Journal of Animal and Feed Sciences, 9, 2000, 727 - 735

Fermentation of glucose, xylose, cellulose and waste paper by the rumen anaerobic fungus *Orpinomyces joyonii* A_4*

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(Received 4 April 2000; accepted 4 October 2000)

ABSTRACT

The rumen anaerobic fungus *Orpinomyces joyonii* A_4 was grown in a complex medium with glucose, xylose, microcrystalline cellulose or waste paper. The fungus produced significantly ($P<0.05$) more formate, acetate and ethanol, but less lactate from xylose than from glucose. Protein content and yields of dry matter and protein in cells grown on glucose and xylose were not significantly different. Fungal cells contained 64.8% of crude protein, but only 34.9-35.2% of true protein determined according to Lowry. In dual-substrate fermentation both sugars were utilized simultaneously. *O. joyonii* A_4 efficiently metabolized microcrystalline cellulose and milled office paper (up to 41.1 and 38.0 mmol of fermentation end-products per l, respectively). Its growth on newspaper was poor (13.4 mmol of fermentation end-products per l). Glucose accumulated in fungal cultures with cellulose (2.55 g/l) and office paper (0.60 g/l) at the end of the incubation.

KEY WORDS: rumen, anaerobic fungi, carbohydrates, waste paper

INTRODUCTION

Anaerobic fungi are inhabitants of the digestive tract of herbivorous mammals, both ruminants and non-ruminants. In the rumen and hindgut, anaerobic fungi se-

* Supported by grant no. 524/97/1221 of the Grant Agency of the Czech Republic

crete a broad range of polysaccharide-degrading enzymes and contribute significantly to the digestion of fibre. Fibrolytic enzymes of anaerobic fungi have high specific activities compared with those of aerobic fungal strains, thus anaerobic fungi could be interesting for several biotechnological applications (reviewed by Teunissen and Op de Camp, 1993). The aim of our study was (*i*) to investigate fermentation characteristics of fibre monomeric components (glucose and xylose) in cultures of a polycentric rumen fungus *Orpinomyces joyonii* A_4, and (*ii*) to compare its growth on microcrystalline cellulose, office paper and newspaper. In addition, (*iii*) we attempted to establish a co-culture of the fungus and a yeast. Presumably, the yeast should metabolize soluble sugars (by-products of cellulose digestion) to ethanol.

Several authors investigated growth of rumen monocentric fungi on glucose and xylose. Mountfort and Asher (1983) found that glucose was the preferred substrate compared with xylose in *Neocallimastix frontalis* PN-1. Xylose utilisation was inhibited in the presence of glucose. In contrast, in experiments of Lowe et al. (1987), glucose and xylose were utilized simultaneously in a culture of *Neocallimastix* spp. R1, although metabolite profiles differed. Williams et al. (1994) reported higher biomass yield of foregut and hindgut isolates *Neocallimastix* spp. and *Piromyces* spp. on glucose than on xylose. There are numerous reports on degradation of plant cellulosic materials, but no reports on digestion of waste paper by anaerobic fungi. A report on digestion of office paper, newspaper and magazine paper by the cellulolytic rumen bacterium *Fibrobacter succinogenes* was published recently (Martin and Martin, 1998).

MATERIAL AND METHODS

Organisms and growth conditions

O. joyonii A_4 was isolated in this laboratory from the rumen fluid of a camel. The strain was subcultured every 3 days. The complex medium for maintenance and growth experiments was medium 10 of Caldwell and Bryant (1966) with 15% rumen fluid, modified as described by Kopečný and Hodrová (1995). The medium was prepared anaerobically, using cysteine hydrochloride (0.5 g/l) as a reducing agent. Fungi were grown in 100 ml serum bottles closed by rubber stoppers, under a CO_2 atmosphere. Ninety ml of the medium containing glucose or xylose at 4 g/l were inoculated with 1 ml of a 3-day-old fungus culture. One bottle of inoculated medium was immediately frozen. Other bottles were incubated at 39°C for 4 days. Cultures were grown in four replicates. The dual-substrate utilisation pattern was investigated using the same growth conditions. Glu-

cose and xylose were supplied at 2 g/l. Again, one inoculated bottle was immediately frozen. Other inoculated media were incubated for 1, 2, 3, 4 and 5 days in four replicates.

Cellulosic substrates included microcrystalline cellulose, analytical grade from Lachema (Brno, Czech Republic), used office paper and newspaper (the Metro daily). Office paper and newspaper were milled using a mill for grinding dry plant samples, type QB 136 (Labor MIM, Esztergom, Hungary). Cellulosic substrates were added at 5 g/l to the same medium. Inoculated cultures (40 ml) were incubated for 1-6 days in two replicates. Metabolite production instead of substrate utilisation was measured in these cultures as the fungal mycelium was inseparable from undigested solid substrate.

Thermotolerant yeast *Kluyveromyces marxianus* var *marxianus* strain CYY 51-1-1 was purchased from the culture collection of the Institute of Chemistry (Bratislava, Slovakia). The yeast was maintained in a medium containing (g/l): $(NH_4)_2SO_4$, 1.5; yeast extract, 5; peptone, 3; enzymatic hydrolysate of casein, 5; glucose, 10. Fungal cultures were inoculated with the yeast 2 days after the start of the incubation. In a separate experiment, the growth of this yeast under aerobic and anaerobic conditions was compared, using medium 10 with 10 g/l glucose and the above-described medium for yeast.

Analyses and calculations

The cultures were centrifuged at 6000 g for 15 min. The cell dry mass was determined after washing with rinsing solutions (Jenkinson et al., 1975), and drying at 105°C overnight. Cell protein was extracted with 1 M NaOH (100°C; 1 h), and determined according to Lowry (Herbert et al., 1971). The remaining analyses were done on frozen samples after thawing. Formate was determined colorimetrically (Sleat and Mah, 1984), ethanol and acetate by gas chromatography on a column of Chromosorb WAW with 15% SP 1220 and 1% H_3PO_4 (Supelco, USA), and lactate in microdiffusion units (Conway, 1957). Residual xylose was estimated by the orcinol reagent (Herbert et al., 1971), glucose, enzymatically using a commercial kit (Lachema, Czech Republic). Carbon and nitrogen contents were determined using a Perkin Elmer 2400 elemental analyzer in freeze-dried cells grown on glucose + xylose. Waste paper was analyzed by standard AOAC techniques (AOAC, 1984).

Growth yields and production of metabolites are expressed per gram of substrate utilized. Carbon recovery was calculated from the metabolic products and carbon content of cells. The calculation was based on the presumption that acetate, lactate and ethanol originate from a common 3-carbon compound (pyruvate). The significance of differences between glucose- and xylose-grown cultures was evaluated by the *t*-test (SAS, 1989).

RESULTS

Whereas glucose was utilized completely (99.8%), significant amounts of xylose (42.3%) were left in four-day cultures of *O. joyonii* A_4 (Table 1). The fungus produced more formate, acetate and ethanol, but less lactate from xylose than from glucose ($P<0.05$). Cell protein content and growth yields of dry matter and protein in cells grown on glucose and xylose were not significantly different. Carbon recovery in *O. joyonii* A_4 cultures was incomplete, i.e. lower than 100%. In dual-substrate cultures both sugars were utilized simultaneously (Figure 1). In these cultures xylose was utilized better, as only 12.6% of the initial amount of xylose was left after 4 days of incubation. The composition of waste paper used as a substrate of fungal cultures is shown in Table 2. It follows from the data in Table 3 that *O. joyonii* A_4 metabolized microcrystalline cellulose and office paper well, but its growth on newspaper was poor. There was no difference in growth on white,

TABLE 1

Parameters of growth and metabolism of rumen anaerobic fungus *O. joyonii* A_4 on glucose and xylose[1]

Parameter	Substrate		
	Glucose		Xylose
Substrate used, mg/l	3950 ± 6		2282 ± 370*
Metabolites, mmol per g substrate used			
formate	4.8 ± 0.1		7.1 ± 1.5*
acetate	2.9 ± 0.1		3.8 ± 0.7*
lactate	3.1 ± 0.2		1.7 ± 0.1*
ethanol	1.9 ± 0.1		3.0 ± 0.6*
Cell dry weight, mg/l	436 ± 8		233 ± 25*
Cell composition, % DM[2]			
protein (Lowry)	35.2 ± 0.2		34.9 ± 0.3
carbon		40.24	
nitrogen		10.37	
Yields, mg per g substrate used			
DM	110 ± 2		102 ± 27
protein	39 ± 1		36 ± 10
Carbon recovery, %	82.5 ± 3.1		86.5 ± 11.9

[1] means of four cultures ± SD

[2] DM, dry matter

* significantly different from the corresponding glucose value at $P<0.05$

black-and-white and coloured parts of the newspaper. At the end of the incubation a high concentration of glucose (2.55 g/l) was found in fungal cultures supplied with cellulose. Glucose was also detected in fungal cultures grown 6 days on office paper, but not in those grown on newspaper. The yeast, *Klyuveromyces marxianus* var *marxianus,* did not grow under strictly anaerobic conditions. Anaerobiosis prevented growth of the yeast both in the medium for yeast and in medium 10.

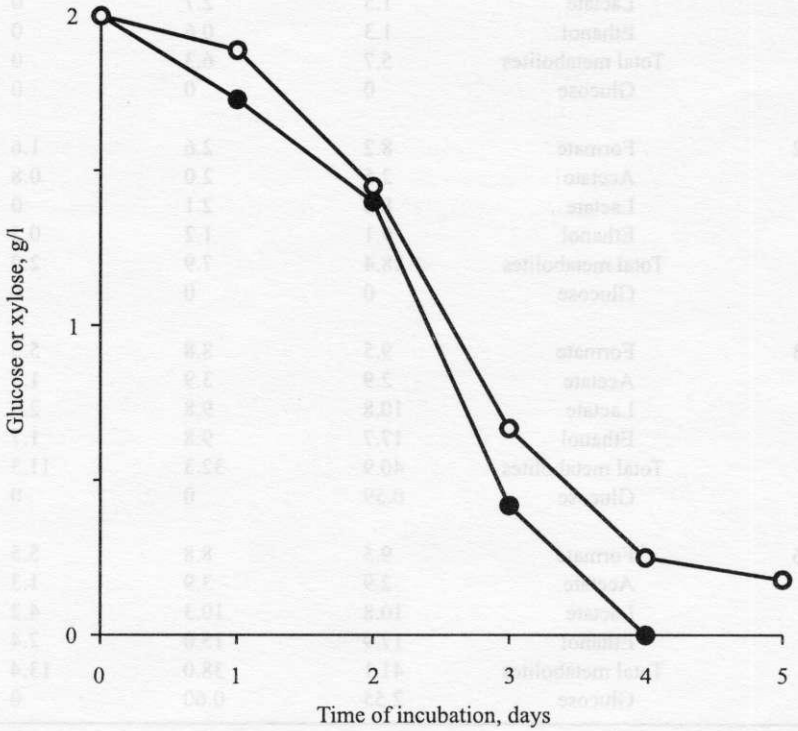

Figure 1. Time course of glucose (●) and xylose (○) utilisation in culture of *O. joyonii* A_4

TABLE 2

Composition of waste paper, g/kg

Fraction	Office paper	Newspaper
Dry matter	970	957
NDF	765	855
ADF	726	753
Hemicellulose (NDF-ADF)	39	102
Lignin	7	170
Ash	147	56

TABLE 3

Production of metabolites (mmol/l) and glucose (g/l) in cultures[1] of *O. joyonii* A_4 grown on microcrystalline cellulose, milled office paper and newspaper[2]

Time of incubation days		Cellulose	Office paper	Newspaper
1	Formate	2.0	2.1	0
	Acetate	0.9	0.9	0
	Lactate	1.5	2.7	0
	Ethanol	1.3	0.6	0
	Total metabolites	5.7	6.3	0
	Glucose	0	0	0
2	Formate	8.2	2.6	1.6
	Acetate	2.5	2.0	0.8
	Lactate	8.6	2.1	0
	Ethanol	9.1	1.2	0.5
	Total metabolites	28.4	7.9	2.9
	Glucose	0	0	0
3	Formate	9.5	8.8	5.5
	Acetate	2.9	3.9	1.3
	Lactate	10.8	9.8	2.8
	Ethanol	17.7	9.8	1.7
	Total metabolites	40.9	32.3	11.3
	Glucose	0.59	0	0
6	Formate	9.5	8.8	5.5
	Acetate	2.9	3.9	1.3
	Lactate	10.8	10.3	4.2
	Ethanol	17.9	15.0	2.4
	Total metabolites	41.1	38.0	13.4
	Glucose	2.55	0.60	0

[1] means of two cultures

[2] substrates were added at 5 g/l

DISCUSSION

Digestion of fibre is the pivotal part of the metabolism of rumen fungi. More attention has been given so far to the fungal decomposition of cell wall polysaccharides than to the metabolism of fibre monomeric constituents. The Embden-Meyerhof glycolytic sequence is the primary pathway of hexose catabolism in *Neocallimastix patriciarum* (Yarlett et al., 1986), *N. frontalis* (O'Fallon et al., 1991),

and undoubtedly also in other species of anaerobic fungi. The involvement of non-glycolytic pathways in carbohydrate metabolism of anaerobic fungi has not been described, but can not be excluded. The metabolism of pentoses is less understood than the metabolism of hexoses in ruminal organisms. In accordance with results of Lowe et al. (1987), growth of *O. joyonii* A_4 on glucose produced more lactate and less acetate than growth on xylose. Whether a 2-3 cleavage of xylose occurred, however, remains to be determined in a future study. Fungal cells contained 64.8% of crude protein (N x 6.25), but only 34.9-35.2% of true protein determined according to Lowry. It follows from these data that cells contained about 46% N as non-protein N, presumably chitin and nucleic acid N. The carbon recovery was incomplete in all cultures, probably because of secretion of soluble products of fungal metabolism, other than formate, acetate, lactate and ethanol, into the medium.

O. joyonii A_4 grew well on office paper, but not on newspaper. Obviously, the printing ink did not inhibit the growth of strain A_4 as the fungus grew poorly both on white and coloured parts of newspaper. Office paper contained more ash (filler) and less hemicellulose than newspaper (Table 2). The hemicellulose content in newspaper does not differ much from that in filter paper (Collings and Yokoyama, 1979). The principal reason, however, why newspaper and office paper were digested so differently, seems to be the much higher content of lignin in the former material (170 vs 7 g/kg). Large amounts of glucose were found in fungal cultures grown on cellulose (Table 3). Apparently the rate of cellulose hydrolysis in these cultures was higher than the rate of utilisation of cellulose degradation products. Our observation is not unique. In previous experiments, the polycentric fungus *O. joyonii* A_4 extensively degraded cellulose and large amounts of glucose and cellodextrins accumulated in the medium (Hodrová et al., 1995). In co-cultures of this fungus with rumen bacteria *Megasphaera elsdenii* and *Eubacterium limosum*, glucose and cellodextrin concentrations decreased by 68-86%, and consequently the extent of cellulose digestion increased. Similarly, Tanaka et al. (1986) found a high concentration of glucose (6.9 g/l) in starch-grown cultures of a mould, *Aspergillus awamori* IFO 4033.

The aim *(iii)* of this study was to establish a co-culture of the fungus and an ethanol-producing thermotolerant yeast. Our attempt was unsuccessful because of the inability of the yeast to grow under anaerobic conditions, which were necessary for growth of the fungus. Mixed cultures that obtained relatively good results were those using starch as a substrate for solvent production. Tanaka et al. (1986) fermented potato starch to ethanol by a co-immobilized mixed culture of an aerobic mould *Aspergillus awamori* and an anaerobic bacterium *Zymomonas mobilis*. Similarly, Abouzied and Reddy (1987) produced ethanol from unhydrolyzed starch using a co-culture of an anaerobic yeast *Saccharomycopsis fibuligera* and a yeast *Saccharomyces cerevisiae*.

In experiments of Martin and Martin (1998), *Fibrobacter succinogenes* S85 efficiently digested filter paper, but was unable to degrade office paper, newspaper or magazine paper. Our results thus indicate that the ability of *O. joyonii* A_4 to digest waste paper was better than that of *F. succinogenes* S85. We can conclude that rumen anaerobic fungi have the potential to convert some waste cellulosic substrates into certain value-added products. This was suggested earlier by other researchers, too: Selinger et al. (1996) proposed improving the *Trichoderma* cellulase system with ruminal fungal cellulases by using recombinant DNA technology, and Nakashimada et al. (2000) converted cellulose to methane by means of co-cultures of a rumen anaerobic fungus *N. frontalis* and methanogens. Further experiments should examine a system for conversion of cellulosic materials to ethanol, based on a co-culture of an anaerobic cellulolytic fungus and an anaerobic ethanol-producing bacterium, e.g. *Zymomonas mobilis*.

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STRESZCZENIE

Rozkład glukozy, ksylozy, celulozy i odpadów papierowych w żwaczu przez beztlenowe grzyby *Orpinomyces joyonii* A_4

Żwaczowe beztlenowe grzyby *Orpinomyces joyonii* hodowano na złożonym podłożu, zawierającym glukozę, ksylozę, mikrokrystaliczną celulozę lub odpady papierowe. Grzyby produkowały istotnie więcej ($P<0.05$) mrówczanu, octanu i etanolu, natomiast mniej mleczanu z ksylozy niż z glukozy. Zawartość białka oraz produkcja suchej masy i białka grzybów rosnących na pożywkach z tymi cukrami nie różniła się. Komórki grzybów zawierały 64,8% białka ogólnego, lecz tylko 34,9-35,2% białka właściwego oznaczonego wg Lowry. Obydwa cukry były rozkładane równocześnie na podłożu zawierającym te dwa substraty. *O. joyomii* A_4 efektywnie rozkładał mikrokrystaliczną celulozę i zmielone odpady papierowe (do 41,1 i 38,0 mmola końcowych produktów fermentacji na litr, odpowiednio). Wzrost grzybów na podłożu z odpadami papierowymi był słaby (13,4 mmol końcowych produktów fermentacji na litr). Końcowe stężenie glukozy w kulturach grzybów rosnących na pożywkach z celulozą wynosiło 2,55 g/l, a z papierem 0,60 g/l.