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Improvement of the biodegradation efficiency by pre-treatment and innovative biological techniques

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1. Introduction

The total biodegradation efficiency of the faecal material in the thermophilic anaerobic reactor is equal to about 40 % if the system is operated at an equilibrium pH of 8, an ammonia concentration higher than 3 g/l due to the feeding of urea and a solid retention time of about 50 days. The biodegradation efficiency decreases to 30 % when the equilibrium pH is lowered to 6.5 due to the lower ammonia concentration and the solid retention time equal to 25 days.

TN41.3 reviewed already well known organisms that may be used to increase the biodegradation efficiency of the faecal material. This document focuses on the possible introduction of anaerobic fungi. Anaerobic fungi are the inhabitants of the digestive tract of herbivorous mammals, ruminants as well non-ruminants. These fungi produce and secrete enzymes to hydrolyse specific compounds such as cellulose, pectin, xylan and other plant derived recalcitrant compounds.

Microscopic evaluation of the content of the demonstration reactor in conditions reported in TN 43.3 showed that no fungi are present in the inoculum. This means that it can be interesting to introduce anaerobic fungi in the first compartment to improve the biodegradation efficiency of fibrous material.

2. Anaerobic fungi

2.1 Introduction

Extracellular microbial cellulase is of major importance for industrial purposes to produce glucose from cellulose. Particulary notable in this regard are the cellulases of *Trichoderma* species, *Penicillium pinophilum/funiculosum* and *Fusarium solani*. These aerobic fungi can be cultivated in axenic cultures to produce the cellulase. The product can be purified so that the pure enzyme cellulase is remaining. This kind of cellulase may be used to pre-treat the faecal material or the recalcitrant fraction of it. Yet, it is not possible to introduce this kind of fungi directly in the reactor because the operation conditions are not favourable for survival and growth of this species.

Fungi can be found in various of natural ecosystems such as soil but some specific genera are known which are associated with the gastro-intestinal tract of herbivorous mammals, ruminants as well non-ruminants. Faeces is the major route for transfer of anaerobic fungi (Wubah et al. 1991b). One of the major characteristics of all anaerobic fungi examined as thus far, is their production and secretion of a range of polysaccharide degrading enzymes, including cellulases, xylanases and glucosidehydrolases. Most of the in vitro studies on the location of fiber-degrading enzymes produced by anaerobic fungi indicate that they are predominantly extra-cellular and free in the culture liquid. Therefore anaerobic fungi and their enzymes could be interesting for many biotechnological applications including saccharafication of lignocellulosic residues.

2.2 Classification

In 1975 it was first found that an ovine rumen inhabitant which was known as a flagellate named *Neocallimastix frontales* was in fact a zoosporic stage of an obligate anaerobic

chytridiomycete fungus. During the eighties research was performed to identify and classify anaerobic fungi. The classification of anaerobic fungi is shown in Table 2–1.

Anaerobic fungi have been isolated from foregut fermentors, ruminants such as cattle as well from ruminant like animals such as the kangaroo. Present results indicate that fungal colonisation in herbivores requires a high fiber diet and a capacious organ for fermentative digestion.

Table 2–1 Classification of the anaerobic fungi (Teunissen & Op den Camp, 1993)

Division	Eumycota
Subdivision	Mastigomycotina
Class	Chytridiomycetes
Order	Spizellomycetales
Family	Neocallimasticaceae

Genus	***Species***	***Host***
Neocallimastix	*N. frontalis*	sheep
	N. hurleyensis	sheep
	N. patriciarum	sheep
Caecomyces	*C. communis*	sheep
	C. equi	horse
Piromyces	*P. communis*	sheep
	P. dumbonica	Indian elephant
	P. mae	horse
	P. rhizinflata	ass
Orpinomyces	*O. bovis*	cattle
	*O. joyonii**	sheep
Ruminomyces	*R elegans*	cattle
	*R. mucronatans**	cattle

*Original names were *Neocallimastix joyonii* and *Anaeromyces mucronatans* respectively

2.3 Cellulose biodegradation by Neocallimastix frontalis

Many microorganisms can grow on cellulose but few synthesize the complete enzyme systems that can effect the hydrolysis of crystalline cellulose material. Those enzyme systems comprise endoglucanases, exoglucanases and β-glucosidases and can be produced both by aerobic and anaerobic fungi. Comparative study has shown that the extracellular cellulolytic enzymes of *N. frontalis* have an higher cellulose digestion capacity for cellulose than the cellulase of the aerobic fungus *T. reesei* (Wood et al. 1986).

The initiation of cellulose degradation (amorphogenesis) by anaerobic fungi differs from the mechanism (oxidative or other non-hydrolytic processes) described for aerobic fungi.

Wood et al (1986) found that the anaerobic rumen fungus *Neocallimastix frontalis* when grown in co-culture with the methanogenic bacteria *Methanobrevibacter smithii* produced a cellulase that was able to solubilize crystalline cellulose to the extent of 98 % in 72 h.

2.4 Cellulose biodegradation by Piromonas

A new anaerobic rumen fungus *Piromonas communis* was selected from sheep digesta and cultured on cotton fibre. This fungus contains an extracellular cellulase that can solubilize hydrogen-bond-ordered cellulose at a rate greater than that shown by the cellulase of the *N. frontalis M. smithii* co-culture. The cell-free culture fluid is also very rich in xylan-degrading enzymes.

The hydrolysis of cellulose and xylan obtained from Graminacea by cell-free cellulase from *Piromonas communis* was compared with cell-free cellulases from *Trichoderma koningii, Trichoderma reesei* and *Penicillum pinophilum* (Wood & Wilson, 1995).

Figure 2-1 compares the solubilisation of cellulose obtained from cotton fibre by *P. communis, P. pinophilum, T. koningii* and *T. reesei*. It appeared that the cell-free cellulase of *P. Communis* hydrolysed cellulose with an efficiency of 85 % within a period of 48 hours. This was significant higher compared to the hydrolyses noticed in the other applications in which only 7% to 12% was hydrolysed. A notable property of the *P. communis* enzyme was the approximate linearity of the rate of hydrolysis of the cellulose substrate. Notice that the experiment was performed under the optimal conditions for each origin of cellulase: 40 °C and pH 6.0 for *P. communis*; 50°C and pH 5.0 for *P. pinophilum, T. koningii* and *T. reesei*.

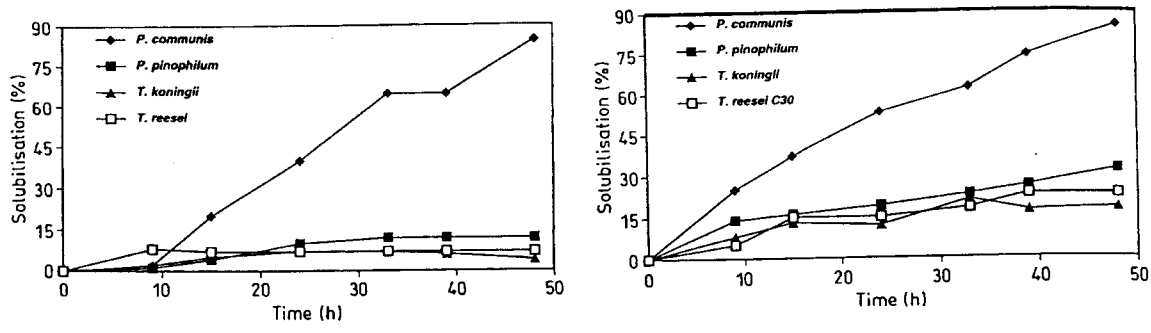

Figure 2-1. Solubilisation of cellulose by cell-free cellulases of *P. Communis, P. pinophilum, T. koningii* and *T. reesei* (Wood & Wilson, 1995)

Table 2–2 reports the results of a comparative experiment in which 5 mg cellulose or xyla n derived from different sources are hydrolysed by the cellulase of *Piromonas communis*. It appeared that cellulose and xylan obtained from plant material, mainly from Graminacea, are quite well hydrolysed.

Table 2–2. Hydrolysis of different cellulose and xylan sources by *Piromonas communis P* (Wood & Wilson, 1995)

Substrate	*Reducing sugar (µg)*	*Relative to cotton fibre*
Cotton fibre	1705	100
Whatman cellulose powder	679	40
Barley straw alpha-cellulose	549	32
Oat straw alpha cellulose	1394	82
Ryegrass alpha cellulose	1136	67
Birchwood xylan	2275	133
Oat spelt xylan	2773	163
Ryegrass cell walls	1136	67

The high activity of cellulase of *P. communis* is presumably due to very specialised environment in which these fungi live. Those fungi exist in an environment containing very high concentrations of predatory microorganisms. Therefore the enzymes of anaerobic fungi must act quickly before the substrate is conolised by other cellulolytic microorganisms. However the activity of the cell-free enzyme is lost in a relatively short time. *P. communis* cellulase is relatively unstable when kept at 4 °C and at -18°C. In the presence of cellulose the cellulase is still very active over a period of several days at 40 °C, over a pH range 6.0-6.8.

2.5 Hemicellolose biodegradation

Hemicellulose is the second most abundant polysaccharide in nature and consists largely of xylan, a β-1,4-linked D-xylose backbone with arabinofuranose, glucuronic acid and methylglucuronic acid. Enzymes degrading xylan are produced by various microorganisms including terrestrial bacteria, algae, rumen bacteria and a variety of invertebrate animals (Dekker & Richards 1976).

Two xylanases obtained from anaerobic fungi are described. A β-xylosidase from *Neocallimastix frontalis* and an endoxylanase from *Piromyces sp.*strain E2. The β-xylosidase has a small activity towards xylan (the substrate for endoxylanase). The endoxylanase of *Piromyces* sp. strain E2 has a high activity on oat spelt xylan. The products of the enzyme were xylo-oligosaccharides and no xylose was found.

2.6 Pectin biodegradation

Pectin can be degraded by some rumen fungi. These microbes produce mainly exo-polygalacturonase, endo- and exo-pectase lyase and pectin esterase (Wojciechowicz and Ziolecki 1984; Paster and Canale-Parola 1985). *Orpinomyces joyonii* A4 was isolated from rumen fluid of a camel, *Neocallimastix sp.* JL3 from faeces of a red deer, *Neocallimastix sp.* OC and *Neocallimastix sp.* H15 from rumen fluid of a sheep. All pectinolytic isolates utilized glucose, cellobiose, cellulose, fructose xylose and lactose. No growth was observed in the presence of galactose, galacturonic acid and arabinose. Pectinase activities found in endocellular and exocellular fractions of *Neocallimastix* sp. H15, JL3 and OC and *O. joyonii* A4 are given in . The highest activity in all strains is found in the case of endocellular pectin lyase and polygalacturonase. The pH optimums of polygalacturonase were observed in endocellular and exocellular fractions (Table 2–4). All exocellular polygalacturonase had pH-optimum at pH 6.0, except the strain H15 which showed another optimum at pH 7.5. In the endocellular fraction the enzyme possesses other optima besides the main one at pH 6.0.

Table 2–3 Activity of enzymes involved pectin degradation (Kopecny and Hodrova, 1995)

	Fungal strain			
Enzyme activity	A4	OC	H15	JL3
Polygalacturonase*				
Exocellular	46±9	29±6	10±3	27±10
Endocellular	251±62	43±3	435±50	167±11
Pectate lyase*				
Exocellular	110±39	83±7	29±17	1±2
Endocellular	69±2	31±1	91±4	49±5
Pectin lyase*				
Exocellular	142±4	185±89	166±41	93±24
Endocellular	258±17	274±5	294±17	908±22

* Enzyme activity is expressed in μg galacturonic acid h^{-1}mg $protein^{-1}$

Table 2–4 Optimal pH of rumen fungal polygalacturonases (Kopecny and Hodrova, 1995)

	Fungal strains			
Fractions	A4	OC	H15	JL3
Exocellular	6.0	6.0	6.0, 7.5	6.0
Endocellular	6.0, 8.1	5.05, 6.0	6.0, 8.1	6.5, 8.55

2.7 Antagonism between chitinolytic organisms and anaerobic fungi

The anaerobic fungi *Orpinomyces joyonii* A4 was cultivated on crystalline cellulose alone and in association with the rumen chitinolytic bacterium *Clostridium* sp. strain ChK5 (Kopecný et al., 1996). Pure culture *Orpinomyces joyonii* A4 showed biphasic cellulolysis which resulted in the release of glucose (Figure 2-2). Co-culture of the fungus with *Clostridium sp.* ChK5 inhibited cellulose degradation and no free glucose was detected.

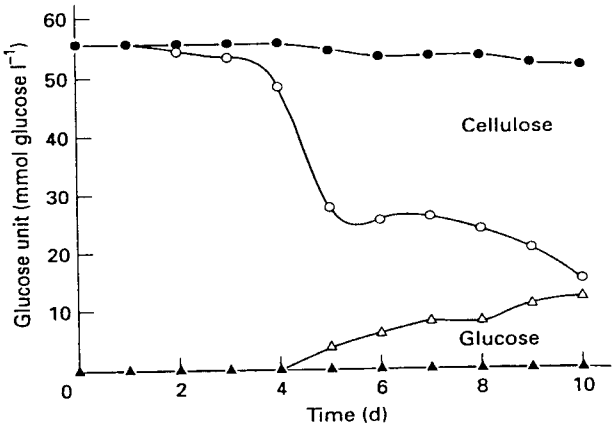

Figure 2-2. Cellulose degradation (O,●) and glucose release (Δ,▲)by monocultures of *Orpinomyces joyonii* A4 (open symbols) and by co-cultures of *O. joyonii* A4 with *Clostridium tertium* strain ChK5 (solid symbols) grown on cellulose (Kopecný et al., 1996)

Pure culture of *O. joyonii* A4 metabolise cellulose to formate, acetate, ethanol and lactate. The presence of stain CHK5 depressed the formation of short-chain fatty acids as shown in figure 2-3.

These results suggest that in addition to protozoal chitinases (Morgavi et al. 1994) chitinases from bacteria may play a role in the regulation of cellulolysis by anaerobic fungi when chitinolytic bacteria are present in significant numbers.

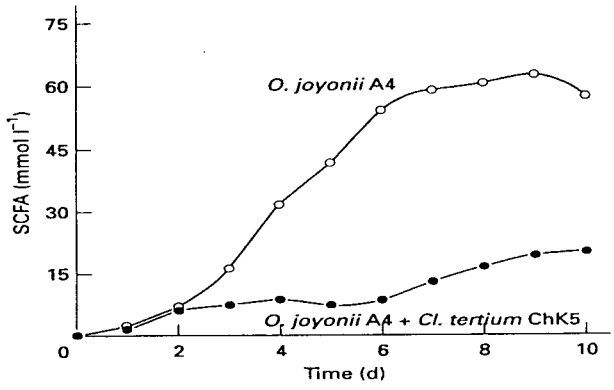

Figure 2-3 Production of short-chain fatty acids (SCFA) by *O. joyonii* A4 (O) and by co-cultures of *O. joyonii* A4 with *Clostridium tertium* strain ChK5 (●) (Kopecný et al., 1996)

2.8 Effect of *Aspergillus oryzae* fermentation extract on anaerobic fungi

Three fungi *Neocallimastic frontalis* EB 188, *Piromyces communis* DC 193 and *Orpinomyces* ssp. RW 206, isolated from cattle, were used to investigate the response of these fungi to the addition of *Aspergillus oryzae* fermentation extract (i.e. Amaferm) (Harper et al., 1996). This fermentation extract is often included in the diet used for cattle to stimulate growth and activity of rumen microflora, to increase fiber breakdown and digestive efficienty and to stabilize rumen pH (www.vitaferm.com). Laboratory studies have suggested that fungi posses accelerated cellulose-degrading activities and more rapid lactate-uptake systems in the presence of soluble extracts of Amaferm. After 48 h of growth, *N. frontalis* EB 188, *P. communis* DC 193 and *Orpinomyces* ssp. RW 206 showed an increase in the secretion of cellulase and in protein and culture mass production in the presence of extract (Table 2–5). The effect of the extract on the volatile fatty acids produced by each of the three fungal species is shown in Table 2–6, where an increase in the production of acetate and total volatile fatty acids is noticed.

Table 2–5 Effects of Amaferm on fungi (Harper et al., 1996)

Fungus	*Increase (%)*			
	Endoglucanase	*Supernatant protein*	*Culture mass*	*β-Glucosidase*
EB 188	135.5	114.3	115.7	113.9
RW 206	127.1	121.7	131.0	99.5
DC 193	120.2	125.8	118.7	115.2

Table 2–6 Analysis of volatile fatty acids (VFA)of cultures (Harper et al.,1996)

Fungal strain	*Treatment*	*Acetate (mmol/ml)*	*Acetate increase(%)*	*Total VFA (mmol/ml)*	*VFA increase (%)*
EB 188	Control	6.94	-	7.01	-
	Extract	7.77	11.96	8.62	22.97
RW 206	Control	7.25	-	7.30	-
	Extract	8.22	13.38	8.70	19.18
DC 193	Control	6.95	-	7.38	-
	Extract	7.16	3.02	8.82	19.51

The growth rate and the secretion of proteins and cellulases were accelerated in the presence of extract. Extract addittion also caused an increase in the production of volatile fatty acids. The components in the extract responsible for the stimulation are soluble.

3. Conclusions

Anaerobic fungi may degrade cellulose, hemicellulose and pectin with a higher efficiency compared to other types of micro-organisms. The cellulose biodegradation rate of the extracellular cellulase of *Piromonas* species is higher than when cellulase of aerobic bacteria or of the *N. frontalis M. smithii* co-culture was used. The use of enzymes of anaerobic fungi may improve the biodegradability of human faecal material or the recycled recalcitrant fraction. Faecal matter of sheep contain anaerobic bacteria and can be used as an inoculum. Isolation and growth of anaerobic fungi can be performed according to the method described by Lowe et al. (Lowe et al., 1985). This method involves the use of plate cultures and the growth of anaerobic fungi on defined media. The feasibility of this method can be questioned because of the difficult procedure used for cultivation and growth. Therefore an additional aerobic reactor proposed in TN41.3, containing aerobic fungi is easier to carry out. Pretreatment of faecal material in an additional reactor containing anaerobic fungi may improve the biodegradability by the anaerobic association of the first compartment. Yet, it was reported by Morgavi et al (1994) that also antagonistic effects between anaerobic bacteria and anaerobic fungi can occur.

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