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FULL PAPER

Survival of anaerobic fungus *Caecomyces* sp. in various preservation methods: a comparative study

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Abstract The present investigation was designed to observe the survival of the anaerobic fungus *Caecomyces* sp. in various routine preservation methods. Among all the treatments, cryopreservation of fungi at −70 °C with glycerol was found to be most effective for long-term maintenance (more than 90 days) of rumen fungi, followed by dimethyl sulfoxide (DMSO) and ethylene glycol (up to 60 days). In contrast, at −196 °C, DMSO showed maximum survival (more than 90 days), followed by glycerol (up to 90 days) and ethylene glycol (up to 30 days). At 39 °C, maximum survival (up to 30 days) was observed with soft agar and wheat straw; at refrigeration temperature, preservation with Orpin's media containing straw showed maximum survival (up to 30 days).

Keywords Anaerobic fungi · Cryopreservation · Fiber degradation · Microbial survival · Rumen microflora

Introduction

It is now well established that anaerobic rumen fungi (Neocallimastigomycota) efficiently take part in fiber digestibility in ruminants, leading to a more rapid degradation of forage entering the rumen (Orpin and Joblin 1988; Lee et al. 2004). These fungi play a key role in plant-fiber degradation in the rumen by releasing various enzymes such as cellulases, hemicellulases, proteases, and esterases, justifying their use as animal feed additives for improved ruminant nutrition (Lee et al. 2004; Thareja et al. 2006; Tripathi et al. 2007a, b; Nagpal et al. 2008, 2009, 2010, 2011). These fungi have been found in all the geographic regions of the world, being ubiquitous among foregut fermenters and ruminants such as cattle, buffalo, goat (Ho et al. 1993a, b; Thareja et al. 2006), red deer, impala (Bauchop 1979), and wild blue bull (*Boselaphus tragocamelus*) (Paul et al. 2004; Tripathi et al. 2007a), as well as from marsupials including kangaroo, wallaroo, and swamp wallaby (Breton et al. 1989). These fungi have also been isolated from fecal samples of horse, zebra, donkey, rhinoceros, and Indian elephant (Breton et al. 1989; Li et al. 1990; Nagpal et al. 2011), all of which are hindgut fermenters. Presently, six genera and 18 species of anaerobic fungi have been identified and described on the basis of morphological characteristics such as nature of growth, sporangia, rhizoid, and zoospore flagellation (Barr 1990; Li et al. 1993; James et al. 2000, 2006a, b; Hibbett et al. 2007).

Because of their role in plant-fiber degradation, it is of utmost importance to maintain the viability of anaerobic fungal inocula for a sufficiently long time so that they can be used as microbial feed additives to improve the nutritional status of ruminants under field conditions. For prolonged maintenance in the laboratory, pure cultures of anaerobic fungi are transferred every 5–6 days into fresh basal anaerobic medium. For long-term maintenance of these fungi, cultures are usually stored at −80 °C using ethylene glycol or dimethyl sulfoxide (DMSO) as a cryoprotectant (Sakurada et al. 1995). Joblin (1981), however, reported that cultures could be maintained for several months on plant tissues, i.e., sisal (*Agave sisalana*), stored at 39 °C without subculturing. When 20 mg sisal fiber was

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added to the pre-gassed roll-tubes (saturated with CO_2) before the addition of the reduced agar medium under a CO_2 atmosphere at 39 °C, the senescent cultures on sisal agar that had been stored at 39 °C for up to 7 months could apparently be reestablished within 3–4 days by the addition of glucose. However, there is no standard method to preserve these ruminal anaerobic fungi for long-term continuance. Therefore, the proper preservation and maintenance of rumen fungi has long been a matter of debate for researchers, and various methods have been proposed by various researchers for prolonged maintenance of these fungi. Hence, the present study was intended to observe the survival of anaerobic fungus *Caecomyces* sp., a monocentric fungus, with various preservation methods at different temperatures and media to provide a comparative analysis and to find the most appropriate and convenient method(s) for cultivation and research.

Materials and methods

Fungal isolate

The anaerobic fungus *Caecomyces* sp. FE_5 was previously isolated from feces of the Indian elephant (Nagpal et al. 2009, 2011); it was identified on the basis of its morphological characteristics such as nature of growth, sporangia, rhizoid, and zoospore flagellation (Thareja et al. 2006; Tripathi et al. 2007a; Nagpal et al. 2011). 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 a carbon source under anaerobic conditions at 39 °C without shaking (Lowe et al. 1985).

To compare the survival of ruminal fungi in various preservation methods, different treatments were made wherein the fungal isolate was maintained in different combinations of media and cryoprotectants at different temperatures.

Survival of active culture

A 5 % inoculum of 3- to 4-day-old active fungal culture was inoculated into pre-gassed serum tubes (saturated with CO_2) with either sterile Orpin's broth containing wheat straw (1 % w/v) or sterile rumen liquor containing wheat straw (1 % w/v; fresh rumen liquor collected in a pre-gassed thermos flask from the rumen of fistulated adult cattle, maintained at the Institute's cattleyard, and fed on a standard diet containing 10 kg green fodder maize, 1 kg concentrate mixture, and wheat straw ad lib, strained through double layers of muslin cloth before being used), or sterile Orpin's soft agar (0.8 %) media. After inoculation, all the tubes (in triplicates) were kept at 39 °C and at refrigeration temperature (5–7 °C). A set of tubes from each temperature treatment was taken out after 7, 15, 30, 60, and 90 days, and the colony counts were taken using the roll-tube method to observe the survival of fungal isolate in terms of thallus-forming units (tfu/ml) (Joblin 1981). In addition, fresh moist fecal samples ($\sim$10 g) were collected, from cows kept at the Institute's cattleyard, in pre-gassed serum tubes and then autoclaved. An active fungal mycelial culture (0.5 ml) was mixed with these samples and placed into sealed (but not airtight) polythene bags, then kept at laboratory ambient temperature (24–27 °C) and at 39 °C in light for 90 days. The anaerobic fungal counts at each temperature treatment were enumerated after 7, 15, 30, 60, and 90 days, using the roll-tube method, to observe the survival of the fungal isolates in terms of tfu/g, as described previously (Thareja et al. 2006; Nagpal et al. 2009, 2010, 2011).

Survival of frozen culture

To observe the cryopreservation of the isolate, a 0.5-ml inoculum of 3- to 4-day-old active fungal culture was put into pre-gassed cryovials (1.5 ml), and the tubes were separately filled with 1 ml cryoprotectant (0.5 ml of either 10 % glycerol, 10 % DMSO, or 10 % ethylene glycol, plus 0.5 ml 10 % sterile cell-free rumen liquor). The tubes (in triplicates) were then placed for about 1 h in the refrigerator (5–7 °C) and then kept at −70 °C. For preservation at −196 °C, tubes were kept for about 1 h in the refrigerator (5–7 °C) and then for 24 h at −70 °C, and were then transferred to −196 °C. A set of tubes from each treatment were taken out after 7, 15, 30, 60, and 90 days, thawed by moving (rolling) between the palms, and 0.5 ml was used to examine the viability of the fungal isolate using the roll-tube method (Joblin 1981).

Results

Survival of active culture

To evaluate various preservation methods, the samples were analyzed for enumeration of fungal counts after 7, 15, 30, 60, and 90 days preservation (Figs. 1, 2). In Orpin's media at 5–7 °C, the fungal counts were 4.77, 4.69, and 4.39 log tfu/ml after 7, 15, and 30 days, respectively; after more than 30 days, no fungal colony was observed in the roll-tubes. At 39 °C, the counts were 4.79 and 4.74 (log tfu/ml) after 7 and 15 days, respectively; after more than 15 days, fungi failed to survive (Fig. 1a). When maintained on sterile rumen liquor containing wheat straw, the fungal counts were further reduced. At refrigeration temperature (5–7 °C), the counts were 4.74 and 4.60

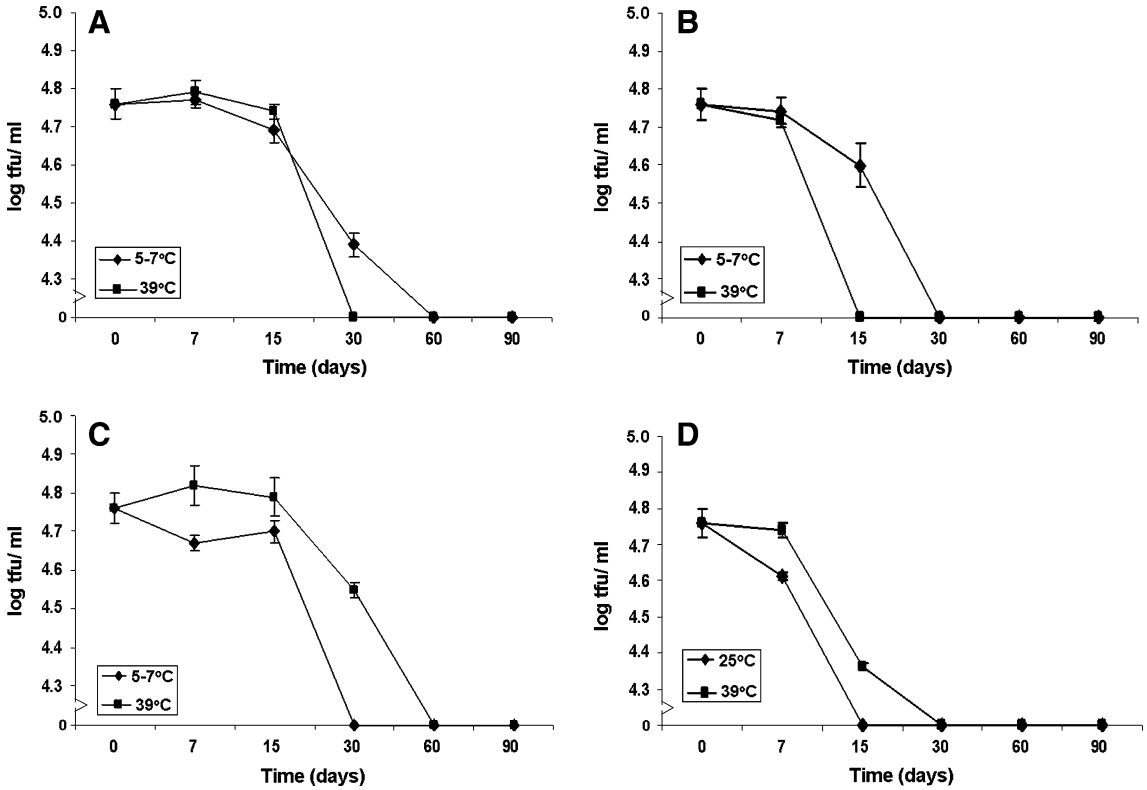

Fig. 1 Survival of *Caecomyces* sp. FE_5 cultured in Orpin's media (**a**), sterile rumen fluid (**b**), soft agar (**c**), and feces (**d**). Each value is mean (±SD) of three replicates. *tfu* thallus-forming units

(log tfu/ml) after 7 and 15 days, respectively, and were reduced to zero afterward; at 39 °C, the count was 4.72 (log tfu/ml) in 7 days, after which fungi failed to survive (Fig. 1b). However, the fungus was able to survive up to 30 days at 39 °C when maintained on soft agar (Fig. 1c). The counts were 4.82, 4.79, and 4.55 (log tfu/ml) after 7, 15, and 30 days, respectively, and reduced to zero afterward. At 5–7 °C, these counts were 4.67 and 4.70 (log tfu/ml) after 7 and 15 days, respectively, and were zero afterward. When mixed and stored with fecal samples, the fungal counts at 39 °C were 4.74 and 4.36 (log tfu/ml) after 7 and 15 days, respectively; and were 4.61 (log tfu/ml) on 7th day at 5–7 °C, but the fungi died afterwards (Fig. 1d).

Survival of frozen culture

Apart from these treatments, freezing preservation at −70 and −196 °C was also tested using different cryoprotectants, i.e., ethylene glycol, DMSO, and glycerol (Fig. 2). In −70 °C preservation with ethylene glycol, the fungal counts were 4.60, 4.55, 4.32, 4.23, and 0.0 (log tfu/ml) after 7, 15, 30, 60, and 90 days storage, respectively; in −196 °C preservation, the counts were 4.69, 4.64, and 4.61 (log tfu/ml) after 7, 15, and 30 days, respectively, beyond which fungi failed to seed a fresh culture (Fig. 2a). During preservation at −196 °C with DMSO as a cryoprotectant, the counts were 4.68, 4.61, 4.55, 4.41, and 4.43 (log tfu/ml) after 7, 15, 30, 60, and 90 days, respectively; these counts were 4.64, 4.63, 4.32, 4.27, and 0.0 (log tfu/ml) at −70 °C preservation (Fig. 2b). Survival was better when glycerol was used as a cryoprotectant (Fig. 2c). At −70 °C preservation, counts were 4.72, 4.71, 4.70, 4.57, and 4.51 (log tfu/ml) after 7, 15, 30, 60, and 90 days, respectively; at −196 °C, these counts were 4.70, 4.69, 4.69, 4.47, and 4.30 (log tfu/ml). It was observed that cryopreservation at −70 °C with glycerol was most effective for long-term maintenance of fungi *Caecomyces* sp. FE_5, followed by DMSO and ethylene glycol, suggesting that it could be applied to other rumen fungi also. At −196 °C, DMSO showed maximum survival, followed by glycerol and ethylene glycol.

Discussion

At 39 °C, maximum survival could be observed with soft agar and wheat straw; at refrigeration temperature, preservation with Orpin's media containing straw showed maximum survival. Hence, these methods could be exploited by researchers for rumen anaerobic fungi, especially

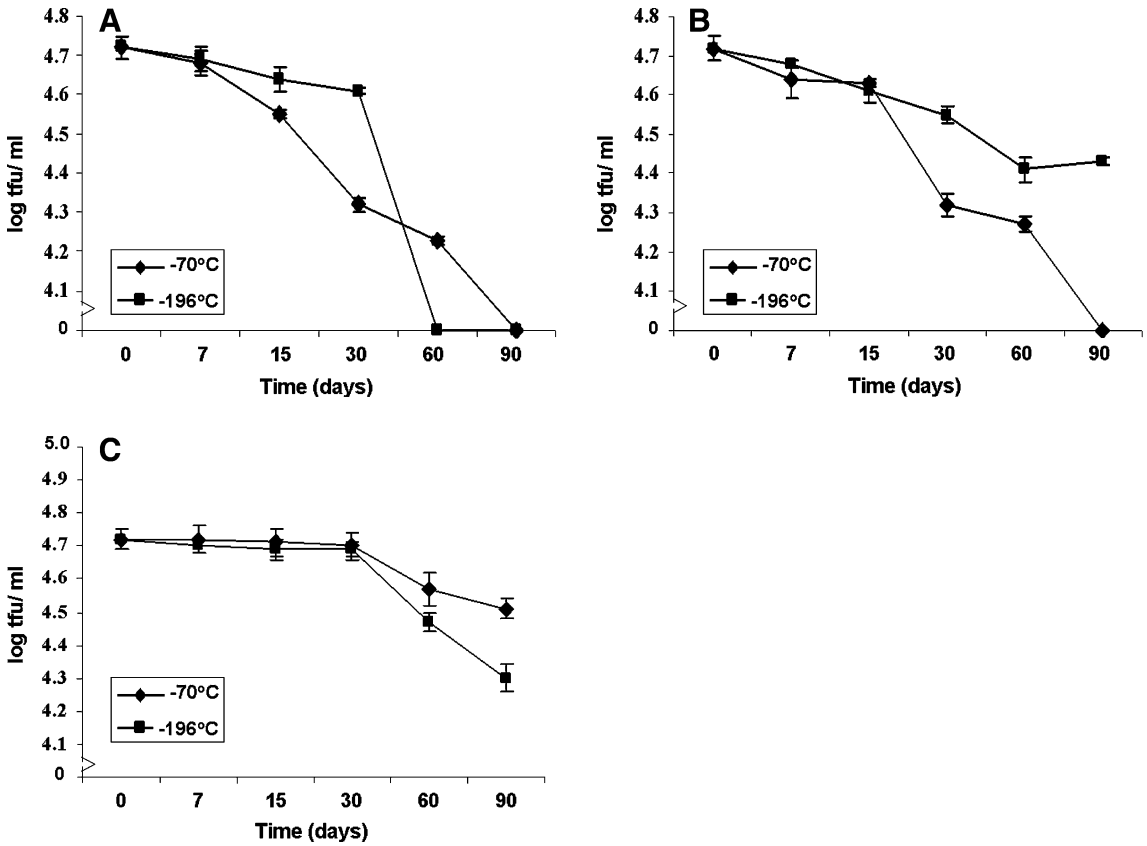

Fig. 2 Survival of *Caecomyces* sp. FE_5 after cryopreservation at −70 and −196 °C with cryoprotectant ethylene glycol (**a**), dimethyl sulfoxide (**b**), and glycerol (**c**) (0.5 ml 10 % cryoprotectant, plus 0.5 ml 10 % sterile cell-free rumen fluid, plus a 0.5-ml inoculum of 3- to 4-day-old active fungal culture into a pre-gassed 1.5-ml cryovial). Each value is mean (±SD) of three replicates. *tfu* thallus-forming units

monocentric, for their routine cultivation and maintenance. However, because of significant differences among various genera of these fungi in terms of zoospores, rhizoids, sporangia, nature of growth, thallus, etc. (Ho and Barr 1995), the ideal method or best cryoprotectant could differ from species to species. For instance, the genus *Neocallimastix* embraces polyflagellated species in which vegetative growth consists of a single sporangium borne on a much-branched rhizoidal system, whereas in the genus *Piromyces*, the vegetative stage is smaller with more highly branched filamentous rhizoids. *Orpinomyces* are characterized by the presence of spherical sporangia developing on simple or branched sporangiophore complexes that arise as outgrowths of hyphae or are terminal. *Anaeromyces* are readily distinguished by the sporangia, which are solitary and frequently have a pointed projection at the apex. Members of *Caecomyces* can be observed to have unique morphology distinguishing them from other genera of rumen fungi by the presence of monocentric rhizoids with either a single or several bulbous holdfast(s), upon which a single globose, ovoid, or ellipsoidal sympodial sporangium develops, with unbranched tubular sporangiophores (Chen et al. 2007). A distinctive correlation has been speculated between the presence of spore-like structures in cultures and the extended viability of the phenotype. For instance, the possible mechanism for the extended survivability of polycentric fungi is that the hyphae produce resistant aerotolerant structures to protect the genetic material, such that new growth is initiated in favorable conditions without the production of zoospores. The spore-like structures form a resting state, enabling the fungus to survive for extended periods in unfavorable conditions before germinating under conditions more advantageous for growth. It has also been speculated that the spore-like structures in these isolates are formed as a consequence of 'deliberate' or programmed hyphal fragmentation. In contrast, the growth patterns of monocentric fungi, which do not contain nuclei in their rhizoids, make it unlikely that they would survive and generate new growth in this manner. Further, the presence of melanized or thick-walled zoosporangia of monocentric fungi could also affect the rate of success of preservation and survival for longer periods.

Joblin (1981) reported that anaerobic fungi cultured on agar or broth, with or without glycerol as a cryoprotectant, failed to maintain viability after storage at −60 °C.

However, Yarlett et al. (1986) reported that successful cryopreservation of the anaerobic fungus *Neocallimastix patriciarum* (Orpin and Bountiff 1978) at −80 °C using DMSO as cryoprotectant showed a survival rate of only 40 % after 1 year. However, in a similar study by Sakurada et al. (1995), a survival of 80 % after 1 year storage at −84 °C was obtained using ethylene glycol and cell-free rumen fluid. At 39 °C, maximum survival was observed with soft agar and wheat straw; at refrigeration temperature, preservation with Orpin's media and straw showed maximum survival. Joblin (1981), however, reported that such fungal cultures could be maintained up to 7 months on plant tissues stored at 39 °C without subculturing. Pure cultures of anaerobic fungi can also be maintained in a medium consisting of centrifuged rumen fluid, tryptone, yeast extract, a carbon source, a buffer, L-cysteine as a reducing agent, and vitamins (Wubah et al. 1991). *Neocallimastix frontalis* was reported to be maintained in a similar medium but without yeast extracts and rumen fluid (Lowe et al. 1985).

The present study advocates that cryopreservation of fungus *Caecomyces* sp. FE_5 at −70 °C with glycerol is most effective for long-term maintenance, followed by DMSO and ethylene glycol. In contrast, at −196 °C, DMSO shows maximum survival followed by glycerol and ethylene glycol. The reason why −196 °C cryopreservation was poorer than −70 °C preservation could be that cryopreservation is a very complex process and is not fully understood even after years of research. The possible explanation could lie in the rate of cooling, which is faster during cryopreservation in liquid nitrogen, ultimately leading to the formation of larger ice crystals that are always detrimental to the cells. On the other hand, during freezing at −70 °C, the rate of cooling is slow and thus freezing outside the cell is faster than inside, which leads to water migration out of the cell as a consequence of osmotic imbalance, thereby resulting in less intracellular ice and less damage to cells. Moreover, the effect of rate of freezing on cells is an individual property of different cell types and has always been advocated to be optimized for each group of cells; this could have been one of the most critical reasons for lower survival at −196 °C than at −70 °C.

The present investigation was particularly designed to compare various methods and observe the most effective procedure for *Caecomyces* sp., because there are few reports regarding the cultivation and maintenance of this monocentric fungus. Therefore, further research is required for demonstrating the appropriate preservation method for various genera of these fungi for a much longer preservation (years or more). Also, study is necessary for examining the possible effect of programming freezing to control the freezing rate, which could reduce ice size inside the cells during the freezing process to improve the survival of cultures in preservation by freezing.

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