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Effects of Live *Saccharomyces cerevisiae* Cells on Zoospore Germination, Growth, and Cellulolytic Activity of the Rumen Anaerobic Fungus, *Neocallimastix frontalis* MCH3

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Abstract. The effects of a live yeast strain of *Saccharomyces cerevisiae* have been investigated on zoospore germination, metabolism, and cellulolytic activity of the anaerobic rumen fungus *Neocallimastix frontalis* MCH3. The addition of yeast cells to a vitamin-deficient medium stimulated the germination of fungal zoospores, increased cellulose degradation and hydrogen, formate, lactate, and acetate production. Responses depended on the concentration of yeast cells added and on their viability. Yeast supplementation provided vitamins such as thiamine, which is essential for fungal growth and activity. These results demonstrate that yeasts could enhance plant cell wall colonization by *N. frontalis*. With certain diets, yeasts could therefore be a good tool to optimize the microbial degradation of lignocellulosic materials, but more research is needed to understand their mechanisms of action, so that they can be used with maximum efficiency as feed supplements.

Various additives have been developed to improve the performance of ruminants, to promote animal health, and to optimize feed utilization and rumen functions. Among these preparations, ionophore antibiotics have been extensively used [22], but during the last decade there has been an increased interest in probiotics. Preparations based on *Aspergillus oryzae* or *Saccharomyces cerevisiae* cultures are the most commonly used for ruminants. In certain feeding conditions, these probiotics are able to alter ruminal fermentation patterns and to increase the numbers of cellulolytic and lactate-utilizing bacterial species [5, 20, 26]. Consequently, stimulation of forage cell wall digestion [4, 25] and modification of rumen pH in animals fed high-grain diets [29] have been observed. Beneficial effects on milk or meat production have also been reported [17, 27]. The mechanisms of action of probiotics are not fully understood: varying results have been obtained by in vivo and in vitro experiments, depending on diet composition [3, 6] and the nature and dose of the probiotic used.

The aim of this study was to investigate the effects of a live strain of *S. cerevisiae* on zoospore germination, metabolism and cellulolytic activity of an anaerobic rumen fungal species, *Neocallimastix frontalis* MCH3. This anaerobic Chytridiomycete readily colonizes, penetrates, and degrades lignocellulosic materials in vitro [1]. It is also able to hydrolyze most of the plant cell wall polysaccharides because of its complex enzymatic equipment, which includes cellulases, hemicellulases, glycosidases, and esterases that have been partly purified and characterized [8, 9].

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Materials and Methods

Origin of microorganisms. *Neocallimastix frontalis* MCH3, which was isolated from sheep rumen, came from the fungal culture collection of our laboratory.

The *S. cerevisiae* (SC) strain C.N.C.M. I-1096 (Institut Pasteur, Paris) was provided by Santel-groupe Agritek. This strain has already been shown to enhance plant cell wall degradation in vitro, in a rumen-simulating technique (Rusitec) [13].

Culture conditions. The culture medium used to grow *N. frontalis* MCH3 was derived from that described by Lowe et al. [15]: in medium V^+ only yeast extract was omitted, while medium V^- was also depleted in vitamins (only 10% of the initial concentration of the vitamin solution was included in the medium). Carbon and energy source in media V^+ or V^- was cellulose filter paper strips (Whatman No. 1; 100 mg/tube) or cellobiose (0.2%). Anaerobic

cultures were performed according to the Hungate technique [10]; media were dispensed into CO_2-filled, 16-ml screw cap Hungate tubes (Bellco Clan Inc, Vineland, New York). In all experiments, the media were inoculated with 0.5 ml of a zoospore suspension containing 10^3–10^4 zoospores/ml. This suspension was obtained from a 60-h fungal culture in serum bottles containing 250 ml of a rumen fluid medium [11]. Cultures were then centrifuged at 1000 *g* for 15 min, and the pellets were diluted in the anaerobic mineral solution of Bryant and Burkey [2].

Saccharomyces cerevisiae cells were grown in a yeast extract–malt extract–glucose–peptone medium at 30°C. Yeast cells were obtained from overnight cultures by centrifugation at 1000 *g* for 10 min. The pellets were then resuspended in an appropriate volume of the anaerobic solution. *S. cerevisiae* cells were counted in a Malassez cell and added under an O_2-free CO_2 atmosphere to the fungal zoospores in medium V^- at the following concentrations: 2×10^5, 2×10^6, or 2×10^7 cells · ml^{-1}. The effect of autoclaved yeasts (120°C, 20 min) was also investigated.

All incubations were performed anaerobically in triplicate at 39°C for 2, 4, and 6 days in cellulose medium, and for 2 and 4 days in cellobiose medium; fungal monocultures in media V^+ and V^- were used as controls. The final culture volume was 10 ml.

Yeast cells were counted at 2, 4, and 6 days of incubation in cellulose V^- medium by being plated on malt–agar (Difco Laboratories, Detroit, Michigan) Petri dishes; CFU were counted after 72 h of incubation at 30°C.

Analytical methods. *Cellulose degradation* was determined by measuring the dry matter (DM) content of filter paper remaining in the tubes at the end of the period of incubation. The cultures were centrifuged for 15 min at 1000 *g*. The pellet was then washed with boiling distilled water for 15 min to eliminate the fungal material adhering to the filter paper; the operation was repeated three times. Afterwards the tubes were dried for 72 h at 80°C and weighed. Supernatants were frozen at −20°C before analysis of fermentation end products.

Analysis of fermentation end products. At the end of incubations, hydrogen production was measured in the headspace of the tubes by gas chromatography. About 2 ml of the gas phase was withdrawn with a syringe through the butyl cap and injected into the chromatograph. Formate, lactate, and succinate were analyzed by enzymatic methods (Boehringer Mannheim France SA); volatile fatty acids (VFA) were determined by gas chromatography [12].

Analysis of reducing sugars. The amount of reducing sugars released from fungal cellulose breakdown and remaining in the cultures was measured according to the dinitrosalicylic acid procedure of Miller [16].

Zoospore germination. Fungal zoospores were prepared as previously described; 10^3–10^4 spores/ml were inoculated in V^+, V^-, and $V^- + 10^7$ SC · ml^{-1} media containing an agar-cellobiose block (25 mm^2 surface) prepared according to the method described by Ushida et al. [24]. After incubation, the culture supernatants were discarded, and the agar blocks were gently washed with an anaerobic mineral solution [2] to eliminate the yeasts and zoospores that had not adhered to the blocks. Germinated spores on the blocks were counted microscopically in a Malassez cell after lactophenol blue staining.

Analysis of SC thiamine (vitamin B1) content. An SC cell suspension (1 ml) was obtained by centrifugation (1000 *g*, 15 min) of an overnight culture; the pellet was homogenized in 0.25 ml of Tris-HCl buffer (0.5 M, pH 7.0), and an equal volume of glass beads (0.45 mm diameter, Sigma Chemical Co., St. Louis, Missouri) was added to the suspension. The mixture was vigorously shaken for 1 min to disrupt yeast cells and then placed in ice for the same time. The operation was repeated three times; the effectiveness of the treatment was confirmed by the microscopic examination of disrupted cells. Cell debris and glass beads were eliminated by centrifugation. The supernatant was recovered and then frozen and lyophylized before analysis. Thiamine content was determined by HPLC [14].

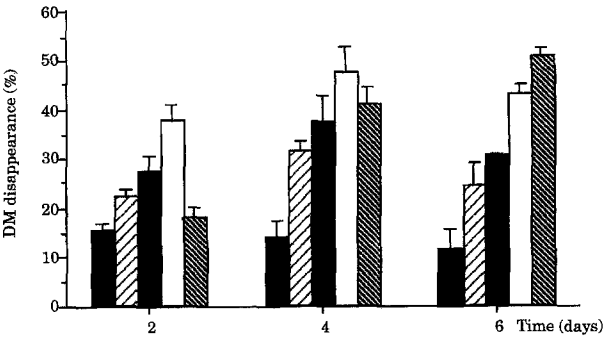

Fig. 1. Filter paper degradation by *N. frontalis* MCH3, grown in a vitamin-deficient medium (V^-), in the absence or in the presence of different concentrations of *Saccharomyces cerevisiae* (SC). ■ Medium V^-; ▨ medium V^- + SC (2×10^5 cells · ml^{-1}); ▩ medium V^- + SC (2×10^6 cells · ml^{-1}); □ medium V^- + SC (2×10^7 cells · ml^{-1}); ▧ complete medium (V^+). 100 mg of filter paper were included in each tube. Results are expressed as mean ± SD (n = 3).

Results

Effects of SC added at different concentrations on cellulolytic activity of *N. frontalis* MCH3. In the vitamin-deficient medium V^-, the cellulolytic activity of MCH3 was low. The amount of cellulose degraded increased regularly with time when the fungus was grown on medium V^+ (Fig. 1). The addition of yeast cells in medium V^- significantly enhanced cellulose breakdown; however, the stimulatory effect depended on the concentration of yeast cells added. At 2 days of incubation, filter paper degradation in the presence of yeasts was higher not only than that measured in the fungal monoculture made in V^- medium, but also than degradation in the fungal monoculture in V^+, evidence that the initial rate of cellulolysis was enhanced in the presence of SC cells. After 4 days of incubation, the three concentrations of SC cells still exerted a stimulatory effect on fungal cellulose breakdown, but the percentages of DM degraded were lower in the presence of yeasts than in V^+ medium, except in $V^- + 2 \times 10^7$ SC · ml^{-1}.

Yeast numbers dramatically decreased at the beginning of incubation (from 2×10^7 to 5.2×10^5 CFU · ml^{-1} within 2 days of incubation), indicating strong cellular lysis. Thereafter the number of viable

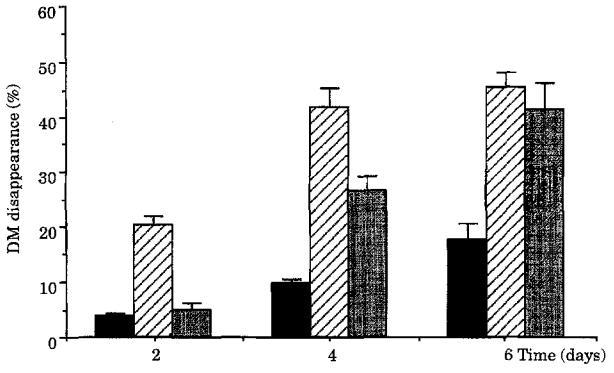

Fig. 2. Effect of live or autoclaved SC cells (1×10^7 cells · ml^{-1}) on the cellulolytic activity of MCH3 grown in medium V^-. ■ Medium V^-; ▨ medium V^- + live SC; ▩ medium V^- + autoclaved SC. Results are the means of triplicate values.

Table 1. Reducing sugars (mM) remaining in cellulose *Neocallimastix frontalis* cultures in medium V^-, in the absence or presence of live or autoclaved SC cells (1×10^7 cells · ml^{-1})

	Reducing sugars (mM)		
Time (days)	2	4	6
N. frontalis MCH3	0.57 ± 0.05[a]	0.88 ± 0.10	0.97 ± 0.23
MCH3 + live SC	5.61 ± 0.50	6.38 ± 2.2	3.86 ± 1.44
MCH3 + autoclaved SC	0.84 ± 0.18	2.28 ± 0.38	9.98 ± 1.44

[a] Results are the mean ± SD of triplicate values.

cells of SC remained stable until the end of culture (about 10^5 cells · ml^{-1}).

The influence of yeast viability on filter paper degradation was determined by the addition of either live or autoclaved SC to V^- fungal cultures. The concentration of 10^7 yeast cells · ml^{-1} was selected; as previously observed, this concentration greatly stimulated cellulolytic fungal activity. The addition of autoclaved cells in V^- had no effect after 2 days of incubation, but led to a slight increase in DM disappearance after 4 days (Fig. 2). As already observed, cellulose breakdown increased sharply in the presence of live yeasts. At the end of the period of incubation, the effects of live or autoclaved SC were similar. Increasing amounts of hexoses were liberated in the presence of SC at the beginning of incubation, but soluble released sugars seemed to be metabolized at the end of culture. This was observed only in the presence of viable yeasts. In contrast, sugars were fermented less in the presence of autoclaved SC (Table 1).

Effects of live SC cells on fungal metabolism. When MCH3 was grown in medium V^- containing cellulose filter paper or cellobiose, the addition of yeast cells

Table 2. Fermentation end products (mM) of *Neocallimastix frontalis* MCH3 grown with either cellulose or cellobiose on vitamin-deficient medium (V^-) in the absence or presence of SC (2×10^7 cells · ml^{-1})[a]

	Cellulose		Cellobiose	
Substrate	−SC	+SC	−SC	+SC
Hydrogen	3.7 ± 0.2	7.7 ± 0.7	1.6 ± 0.2	4.3 ± 0.3
Acetate	2.9 ± 0.3	6.6 ± 0.4	ND[b]	ND[b]
Formate	3.9 ± 0.6	12.1 ± 0.2	2.8 ± 0.5	9.6 ± 0.7
Lactate	1.9 ± 0.0	8.7 ± 0.8	0.6 ± 0.1	3.7 ± 0.2
Succinate	0.4 ± 0.0	0.9 ± 0.1	0.5 ± 0.1	0.9 ± 0.0

[a] Analyses were performed after 6 days of incubation on cellulose, and after 4 days on cellobiose. Each value represents the mean ± SD (n = 3).
[b] ND, not determined.

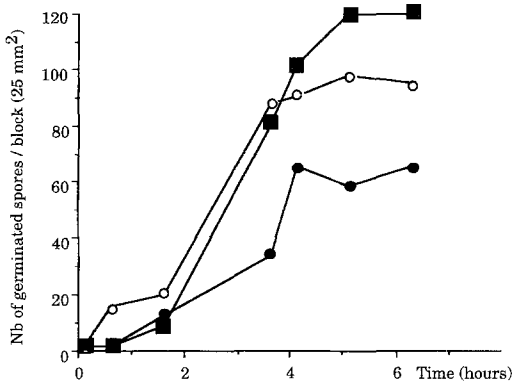

Fig. 3. Germination of *N. frontalis* MCH3 zoospores in the absence or presence of SC (10^7 cells · ml^{-1}). MCH3 zoospores were grown in media: ○ V^+; ● V^-; ▩ V^- + SC cells.

(2×10^7 cells · ml^{-1}) stimulated fungal metabolism: the concentrations of all fermentation end products were higher than in the absence of SC (Table 2). Formate and hydrogen productions were particularly stimulated, with the effect being dependent on the concentration of SC cells added (data not shown). Lactate, succinate, and VFA (especially acetate) concentrations were also increased in the presence of yeasts.

Effects of SC on fungal zoospore germination. The zoospores of *N. frontalis* MCH3 were able to germinate on the agar-cellobiose blocks in media V^+ or V^- and developed a rhizoïdal system allowing them to penetrate into the agar block. Germination was much higher in medium V^+ than in medium V^- (Fig. 3). After 2 h of incubation in medium V^-, the presence of yeasts strongly stimulated germination. After 4 h, the positive effect of SC was also observed, even in comparison with medium V^+. The experiment was

performed twice more, but only the results from one count are shown in this paper. Nevertheless, the same trend was observed in all the experiments.

SC thiamine content analysis. The initial concentration of thiamine in medium V^- was 100 ng · ml^{-1} while in $V^- + 2 \times 10^7$ SC · ml^{-1} it reached 440 ng · ml^{-1} (each value was the mean of duplicate analysis). After 6 days of incubation in cellulose medium V^-, the vitamin B1 content of the fungal supernatants was low (23.9 ng · ml^{-1} and 27.3 ng · ml^{-1} in the absence or presence respectively of SC).

Discussion

Our results show that the addition of S. cerevisiae cells to a vitamin-depleted medium stimulated the germination of *N. frontalis* zoospores and cellulose degradation. As a result, the concentration of the end products of fermentation also increased. Welch and Calza [28] have also shown that cellulase production and growth of *N. frontalis* EB 188 was stimulated by an *Aspergillus oryzae* fermentation extract. However, this probiotic was not effective when tested in vitro with the other rumen fungal species *N. frontalis* RE1, *N. patriciarum* CX, and *Piromyces communis* P [18].

The effect of live yeasts on the degradation of filter paper was particularly marked at the beginning of the period of incubation, which indicates that they stimulate the rate of cellulolysis. The extent of degradation is governed by *S. cerevisiae* viability since live yeasts had a greater effect than autoclaved yeasts. Stimulation depends on the concentration of yeast cells added. Yeasts therefore provide the fungus with vitamins, in particular thiamine, since in the presence of yeasts the vitamin deficiency induced in the medium V^- was largely made up for. The highest yeast concentration used in this study (2×10^7 cells · ml^{-1}) supplied four times as much thiamine as in the V^- medium. The great decrease in the number of live yeast cells over 48 h suggests that thiamine becomes rapidly available to the fungus. At the end of culture, the supernatants contained practically no more of this vitamin. This shows that thiamine, which occurs in concentrated amounts in yeast cells, was used by the fungus. Orpin and Greenwood [21] have reported that *N. patriciarum*, which is a rumen fungal species close to *N. frontalis*, has particularly high B-vitamin requirements for its growth and metabolism. In addition, it is known that there are great concentrations of thiamine in the cytosol of yeast cells [23].

In autoclaved yeasts, thiamine and other vitamins are partly destroyed by heat. This could explain why live yeasts have a greater effect. The implication of other mechanisms of action that require yeast viability is also possible. According to Newbold et al. [19], the positive effect of yeasts is related to their oxygen-scavenging ability; stimulation may also involve their use of soluble sugars, which can avoid cellulose breakdown inhibition by these sugars.

The effect on cellulose degradation observed in medium V^- was also evidenced when the medium was not vitamin deficient (V^+) in the early stages of growth. Hence, even in optimal growth conditions for the fungus, yeasts are able to stimulate cellulolysis at the beginning of fungal development. Such an effect on the rate of cellulolysis has also been observed during measurements in vivo of forage degradation *in sacco* by Williams et al. [29] and Chademana and Offer [4].

Nevertheless, it was in comparatively unfavorable growth conditions for *N. frontalis* that the yeasts had the greatest and most lasting effects. It seems clear that the addition of a probiotic to animal feed is mainly warranted when environmental conditions are unfavorable or harmful to the microorganism. The vitamins supplied by yeasts and their potential stimulating effect on fungal growth and activity may help to improve cellulolysis in the rumen quite markedly. Fungi live attached to plant fragments and in particular on lignocellulosic tissues that are difficult to degrade such as sclerenchyma or cribro-vascular bundles, which are colonized as soon as they enter the rumen [7]. Hence, even when the animal has an adequate food supply, the fungi may have limited access to substrates and growth factors, either because of the nature and quality of the substrates colonized, or as a result of competition with other microorganisms in the ecosystem for use of these substrates. Stimulation of fungal growth by yeasts in the rumen may thus enhance the degradation of forages rich in lignocellulose.

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