In vitro fibrolytic activity of the anaerobic fungus, *Caecomyces* sp., immobilized in alginate beads

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ABSTRACT

In the present study, *Caecomyces* sp., an anaerobic rumen fungus from faeces of the Indian elephant, was immobilized using calcium-alginate. The resulting beads were found to solubilize within 4-5 h in culture medium at 39°C (pH 6.9), indicating their possible dissociation in the rumen, if used as a feed additive. Since media was also used as a component of the immobilization material, the fungi were not only found to survive, but also to grow, as mycelial growth was visible macroscopically on the surface of the beads. Incubation of immobilized fungi with wheat straw at different temperatures was found to extend the viability of immobilized fungi. Immobilization of rumen fungi and their subsequent incubation with an appropriate substrate (e.g., wheat straw, etc.) could be used to enhanced their survival, industrial use as propagules, and as direct-fed microbials/ feed additives for domesticated ruminants.

KEY WORDS: rumen, anaerobic fungi, fibre degradation, alginate beads, direct-fed microbials

INTRODUCTION

There is evidence supporting the existence of a definite positive relationship between the presence of anaerobic fungi in the rumen and the voluntary intake of herbage diets of low digestibility (Gordon and Phillips, 1989; Lee et al., 2000; Thareja et al., 2006). One potential means to take advantage of this relationship may involve inoculation of efficient anaerobic fungal strains into the ruminants to increase fungal activity in the rumen. Anaerobic fungi, found in the rumen and

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other parts of the gastrointestinal tract of herbivores, play a vital role in plant-fibre degradation by releasing various (hemi-) cellulolytic enzymes like cellulases and hemicellulases (Orpin, 1975; Lee et al. 2000; Thareja et al., 2006; Dayanand et al., 2007; Tripathi et al., 2007a), justifying their use as animal feed additives for improved ruminant nutrition. Feeding of animal probiotic cultures as direct fed microbials (DFM) could be the safest method for improving rumen ecological conditions, where these efficient organisms bring favourable changes (Dey et al., 2004; Tripathi et al., 2007b; Sehgal et al., 2008). Due to their anaerobic nature, however, these fungi need to be maintained under an atmosphere of CO₂ during growth, making their cultivation difficult. Therefore, in the present investigation, an attempt was made to immobilize a strain of *Caecomyces* sp. and to observe its subsequent activities, with the aim to determine if immobilization could be a simple method allowing the use of these fibrolytic fungi as DFM or microbial feed additives.

MATERIAL AND METHODS

Source of organism

The anaerobic fungus, isolated from faeces of the Indian elephant, was identified as *Caecomyces* sp. on the basis of its morphological characteristics such as the nature of growth, sporangia, rhizoid and zoospore flagellation (Thareja et al., 2006; Tripathi et al., 2007a). The fungal culture was maintained in Orpin's medium (Orpin, 1975) with either cellobiose (2.5 g/l) or milled wheat straw (5 g/l) as the source of carbon, under anaerobic conditions, at 39°C without shaking.

Encapsulation of anaerobic fungi

For encapsulation, sodium alginate was prepared as a 3% solution dissolved in Orpin's medium containing 0.25% glucose. The mixture was thoroughly homogenized, dispensed into pre-gassed serum tubes, and the tubes containing the alginate-medium mixture were then sealed and autoclaved. A 30 ml solution of calcium chloride (0.1 M) was prepared and poured into100 ml serum bottles and autoclaved. A 4-5 day old fungal culture (1 ml) was transferred to 4 ml of alginatemedium mixture, and the mixture was mixed using a vortex and then withdrawn using a 5 ml syringe fitted with an 18-gauge needle. This suspension was added dropwise to a sterile CO_2 -flushed $CaCl_2$ solution through the same syringe fitted with a 21-gauge needle. Each drop formed a spherical bead (diameter 2-3 mm) after contacting the electrolyte solution, and each 5 ml of alginate-zoospore mixture yielded about 100-120 beads. The immobilized cells were kept in calcium chloride for 10-15 min, after which the $CaC1_2$ solution was removed and the beads were washed with formal saline (9 gm NaCl dissolved in 90 ml distilled water and 1 ml 40% formaldehyde, and autoclaved) (McCabe et al., 2001).

In order to check the viability of the fungal culture the beads, these beads were kept in fresh Orpin's broth (pH 6.9) at 39°C for 5-6 h in pre-gassed tubes and fungal mycelia were harvested by centrifugation at 5000 rpm for 15 min at 4°C. The pellet was subsequently dissolved in 10 ml of fresh medium and was used for fungal enumeration by the Hungate roll-tube method (Hungate, 1969; Joblin, 1981) or for chitin estimation (Gordon and Phillips, 1989).

In-vitro fibrolytic activities

For determination of the *in vitro* dry matter digestibility (IVDMD) of wheat straw by the fungal isolates in terms of percent IVDMD, wheat straw (500 mg) was taken in a flask (100 ml) along with 40 ml McDoughall's buffer, 10 ml strained rumen liquor (SRL; freshly collected rumen liquor from fistulated cattle, strained through a double layer of muslin cloth, clarified through centrifugation at 12000 *g* for 20 min, stored at 7-8°C before adding to the medium and immobilized fungus (\approx 500 beads, made out of 5 ml of fungal cultures (\approx 106 thallus forming units/ml)). In addition, a treatment containing 5 ml of free fungal culture was also made. Control beads were made from 5 ml of culture medium without fungal culture. The beads were used as follows:

- Control = straw + buffer + strained rumen liquor + Ca-alginate beads made from 5 ml broth (control)
- Free fungi = straw + buffer + strained rumen liquor + 5 ml *Caecomyces* sp. culture
- Immobilized fungi = straw + buffer + strained rumen liquor + Ca-alginate beads made from immobilization of 5 ml *Caecomyces* sp. culture

The samples were analysed for IVDMD according to the method of Tilley and Terry (1963).

The culture supernatant from the IVDMD reaction mixtures (after 96 h) was analysed for hydrolytic enzyme activities using Orpin's broth supplemented with 1% each of carboxymethyl cellulose (CMC), Whatman No. 1 filter paper (6×1 cm ≈ 50 mg), and xylan (from oat spelts; Sigma) separately, for assaying the activity of CMCase, filter paper cellulase (FPase) and xylanase activities (Mandels et al., 1976; Srinivasan et al., 2001), respectively.

760

NAGPAL R. ET AL.

Estimation of fungal biomass

Fungal chitin was estimated as the total hexosamine content of cultures after acid hydrolysis as described by Gordon and Phillips (1989). In experiments involving biomass estimation of immobilized fungus, beads (approximately 120; made out of 5 ml alginate-culture mixture) were washed thoroughly in distilled water and placed in 10% hexametaphosphate (100 ml) overnight to dissolve the alginate. The resulting free fungal mycelium was sedimented by centrifugation at 5000 rpm for 15 min at 4°C, and the pellet was washed with distilled water. Samples were then hydrolysed for 4 h at 100°C with 6 ml of 6N HCl contained in sealed glass tubes. The hydrolysate was cooled and allowed to settle before filtering through Whatman filter paper. Sub-samples of the hydrolysates were dried at 39°C for 18-24 h. Hexosamine was measured colorimetrically with Erhlich reagent by using D-glucosamine hydrochloride as the standard (Chen and Johnson, 1983) at 530 nm. The chitin content of the samples was calculated as the 1, 4-anhydro-N-acetyl-2-deoxy-D-glucopyranose equivalent.

Direct-fed microbial formulation

An aliquot (5 ml) of actively growing *Caecomyces* sp. culture was immobilized, which produced about 500 beads. These beads were mixed with 10 g sterile wheat straw (mesh-ground through a 1 mm sieve) and the mixture was placed into sealed (but not airtight) polythene bags, and the bags were kept at ambient temperature (25-30°C), refrigeration temperature (5-8°C), and 39°C for 60 days. The fungal counts (tfu/g) from each temperature treatment were obtained after 7, 15, 30, 60 and 90 days, using Hungate's roll-tube method to observe the survival of the isolate. For this, the sample (5 g) was dissolved in 250 ml fresh broth and incubated for 24 h at 39°C for dissociation of beads and also for resuscitation of immobilized fungi. After incubation, 1 ml was used for roll-tube counts, and the remaining fraction was used for chitin estimation (Gordon and Phillips, 1989).

Statistical analysis

The data were expressed as the mean (\pm standard deviation) of three replicates using the randomized factorial design of analysis of variance (ANOVA) according to the General Linear Models procedure of SYSTAT Version 6.0.1 (1996, SPSS Inc.) for testing significance (P<0.05), according to the method of Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Entrapment using polymer gels or microcapsules is a suitable method for immobilization of living cells, and hence, was the method of choice in this project. The preparation of gel beads is relatively simple with alginate, as only aqueous gelling solutions are required. For this reason, the immobilization procedure as described by McCabe et al. (2001) was tried using sodium-alginate and calcium chloride. In order to determine their fate in the rumen, if used as feed-additives, these beads were kept in Orpin's media at different temperatures and pH, and observed intermittently. The beads were found to dissociate within 4-5 h at 39°C and pH 6.9 (Table 1). The time needed for dissociation at different temperatures

Item	Parameter(s)	Time
рН 6.9		
*	39-40	4-5 h
temperature, °C	25-27	9-10 h
	5-7	>20 h
temperature, 39°C		
* -	2	>30 days
	3	>30 days
рН	4	>30 days
r	5	>20 days
	6	7-8 h
	6.9	4-5 h

Table 1. Dissociation of alginate beads in Orpin's media at different pH and temperatures

and pH was, however, greater. These results suggested that the beads could dissociate and release free fungi in the rumen within 4-6 h if used as feed-additives. Moreover, the fungus was able to survive during the immobilization. After the dissociation of beads, the media was used for obtaining fungal counts (Table 2). In case of immobilized fungal hyphae, 58% recovery was observed; on the

Table 2. Survival and recovery of encapsulated *Caecomyces* sp. Values are mean (\pm SD) of three replicates

	Before	After	Percent
Parameter	encapsulation	encapsulation	recovery
Fungal count, tfu x 10 ⁴ / ml	3.6 ± 0.3	2.1 ± 0.6	58
Zoospore count, $\times 10^4$ / ml	2.7 ± 1.2	2.6 ± 0.4	96

other hand, 96% recovery was observed when counts were done only for separated immobilized zoospores. Immobilization studies carried out by Deo and Gaucher (1983) and Kopp and Rehm (1983) also showed that spores were better propagules than hyphae. This could be due to mechanical damage of fungal hyphae or rhizoids during immobilization and subsequent loss of viability. Moreover, immobilized

762

spores might have shown better recovery because viable counts of zoospores can be more accurate and reproducible than fungal hyphae or mycelia.

The IVDMD of wheat straw was 43.2% after 48 h and 46.8% after 96 h when a free fungus was used. In the case of immobilized fungi, the IVDMD (%) was 39.9% and 45.1% after 48 and 96 h, respectively (Figure 1). Although these values were less than that of free fungi, they were, however, quite high when compared with the control, i.e. 38.4 and 38.7% after 48 and 96 h, respectively. Additionally, there was a 5.2% increase from 48 to 96 h in IVDMD of immobilized fungi, which was only 3.6% in the case of free fungi. This clearly suggests that immobilized fungi started acting on straw with a delay since it took time for the beads to dissociate, and that as soon as the fungi were released from the beads, they started growing actively and acting on the wheat straw, which is clear from the rapid increase in IVDMD (%) after 48 h.



Figure 1. Digestion of wheat straw by free and immobilized fungi (*Caecomyces* sp.). Control - straw + buffer + strained rumen liquor + Ca-alginate beads made from 5 ml broth (control); Free fungi - straw + buffer + strained rumen liquor + 5 ml *Caecomyces* sp. culture; Immobilized fungi - straw + buffer + strained rumen liquor + Ca-alginate beads made from immobilization of 5 ml *Caecomyces* sp. culture. Values are mean (± SD) of three replicates

In free fungi, the CMCase and FPase activities after 96 h were 14.2 and 17.7 mIU/ml, respectively; while in immobilized fungi, these values were 12.1 and 14.5 mIU/ml. Additionally, xylanase activities were also estimated, which were 22.8 mIU/ml in free fungi, and 22.5 mIU/ml in immobilized fungi (Figure 2). The results from IVDMD and enzyme activity assays showed that the fungi could be successfully immobilized and subsequently used as feed additives, since immobilized fungi showed significant *in vitro* fibrolytic potential, as is evident from Figures 1 and 2.



Figure 2. Hydrolytic enzyme activities (mIU/ ml/h) of free and immobilized fungi (*Caecomyces* sp.) after 96 h of incubation with wheat straw. Values are mean (\pm SD) of three replicates. Treatments are culture supernatants of IVDMD reaction mixtures after 96 h (see Figure 1)

These enzyme activities are certainly responsible for the enhanced IVDMD of wheat straw using immobilized fungi. McCabe et al. (2001) also reported β -glycosidase activity of 20 mIU/ml using immobilized *Piromyces* sp. The successful survival and growth of fungi within the alginate beads were verified by estimating the total biomass (in terms of cell-wall chitin content) of fungi immobilized and kept at 39°C in pre-gassed sealed serum tubes for a period of 10 days (Figure 3). The fungal biomass was found to increase from 0.3 mg chitin on day 2 to 5.0 mg on day 8, clearly indicating that the fungi were not only able to survive but also grow within the alginate beads, as mycelial growth was also visible macroscopically. The growth of fungus in the Na-alginate beads was attributed to the use of culture media in their preparation in order to get even



Figure 3. Chitin content of encapsulated *Caecomyces* sp. Values are mean (\pm SD) of three replicates. Beads were incubated in fresh Orpin's broth (pH 6.9) at 39°C in pre-gassed tubes

colonization within them. The zoospores formed micro-colonies inside the matrix and also at the periphery of the beads. Kuek (1991) also observed that *Aspergillus phoenicus* was able to grow within the calcium-alginate gel matrix for a period of about six weeks, and the immobilized mycelia remained completely confined to the surface of the bead. Such distribution of anaerobic fungal biomass within the immobilization matrix was observed when immobilized fungi were used as propagules for enzyme production (Honecker et at., 1989; McCabe et al., 2001). However, there are no previous reports of using immobilized anaerobic rumen fungi as feed additives, and the present study investigates the survival and *in vitro* fibrolytic activities of these encapsulated fungi for their possible use as DFM.

In order to make DFM formulation based on immobilized fungi more effective. the fungal beads were further mixed with wheat straw, sealed into polythene bags, and kept at different temperatures to check the viability of immobilized fungi in the formulation. The fungi failed to survive after 30 days at refrigeration temperature, and after 40 days at room temperature; however, the fungus survived up to 50 days at 39°C (Figure 4). These results are quite significant, since, it is relatively difficult to maintain these fungi at such environmental temperatures. Moreover, the conditions in the DFM were not strictly anoxic, hence the ability of these fungi to survive in such an environment led to the suggestion that they produce aero-tolerant survival structures, i.e. zoospores (Davies et al., 1993). The survival and occurrence of these structures in the life cycle of rumen fungi may be of great significance for the exploitation of these microbes as DFM/feed additives. At room temperature and refrigeration temperature there was a gradual decrease in the chitin content of the sample. However, at 39°C, biomass increased up to 20 days, after which it started decreasing up to 60 days (Figure 5). This could again be attributed to the use of media in Na-alginate mix, which could support the growth



Figure 4. Survival of encapsulated *Caecomyces* sp. (log tfu/g) in direct fed microbials. Each value is mean \pm SD (n = 3); RT - room temperature



Figure 5. Biomass of encapsulated *Caecomyces* sp. in direct fed microbials. Each value is mean \pm SD (n = 3); RT - room temperature

of the fungi for some time in the beginning. Milne et al. (1989) reported, however, that anaerobic fungi survived up to 128 days in air-dried samples of faeces; however, similar samples that were kept moist did not yield any fungi after 24 h. In the present study, the moist conditions in DFM formulation must be the reason why the fungi could not survive after 50 days, as the samples were sealed and there was no way for moisture to escape, thus creating conditions similar to those of air dried faeces. McGranaghan et al. (1999) also studied the survival of anaerobic fungi in faeces at 39°C following air-drying and sealing in polythene bags, and reported enhanced survival for more than 50 days in un-sealed bags. In sealed bags, fungi were able to survive for only 5 days, indicating that, besides temperature, the moisture content of the sample also has a significant effect on propagule survival. Milne et al. (1989) also found that fungi could not survive beyond 24 h under moist conditions (i.e. sealed in a polythene bag at 39°C). In the present study, however, sealing was necessary in order to avoid contamination of the bags. Moreover, survival was observed up to 50 days at 39°C, hence, the observation that the isolate is a monocentric fungus that produces aero-tolerant zoospores is significant with respect to their survival. Although the physiology of rumen fungi under anaerobic conditions has been the subject of extensive investigations, their activities and survival outside their hosts is still poorly appreciated.

CONCLUSIONS

In conclusion, the results of survival of immobilized fungus in a strawbased formulation, and *in vitro* fibrolytic activities suggest that incubation of

NAGPAL R. ET AL.

these fungi with wheat straw at ambient temperatures extended their viability. Hence, immobilization of ruminal fungi and their incubation with an appropriate ligno-cellulosic substrate could be exploited as an innovative method for enhanced survival, commercial enzyme production, and directly fed microbials for domesticated ruminants. However, more *in vitro* as well as *in vivo* trials are needed to authenticate the possibility of their successful inter-species transfer and establishment in the rumen before exploiting these fungi as animal feed additives.

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