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ORIGINAL PAPER

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Characterization of *Aspergillus oryzae* fermentation extract effects on the rumen fungus *Neocallimastix frontalis*, EB 188. Part 1. Zoospore development and physiology

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Abstract Experiments were performed to determine the effect of *Aspergillus oryzae* (AO) fermentation extract on zoospore development in the rumen fungus *Neocallimastix frontalis* EB 188. Powdered product, or liquid extract prepared from such powder, was added at the recommended value for supplementation in dairy cattle. Stationary and stirred cultures were periodically sampled and assayed for extracellular and intracellular protein and enzymes, gas production, zoospore production and maturation, and carbon source utilization. Soluble extract increased fungal physiology when grown in stirred vessels or stationary cultures. Treated cultures produced higher levels of enzymes (nearly double). Mobile zoospores matured into germination entities more rapidly in treated cultures, and when powdered product was used, nearly 3 times more motile zoospores were produced at 56 h of fungal growth. Levels of the intracellular enzyme malate dehydrogenase increased by 6-fold in the presence of powdered product. Product wheat bran carrier used as soluble extract or powder had very little effect on fungal cultures. Medium cellulose was completely hydrolyzed in all cultures but this occurred earlier in those containing AO treatment.

Introduction

Several factors control ruminant animal feed intake including degradation of plant materials, rumen retention time, and rumen pool size. Changes in the chemical/physical nature of the roughage, factors related to rumen microbiology, and host animal effects such as rumen contractility can severely alter the intake of the animal (Hungate 1966; Van Soest 1994). Microbial-based supplements may be useful as production management tools. In the *Direct-fed microbial, enzyme & forage additive compendium*, several products containing *Aspergillus oryzae* (AO) or extracts thereof are listed for animal supplementation (Muirhead 1998). Nearly 100 research papers have been published describing the in vivo or in vitro effects on ruminants or their microflora of products based on *Saccharomyces cerevisiae* or AO. Beneficial—but highly variable—alterations in cattle milk production, heat tolerance, daily weight gain, animal wellness, and disease resistance have been reported (Chiquette 1995; Denigan et al. 1992; Gomez-Alarcon et al. 1991; Higginbotham et al. 1994; Kellems et al. 1990; Sievert and Shaver 1993; Yu et al. 1997). Furthermore, in vitro studies have shown that certain products can increase parameters thought to be related to high animal performance such as increased volatile fatty acid (VFA) production, increased populations of rumen bacteria, protein sparing in the rumen, and increased rumen fiber fermentation (Beharka and Nagaraja 1993; Caton et al. 1993; Chiquette 1995; Martin and Nisbet 1990; Newbold et al. 1992; Nisbet and Martin 1990; Oellermann et al. 1990; Varel and Kreikemeier 1994; Varel et al. 1993; Wiedmeier et al. 1987; Yoon and Stern 1996). Certain products stimulate the growth/physiology of fiber-degrading bacteria or fungi in the rumen (Beharka and Nagaraja 1993; Chang et al. 1999; Harper et al. 1996; Newbold et al. 1992; Nisbet and Martin 1990; Welch et al. 1996). Stimulation of rumen fungi in vitro has been shown in both the presence and absence of rumen fluid (Harper et al, 1996; Chang et al. 1999). The small supplementation amount of direct-fed microbial (DFM) products fed to production animals suggests few feasible modes of action can exist. The products do not likely act as a significant source of fiber, fiber-degrading enzymes, flavor enhancers, or oxygen scavengers.

It is important to conduct a study of AO-based DFMs on a rumen fungi model system. Rumen fungi are found in all domestic ruminants and are the most cellulolytic

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microorganisms known (Mountfort et al. 1982; Weimer 1992; Wood et al. 1986). Fungal mass is strategically located deep within the plant fiber and such invasion weakens (solubilizes) the fiber (Akin et al. 1983; Bauchop 1975; Orpin 1976, 1979). Without fungi, rumen function stops, whereas reintroduction of fungi restores rumen function and increases feed breakdown (Gordon and Phillips 1993). Finally, all the major species of rumen fungi are physiologically accelerated in a dose-dependent manner in the presence of certain DFMs (Harper et al. 1996; Welch et al. 1996).

In this paper we provide information that significantly expands our understanding of the mode of action of Amaferm (an AO-based product) as it relates to in vitro growth of rumen fungi. We provide the first information describing the direct effects of this product on fungal zoospore production. Such evidence is consistent and supportive of earlier findings of increased production of fungal rhizoidal structures in vitro (Chang et al. 1999) and may relate to in vivo rumen interactions.

Materials and methods

Chemicals

All chemicals, unless otherwise stated, were purchased from Sigma (St. Louis, Mo.) or US Biochemicals (Cleveland, Ohio), and were of the best grade available. AO fermentation extract was obtained from BioZyme Incorporated (St. Joseph, Mo.) and supplied as a dry wheat-bran-based powder called Amaferm.

Culture and growth conditions

Neocallimastix frontalis EB 188 was used throughout the work and was obtained from the American Type Culture Collection (Rockville, Md.) as acquisition number 76100. This rumen fungus was first isolated from a cow at the Ensminger Beef Center at Washington State University (Barichievich and Calza 1990). The composition of the semi-defined medium has been described previously (Lowe et al. 1985) but rumen fluid was added to a final volume of 5.0% (v/v). Cultures were grown at 39°C in either 28 ml glass tubes (containing 10 ml medium) or 300 ml glass round-bottom flasks (containing 225 ml medium) using the anaerobic transfer methods of Hungate (1966). Zoospores from 3 to 5-day-old cultures were added at 700–900/ml as needed to start growth. The carbon source was either cellulose (Sigmacell 100) or as indicated. Prevention of bacterial contamination was accomplished via addition of antibiotics as in the method of Joblin (1981) with the addition of chloramphenicol.

Preparation of fermentation extracts

The methods for preparation of soluble extracts from Amaferm powder have been described previously (Welch et al. 1996). Extract was filtered through a 0.22-micron membrane before use unless otherwise stated. The powdered product was used as supplied by the manufacturer. The maximal amount of powder or *Aspergillus* ferment added to culture represents less than 1 part per 20,000 or 555,000 by weight, respectively. We have found that *Aspergillus* spores (approximately 3,300/g) contained within the product are not viable (do not germinate) under anaerobic growth conditions (unpublished results).

Culture preparation

A single batch of zoospores was used to inoculate control and Amaferm-treated cultures. This was necessary because zoospore robustness has an impact on the physiological response of the fungi (Welch et al. 1996). All cultures were observed microscopically each day for mycelium growth, zoospore numbers, and cellulose disappearance. Sample preparation for visible light and scanning electron microscopy have been previously described (Chang et al. 1999).

Assay methods

Methods for the measurement of protein, β-glucosidase, and reducing sugar have been provided previously (Calza 1991). Carboxymethyl cellulase (CMCase) was determined by production of reducing sugar (Barichievich and Calza 1990). Alpha amylase was determined as presented in the *Worthington enzyme manual* (Decker 1977). The method for determination of Avicelase is the same as that for CMCase except that Avicel (type PH-102; FMC, Philadelphia, Pa.) is used as the substrate rather than carboxymethylcellulose. For CMCase, Avicelase, and alpha-amylase, 1 U activity is equal to 1 μmol reducing sugar liberated per minute. The liberation of 1 μmol *p*-nitrophenol/min defines 1 U β-glucosidase activity. Glucose served as a standard for determination of reducing sugars, and bovine serum albumin was used as a standard for protein determination. Malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) were determined as presented in the *Worthington enzyme manual* (Decker 1977). Both assays are based on the change in absorbance at 340 nm resulting from the oxidation of NADH. Methods for extraction and preparation for thin layer chromatography have been described previously (Chang et al. 1999). Proteins were analyzed by the method of Lowry et al. (1951). Cellulose concentration was determined by the method of Hodge and Hofreiter (1962). Zoospores were enumerated using an Olympus light microscope and a hemacytometer. Gas production was determined by the displacement of the plunger of a 50 ml syringe. VFAs were determined using gas chromatography (Erwin et al. 1961). All data as reported are corrected for background and experimental cultures, and were run in at least triplicates of five cultures for each treatment. The standard errors of measurement were less than 5%. Where appropriate, statistical methods were used to determine observational significance (SAS 2001) and held to 95% confidence level (error bars in line graphs) unless otherwise stated. In graphing, points were fitted to a line using the least-square method or the best line program in Microsoft Excel.

Preparation of cellular lysates

Fungal zoospores were chilled at 0–4°C throughout spore collection. The cultures were filtered through two layers of cheesecloth to remove the larger pieces of mycelial mat. The spores were then collected by centrifugation, rinsed at least three times in PIPES (pH 6.8, 1× buffer), and finally resuspended in a small volume of the buffer. The spores were then lysed on ice using sonication (3 min, 50% duty cycle, micro-horn, Sonics and Materials, Danbury, Conn.). Spores lysates were re-centrifuged to remove cellular membranes and supernatant was assayed as required to determine intracellular characteristics.

Results

Light microscopy and scanning electron microscopy were used to determine the extent of substrate attachment, fungal growth, and the disappearance of insoluble cellulose in the presence or absence of soluble product extract. Electron micrographs demonstrate that by 24 h in both treated (and untreated, control data not shown) cultures,

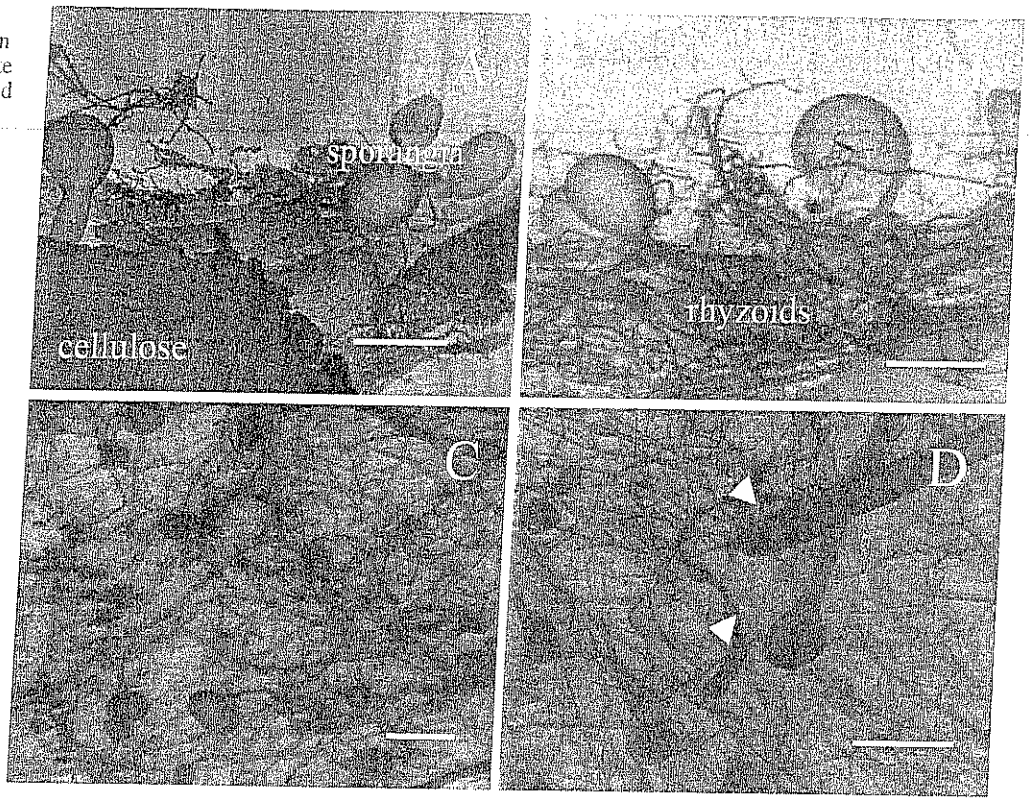

Fig. 1A–D Scanning electron microscopy of cellulose-grown cultures. **A** Fungi and substrate after 24 h growth. **B** Fungi and substrate after 48 h growth. **C** Fungal cultures after 72 h growth. **D** Fungal culture after 120 h growth. *Bars* 20 μm

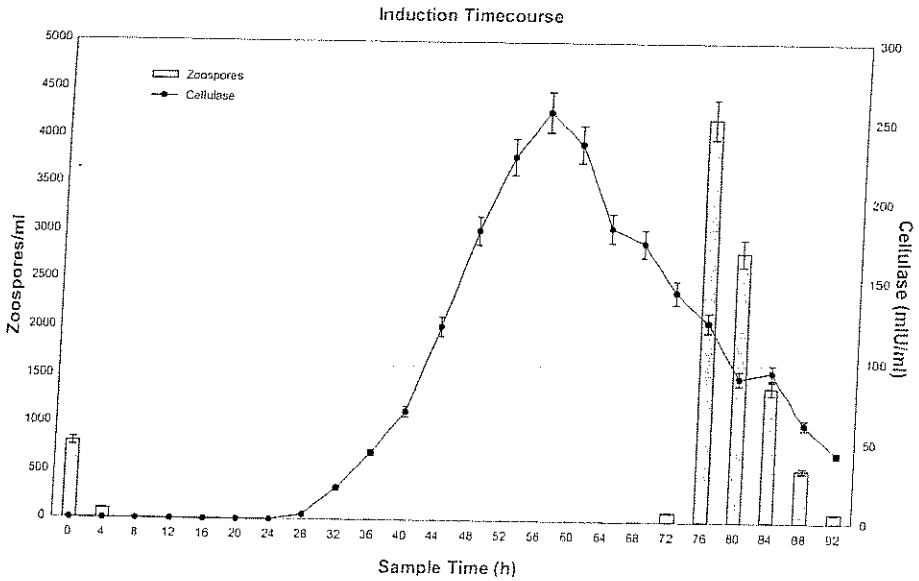

Fig. 2 Relationship of culture cellulase secretion and zoospore production. The numbers of zoospores (motile) and cellulase are plotted against sample time. Samples were taken every 4 h. *Error bars* 5% ± variance

fungal sporangia are well formed and rhizoidal penetration of culture cellulose is apparent (Fig. 1). Within 48 h the majority of cellulose within cultures had been solubilized, rhizoidal development is much more advanced, and sporangia are further enlarged. At 72 h incubation, all visible signs of cellulose are absent and rhizoidal thickening is maximal. Also, fewer sporangial bodies are evident; those that are present are large and many are ruptured (having released their developing zoospores). In the most mature cultures (120 h incubation), the presence of greatly thickened rhizoids and unidentified structures is evident.

The relationship of zoospore production and cellulase secretion in cultures supplied with cellulose as carbon source is shown in Fig. 2. Detection of motile zoospores at early timepoints reflects the initial inoculum. As the zoospores encyst and attach to the carbon source they are no longer detected in the medium liquids. By 32 h the culture possessed measurable cellulase in the medium liquids, achieving a maximum at about 52 h growth, with cellulase

levels decreasing thereafter. Zoospores were produced soon after the peak of cellulase and are at a maximum by 76 h growth. The secretion of cellulase preceded the appearance of the next generation of zoospores.

The effect of product carrier (wheat bran) and Amaferm solubles was determined in cultures of growing fungi. In Fig. 3 the impact of treatment on the secretion of cellulase is reported and compared to untreated cultures. As early as 40 h incubation, the AO-treated cultures possessed higher levels of cellulase than either wheat-bran-treated or control cultures. The peak of secretion occurred in Amaferm-treated cultures at about 70 h of growth and obtained levels up to 150% of controls. The wheat-bran-treated cultures were not different from controls except at 90 h growth when they achieved levels approximately 8% higher than controls.

The presence of fungal zoospores, secreted enzymes and protein, and culture reducing sugar (equivalents) were measured in closed, stirred fermentor vessels in the presence or absence of soluble product extract. These vessels provided a large culture volume that could be sampled numerous times without disturbing the culture solids. The panels in Fig. 4 demonstrate that the treatment

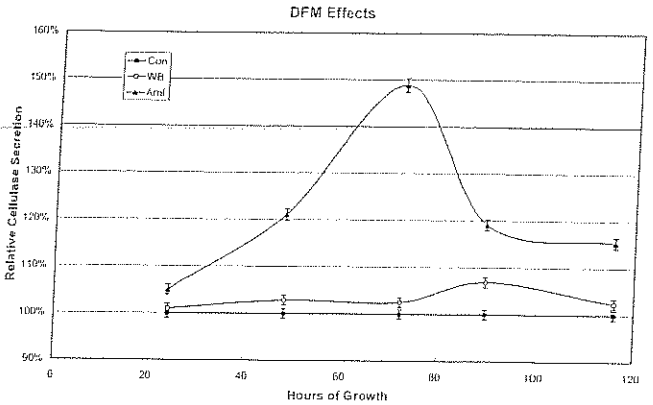

Fig. 3 Effect of soluble product carrier (wheat bran, *WB*) and Amaferm extract (*Amf*) on culture secretion of cellulase. Relative (to control) percent cellulase secretion is plotted against hours of culture growth. *Solid circles* Control, *open circles* wheat bran extract, *solid triangles* treatment extract

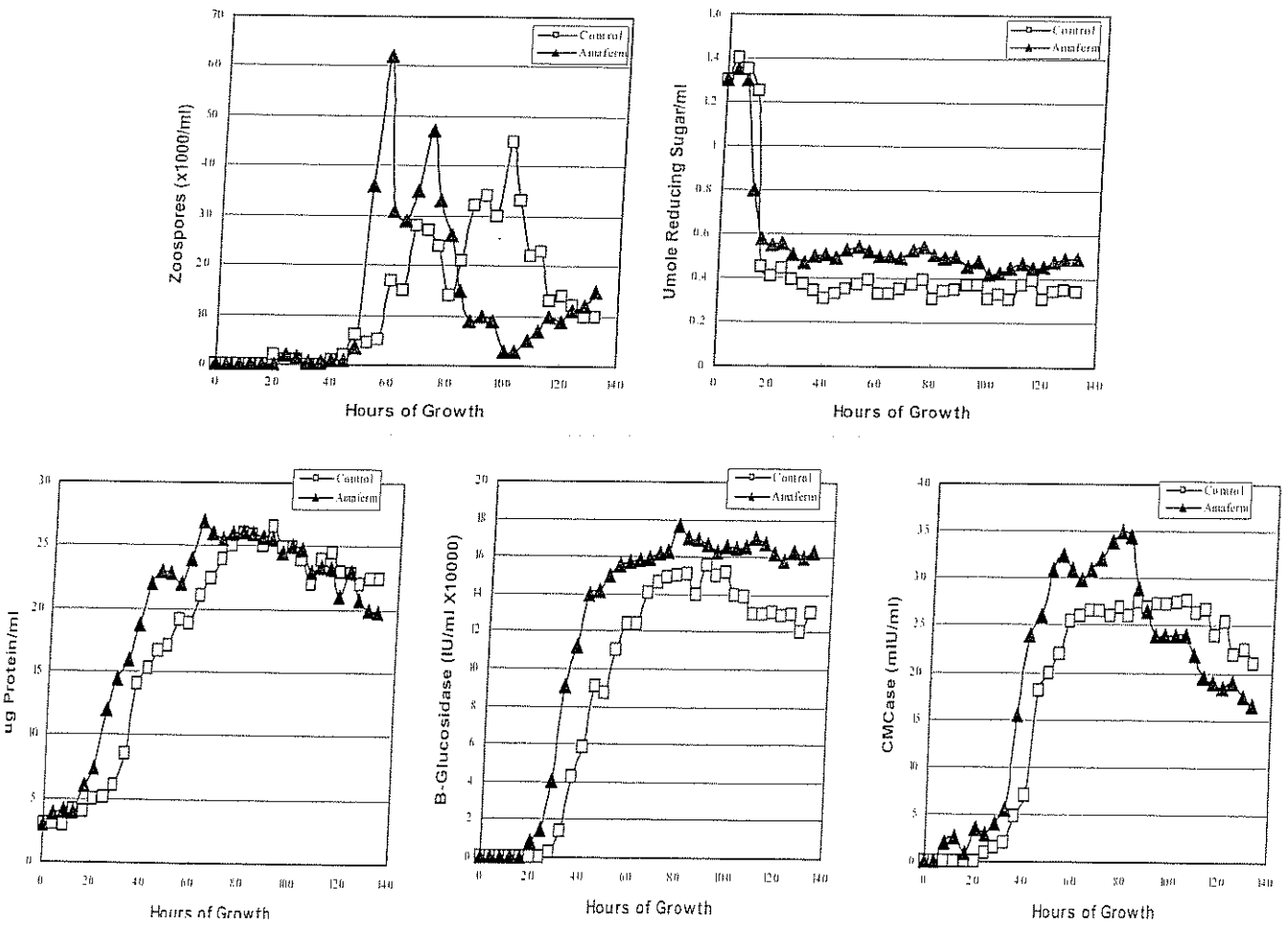

Fig. 4 Effect of product soluble extracts on fungal growth and physiology in sealed stirred reaction vessels. Absolute values of characteristics are plotted against sampling time (h)

greatly shifts the production of viable zoospores, secretion of enzymes and protein, and increases the reducing sugar levels found in the culture supernatants. With the exception of CMCase secretion, the overall curves of entities measured are similar. When comparing zoospores, there were more spores produced at earlier timepoints in AO-treated cultures ($P \leq 0.01$). The double cycle of zoospore production that we invariably record only in stirred cultures is fully complete in treated cultures before the untreated second burst (or cycle) is even at a maximum. Reducing sugars for the treated cultures tended to be 0.1–0.2 μmol/ml higher after 20 h than the control cultures. This indicates a greater fermentation and release of soluble sugars from the breakdown of cellulose. Protein secretion occurs at the same level between treatments; however, secretion from the treated culture occurs about 15 h earlier. Like protein secretion, β-glucosidase secretion occurs earlier; however, total production of the enzyme is also higher throughout the growth period. The curve for CMCase production is different from the others. The treated cultures produce enzymes at an earlier timepoint and achieve a higher production of enzymes (like β-glucosidase). However, the level of CMCase in treated cultures drops below that produced by the control at 80 h. This may represent a timepoint where cellulose is completely immobilized or utilized (Fig. 1) by the fungus.

Within stationary (unstirred) cultures it was possible to obtain multiple samples and use light microscopy to determine culture morphology as it related to zoospore maturation in the presence and absence of soluble product extracts. It is expected that motile zoospores mature first by losing their flagella and finally forming germination tubes (or rhizoids). This provides for three classifications of zoospores that can be utilized to determine culture maturation (motile, non-motile/non-germinated, and germinated). With cellulose as a sole carbon source, it was evident that product stimulated earlier zoospore production and maturation of such zoospores into germination sporangial foci. In Fig. 5 treated and untreated cultures are scored with respect to motile, non-motile/non-germinated, and germinated zoospore counts. The appearance of motile zoospores is several hours earlier in treated cultures and counts are also increased. The same is true for the appearance of germinated spores. The numbers of non-motile/non-germinated spores are constant across treatments except at the last time point where treated numbers are twice those of the control. This may indicate a time where further rhizoidal development is inhibited by lack of carbon source. Within these experiments, we compared the relative total numbers of scored entities in treated and control (untreated) cultures. In treated cultures, motile, non-motile, and germinated zoospores are 120.0%, 151.1%, and 197.0%, respectively, of the control cultures (at least $P \leq 0.01$).

Changes to experiments were designed to determine intracellular characteristics of zoospores in the presence or absence of product. Characteristics of zoospore lysates are listed in Table 1. Extract treatment increased the

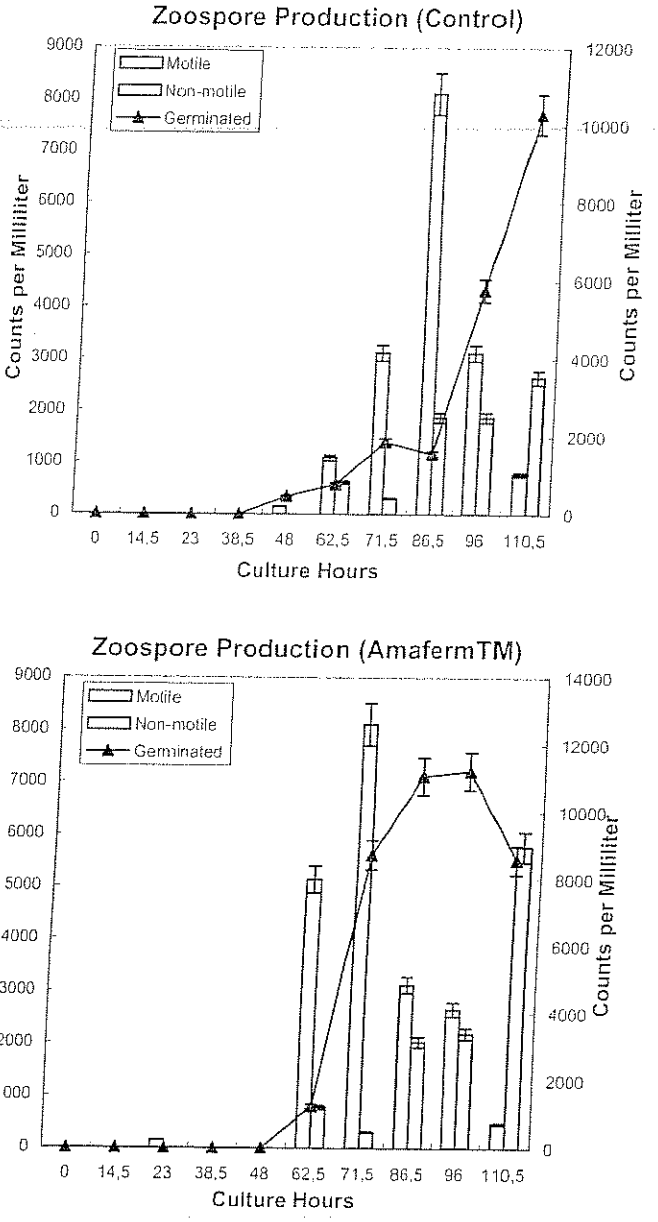

Fig. 5 Effects on fungal production of zoospores in the presence or absence of soluble product extracts. Counts are plotted against culture observation (sampling) time

amount of protein and the numbers of total zoospores by as much as 2-fold (protein). Production of total spores tended to be higher in treated cultures but values were not significantly different. Zoospores possessed measurable levels of some cellulose-degrading enzymes and amylase but not Avicelase (data not shown). Also, all enzymes measured were found in greater amounts in cultures containing extract. The two enzymes expected to be exclusively within the cell, MDH and LDH, increased by greater than 6- and 2-fold, respectively. Reducing sugars were also considerably higher in the treated spores but the values were not significantly different. Levels of protein and amylase were significantly higher in treated cultures (both $P \leq 0.1$). CMCase, MDH and LDH enzyme produc-

Table 1 Measurements of intracellular (via lysates) components from zoospores after 50 h culture. *CMCase* Carboxymethyl cellulase, *MDH* malate dehydrogenase, *LDH* lactate dehydrogenase, *BSA* bovine serum albumin

Protein [μg/ml (as BSA)]		Zoospores (10^6)		CMCase (mIU/ml)		β-Glucosidase (mIU/ml)		Amylase (mIU/ml)		MDH (mU/mg)		LDH (U/l)		Reducing sugar [μmol/ml (as glucose)]	
Amaferm		Amaferm		Amaferm		Amaferm		Amaferm		Amaferm		Amaferm		Amaferm	
−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
136	274	2.55	3.44	0.215	0.295	0.761	1.992	4.19	17.0	4.36	27.76	0.354	0.962	0.284	0.439

Table 2 Powdered product effect on supernatant cellulase, culture gas, culture protein production, and culture acetate production. *VFA* Volatile fatty acid

Powdered Amaferm effects (at 66 and 74 h growth)				
		IU/ml supernatant	IU/mg protein	Percent of control
Avicelase 66 h	Control	0.0467	6.89	
	Amaferm	0.06	4.54	128.48
Gas production/h		ml h^{-1} $culture^{-1}$		
	Control	0.4141		
	Amaferm	0.5303		128.06
Protein 66 h		μg/ml supernatant		
	Control	6.78		
	Amaferm	13.21		194.8
VFA (acetate) production 74 h		μMol/ml supernatant		
	Control	6.44		
	Amaferm	8.06		125.2

tion was also significantly higher ($P \leq 0.01$) in those cultures.

In an effort to closely mimic addition protocols used by herd managers, experiments were conducted to test the effects of powdered, un-autoclaved product on fungal growth and physiology. Because of the difficulty in measuring less than 0.05 mg powder, large (250 ml) stationary pots were used. Aerobic fungi and bacteria present in the product did not grow under the anaerobic conditions used for the rumen fungi. Direct powder contributions to characteristics measured in culture were either below the detection limits of the assays used or represented a small value compared to the actual assay values (data not shown). When the powder was added to fungal cultures it caused a strong increase in several recorded parameters. Gas production is higher in the treated cultures at all time points measured (Fig. 6). Gas production was significantly different at the first two collection points ($P \leq 0.0003$ and $P \leq 0.06$ for 48 h and 66 h, respectively) but not at later times. Cellulase production (as Avicelase) and protein production were also measured in this experiment at 66 h and VFA production at 74 h (Table 2). Acetate production was higher ($P \leq 0.015$) at 74 h in treated cultures. Gas production per hour was also significantly greater ($P \leq 0.04$) for Amaferm-treated cultures. Avicelase, gas production and acetate production were 28, 28, and 25% higher, respectively, in treated cultures than in controls. Protein production was nearly doubled.

The effect on zoospore production by direct powder addition was determined. Measurements of protein, cellulase, and zoospore counts are recorded in Fig. 7 for

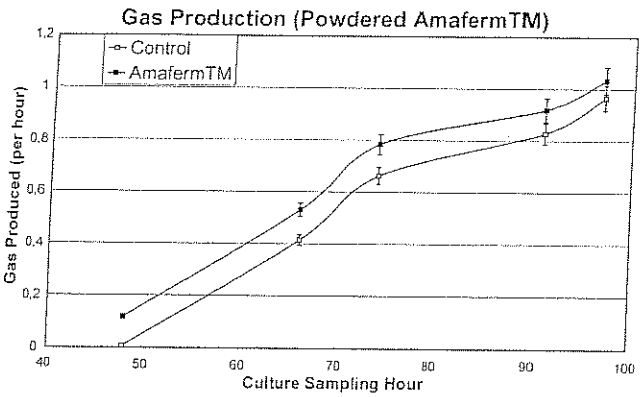

Fig. 6 Gas production in cultures using powdered product. *Open squares* Control cultures, *solid squares* treated cultures. Gas production per hour is plotted against culture sampling time (h)

three timepoints that were the most influenced by AO treatment. Treatment slightly increased protein throughout the course of the experiment and greatly increased CMCase at the 48 and 56 h timepoints. CMCase was only slightly increased at 71 h. β-Glucosidase was significantly higher ($P \leq 0.07$) in the treated cultures throughout the timepoints investigated. Protein values in treated cultures were significantly higher ($P \leq 0.08$) at all timepoints. CMCase values were significantly higher ($P \leq 0.08$) in treated cultures at 48 h but not at later times. The numbers of motile zoospores and their maturation into germination entities was more rapid in treated cultures. The decrease in motile zoospores (87% of control) in treated cultures at 71 h is also consistent with a conversion to mature entities

Table 3 *Powdered product effects on zoospore maturation. M Motile, NM non-motile, and G germinated spores were enumerated*

Culture sampling time (h)																		
	46			56			71			81			92			101		
Culture	M	NM	G	M	NM	G	M	NM	G	M	NM	G	M	NM	G	M	NM	G
Control	400	0	225	375	75	200	3,775	625	2,725	4,150	800	4,475	2,050	2,125	8,550	2,025	2,950	6,450
Amaferm	475	0	275	1,025	300	400	3,275	775	6,650	2,125	600	10,975	1,675	1,750	10,875	700	1,875	15,275
	M/G ratio			M/G ratio			M/G ratio			M/G ratio			M/G ratio			M/G ratio		
Control	1.77			1.88			1.38			0.93			0.24			0.31		
Amaferm	1.72			2.56			0.49			0.19			0.15			0.05		

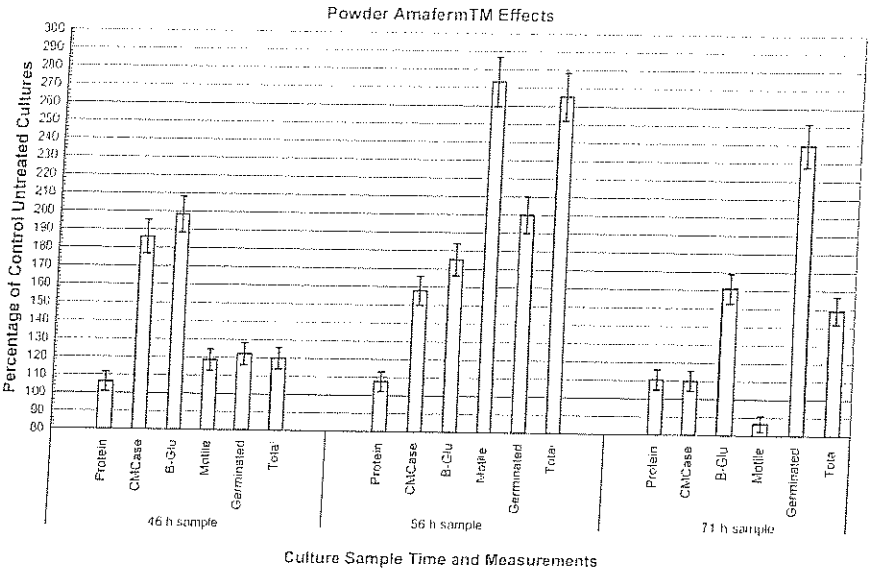

Fig. 7 *Effect of powdered product on zoospore development and fungal physiology and enzymology. Three culture timepoints are shown. All values shown are for treated cultures and are normalized to percentage of control (untreated) cultures*

at a more rapid rate. The effect of product added as a powder on zoospore production is listed in Table 3. A calculation of the ratio of relative numbers of immature zoospores against mature germination entities is also provided. Spore production was significantly higher ($P \leq 0.07$ for motile and $P \leq 0.01$ for non-motile) at 56 h in treated cultures when considering motile and non-motile types. Significantly higher numbers ($P \leq 0.04$) of germinated spores were present in treated cultures at later times (e.g., 81 h). These ratios indicate the ability of treatment to cause increased production of motile spores and a faster or more complete conversion to germinated spores.

Discussion

At the recommended supplementation levels for ruminants, the AO DFM Amaferm caused a stimulation of fungal physiology. Treated cultures matured significantly faster and possessed more extensively immobilized medium cellulose. We provide the first evidence of accelerated production and maturation of zoospores. Several enzymes, including a true measure of cellulase (Avicelase), were elevated in cultures containing product extract. Furthermore, when powdered product was used, levels of enzymes, gas, and zoospore parameters were greatly accelerated, suggesting that components necessary for maximal stimulation of rumen fungi may be contained within the soluble and insoluble fractions of the product. This research was consistent with earlier reports of similar soluble extract increasing the rate of growth and branching extent of rhizoid tissues in rumen fungi (Chang et al. 1999). Earlier research has shown that soluble extract causes increases in fungal mass and the acceleration of secreted cellular components including protein (Welch et al. 1996). If rumen physiology is similarly accelerated in vivo in the presence of this product, we suggest that animal performance may also be increased. However, other microbial physiologies likely complicate the availability and metabolism of such products for rumen fungi in vivo. The relatively small amount of such products recommended as supplement suggests the mode of action is not related to direct effects such as endogenous enzymes, flavor enhancers, pathogen exclusions, rumen broth oxygen scavenging, or viable AO mold spores or physiologies. The process may relate to providing essential and lacking nutrients to reduce microbial growth lag time (Dawson 1987), rumen buffering via microbial uptake systems (Martin and Nisbet 1990), or the physi-

ological acceleration of a population of rumen microorganisms that has been shown to be key to the fiber fermentation process such as the rumen fungi (Welch et al. 1996). We believe this work supports a mechanism related to the growth enhancement of rumen fungi and their influence on fermentation.

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