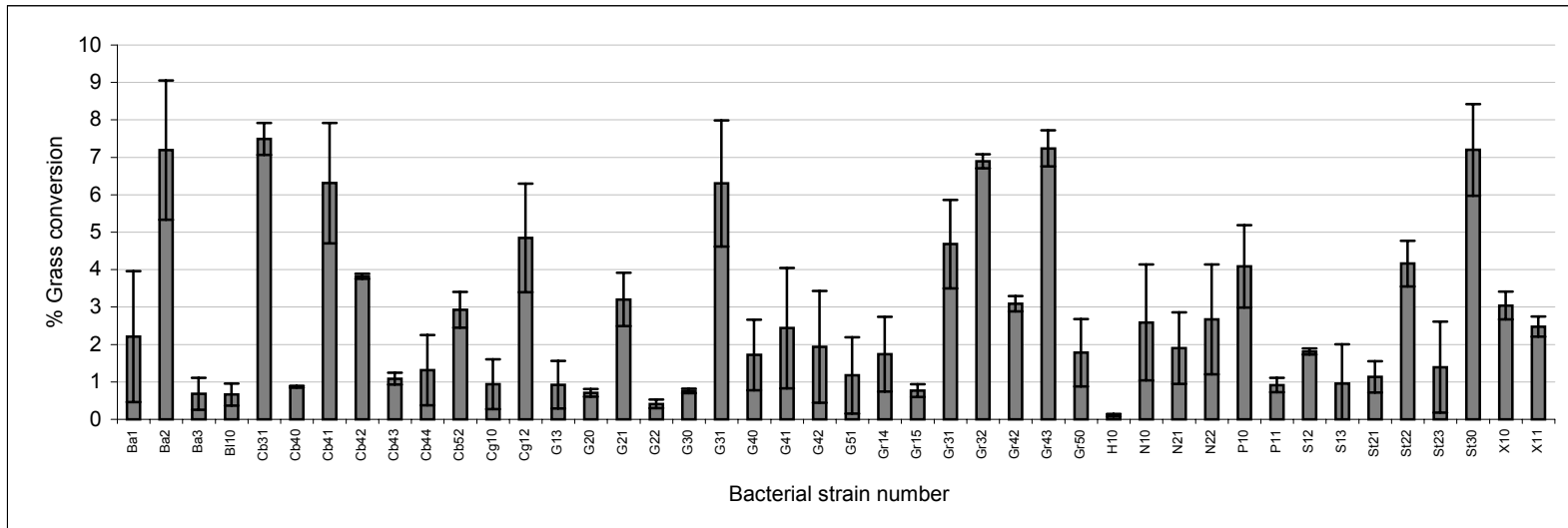


Fig. 1. Bacterial grass conversion.

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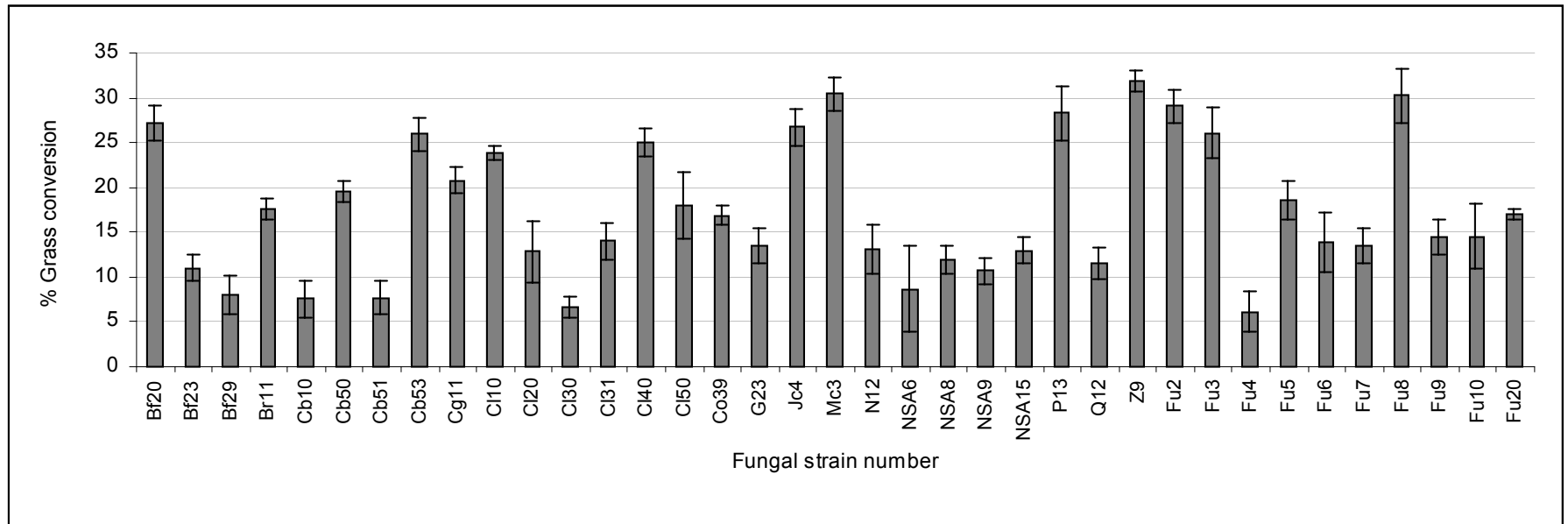


Fig. 2. Fungal grass conversion.

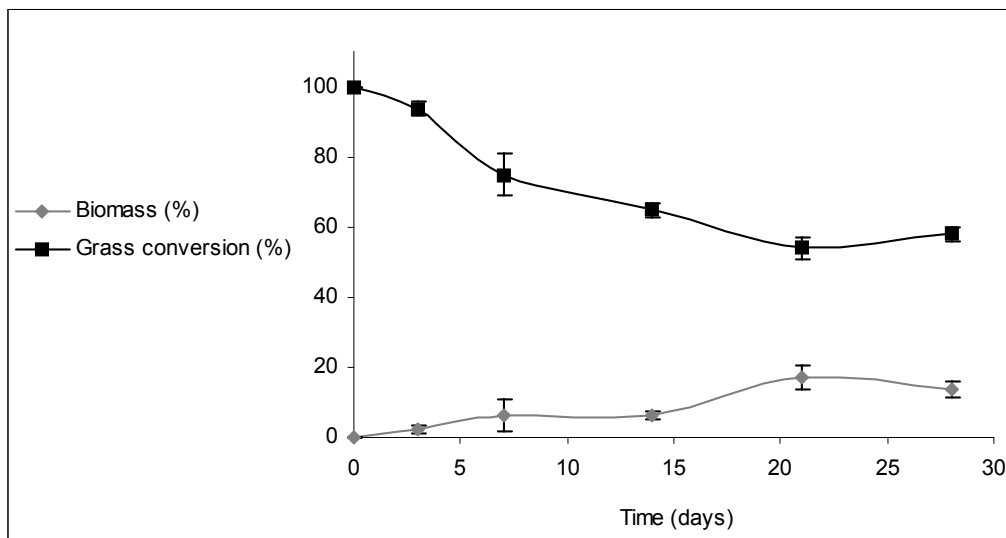


Fig. 3. Fungal biomass formation and substrate degradation during growth on grass.

37°C. The fungus showed good growth at pH 7 up to pH 9, while no growth occurred at pH 5.

Growth and enzyme synthesis profiles of the fungus, when grown on various cellulosic and hemicellulosic substrates were determined (Fig. 4). Good growth was noticed on substrates such as microcrystalline cellulose, cotton, Whatmann paper, unprinted newspaper and xylan. According to the type of cellulosic or hemicellulosic substrate, used as carbon source in the growth medium, enzyme synthesis profiles differed considerably; not only did the level of enzyme activity differ, also the type of enzyme produced differed. Synthesis of the following enzymes was checked: endocellulase, cellobiohydrolase, β -glucosidase, β -1,4-xylanase, β -xylosidase, β -1,4-mannanase, β -mannosidase, endopolygalacturonase, β -1,3-glucanase (laminarase), β -galactosidase, α -galactosidase, α -arabinosidase, α -glucosidase, acetylsterase, ligninases and protease.

None of the tested enzymes was synthesized during growth on glucose, indicating the inducible nature of the enzymes. During growth on grass and newspaper, almost the same types of enzymes were produced. The more pure cellulosic or hemicellulosic substrates induced the synthesis of cellulolytic or xylanolytic enzymes respectively and to a lesser extent, a mixture of both.

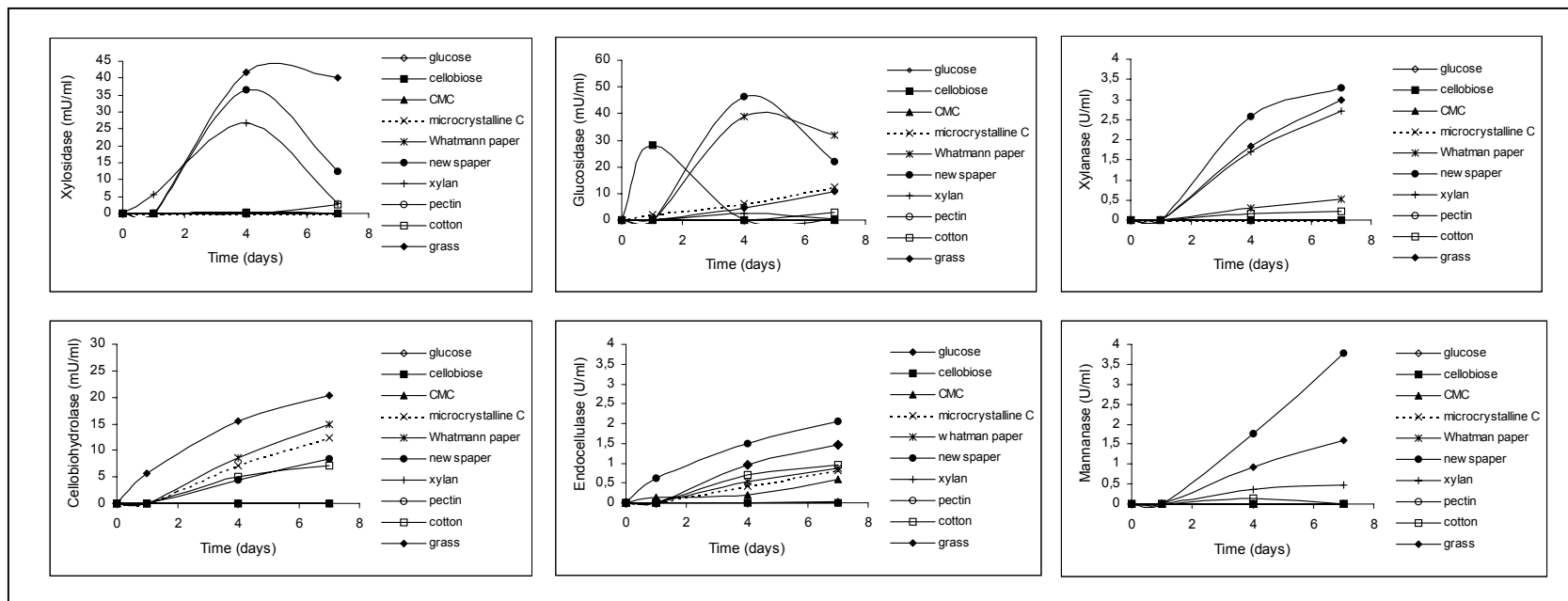


Fig. 4. Enzyme synthesis profiles during growth on different substrates of strain P13.

During growth on grass and newspaper a small amount of protease and α -arabinosidase might be synthesized as well. Sin *et al.* (2002) revealed that fungi encountered on monocotyledonous substrates produce carbohydrases such as cellulases and xylanases on lignocellulosic substrates, indicating the ability of soft rot type decay, often they show little potential for breakdown of lignin in contrast to wood decay fungi.

pH and temperature profiles of the fungal enzymes

The pH and temperature profiles for enzyme activities present in the crude supernatant were determined. Most enzymes function optimally at 40-60°C and at neutral pH. The temperature profiles of the endocellulase, xylanase and mannanase showed an optimum at 60°C, with the endocellulase and the mannanase displaying higher activity over a wider temperature range, especially the range 40-70°C. The temperature profiles of the cellobiohydrolase, glucosidase and xylosidase showed optima both at 40°C and 60°C. These results might suggest that multiple forms of these enzymes are synthesized.

Stability of the fungal enzymes

The thermal stability of the enzymes was compared at 25°C and 60°C (Table 2). The most stable enzymes at 60°C were the endocellulase, the xylanase and the β -glucosidase; all other enzymes were less thermostable. Addition of glycerol (30%) might stabilize some of the enzymes from thermal inactivation.

Table 2. Thermal stability of the enzymes (* = no activity loss after 3 hours of incubation).

Enzyme	$t_{1/2}$ (h)	
	25 °C	60 °C
Endocellulase	*	5.54
Cellobiohydrolase	13.9	0.48
β -Glucosidase	35.7	0.43
Xylanase	*	0.53
β -xylosidase	55.6	0.48
Mannanase	*	0.46

The inhibitory effect of several ions and of EDTA was also assessed, they were all tested at 1mM (Table 3). Cellobiohydrolase, xylosidase and

mannanase were almost completely inhibited by Cu^{2+} , while xylosidase was also inhibited by Zn^{2+} and cellobiohydrolase and mannanase by Mn^{2+} . Cellobiohydrolase was almost completely inhibited by Mg^{2+} . The information that is provided by these kind of tests, might be interesting upon further purification and application of the enzymes.

Table 3. Enzyme inhibition by several ions and EDTA.

Enzyme activity (%)	Blank	Cu^{2+}	Mn^{2+}	Mg^{2+}	Cl^-	Ca^{2+}	Fe^{2+}	Zn^{2+}	EDTA
Endocellulase	100 ± 0.01	64 ± 1.05	79 ± 0.36	99 ± 0.05	99 ± 0.41	97 ± 0.05	132 ± 0.01	100 ± 0.01	95 ± 1.10
Cellobiohydrolase	100 ± 22.1	20 ± 17.3	43 ± 43.3	5 ± 0.06	117 ± 1.56	119 ± 0.89	121 ± 11.5	120 ± 9.6	108 ± 7.89
β -Glucosidase	100 ± 2.03	132 ± 1.09	122 ± 1.56	120 ± 6.22	135 ± 0.03	121 ± 4.23	132 ± 3.68	142 ± 1.82	107 ± 0.51
Xylanase	100 ± 0.81	100 ± 1.21	130 ± 0.07	88 ± 1.35	88 ± 1.11	87 ± 0.03	101 ± 0.65	102 ± 0.77	80 ± 1.09
β -Xylosidase	100 ± 1.32	2 ± 7.58	84 ± 7.22	100 ± 0.06	100 ± 2.06	115 ± 1.36	120 ± 4.21	38 ± 2.65	122 ± 0.02
Mannanase	100 ± 10.2	19 ± 1.22	20 ± 1.25	100 ± 3.69	83 ± 4.56	103 ± 4.81	97 ± 2.55	82 ± 1.49	62 ± 2.09

Hydrolysis of grass cell wall polysaccharides

The hydrolysis rate of different cellulosic and hemicellulosic substrates by the crude supernatant of strain P13 grown on grass, was followed by HPLC-analysis (Fig. 5). The highest degree of conversion was observed with substrates such as unprinted newspaper (72%), xylan (61%), carboxymethyl cellulose (60%), Whatmann paper (53%) and cotton (44%). Microcrystalline cellulose and grass were hydrolysed to a lesser extent.

The sugars released from the cellulosic substrates could be used directly in animal/human food. Alternatively, the sugars could be used as a substrate for subsequent fermentations or other processes, which could yield valuable end products. Cellulolytic and hemicellulolytic enzymes could also be used directly

to increase the digestibility of food having a high-fiber content, and to enhance food flavor, texture or other qualities (Walsh, 2002).

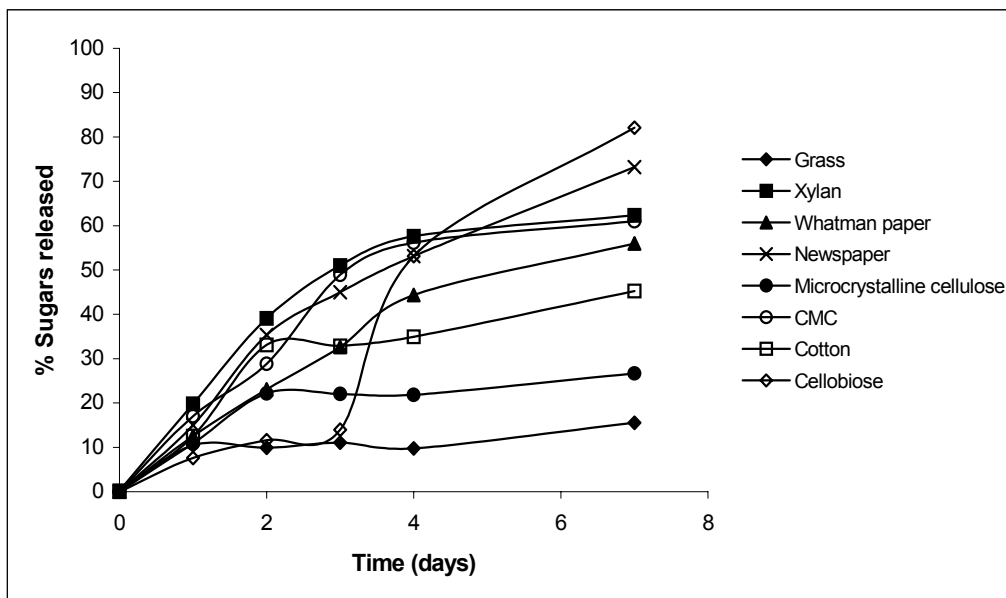


Fig. 5. HPLC-analysis of the hydrolysis of different substrates by enzymes present in the crude supernatant (sugars detected are: D-glucose, D-galactose, D-arabinose, L-rhanose, D-fructose, D-xylose, sucrose, cellooligosaccharides up to cellopentaose and galacturonic acid).

Perspectives

Characterization of fungal strain P13 and optimization of the enzyme synthesis is currently under study, together with further characterization and purification of its enzymes. Further research will also focus on possible applications of the enzymes in several industrial and environmental sectors.

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(Received 18 November 2002; accepted 10 April 2003)