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Growth and Fermentation of an Anaerobic Rumen Fungus on Various Carbon Sources and Effect of Temperature on Development

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An anaerobic fungus (strain R1) resembling *Neocallimastix* spp. was isolated from sheep rumen. When grown on defined medium, the isolate utilized a wide range of polysaccharides and disaccharides, but of the eight monosaccharides tested only fructose, glucose, and xylose supported growth. The organism had doubling times of 5.56 h on glucose and 6.67 h on xylose, and in each case fermentation resulted in production of formate, acetate, lactate, and ethanol. During active growth, formate was a reliable indicator of fungal biomass. Growth on a medium containing glucose and xylose resulted in a doubling time of 8.70 h, but diauxic growth did not occur since both sugars were utilized simultaneously. The optimum temperature for zoospore and immature plant development was 39°C, and no development occurred below 33°C or above 41°C.

Heath et al. (13) assigned *Neocallimastix frontalis* PN1 (4) to a new family, the *Neocallimasticaceae*, within the *Spizellomycetales* of the *Chytridiomycetes*. After their classification, an anaerobic fungus isolated by Orpin and originally referred to as *N. frontalis* (26, 28, 30) was renamed *N. patriciarum* (31). To distinguish between these two species in the present text, the more recent name has been adopted when referring to the organism which was previously called *N. frontalis* and subsequently classified as *N. patriciarum*. The original nomenclature has been preserved in the Literature Cited.

Studies on rumen anaerobic fungi indicate that these organisms have a role in the initial colonization of lignocellulose in the rumen (2, 3), and several isolates have been shown to produce extracellular enzymes (cellulases and xylanases) important in the breakdown of plant polymers (24, 33, 35). Bauchop and Mountfort (4) and Mountfort et al. (25) showed that *N. frontalis* formed stable cocultures with methanogens. When the fungus was grown on cellulose, the end products (formate, acetate, lactate, ethanol, CO_2, and H_2) were typical of mixed-acid fermentation. In coculture, however, formate and H_2 were used as substrates for growth of the methanogens. *N. patriciarum* also has mixed-acid fermentation, but it differs quantitatively from that of *N. frontalis* in that only trace amounts of formate and pyruvate are produced (27).

The zoospores, rhizoids, and sporangia of *N. patriciarum* contain membrane-bound hydrogenosomes (36). Glycolysis via the Embden-Meyerhof-Parnas pathway, which results in production of acetate, lactate, ethanol, H_2, and CO_2, was proposed as the sole mechanism of glucose metabolism in *N. patriciarum*. The presence of the Embden-Meyerhof-Parnas pathway in *N. patriciarum*, has been confirmed by Yarlett et al. in a study of the enzymology of the carbon and electron flow pathways in this organism (36).

We have isolated a *Neocallimastix*-like organism from sheep rumen (20) which differs morphologically from *N. frontalis* and *N. patriciarum* (S. E. Lowe, G. G. Griffiths, A. Milne, M. K. Theodorou, and A. P. J. Trinci, submitted for publication). The isolate was studied to determine whether its growth and metabolism differed from those of the two described species of *Neocallimastix*. An investigation of the effects of temperature on growth and development of the fungus was also made.

MATERIALS AND METHODS

Organism. The rumen fungus was isolated from sheep by Lowe et al. (20) at The Animal and Grassland Research Institute. To ensure that the culture consisted of only a single fungus, a suspension of zoospores was used to inoculate roll tubes (16) containing molten defined medium B agar (20) modified as described below. The tubes were incubated at 39°C for 2 days, and after examination with a Leitz Diavert inverted microscope, a single colony was selected and cultured in an anaerobic tube (18 by 142 mm; Bellco Glass, Inc., Vineland, N.J.) containing 10 ml of liquid defined medium B. All subsequent cultures were derived from this isolate. In this report, the *Neocallimastix*-like organism isolated at The Animal and Grassland Research Institute is referred to as strain R1.

Culture conditions. The culture techniques used were as described by Hungate (16), Bryant (7), and Miller and Wolin (22). All chemicals were obtained from Sigma Chemical Co., Poole, Dorset, United Kingdom, unless stated otherwise. Medium A contained rumen fluid and was prepared as described previously (20) except that yeast extract, peptone, and antibiotics were omitted. Defined medium B was the medium B described by Lowe et al. (20) except that coenzyme M, peptone, yeast extract, and antibiotics were omitted. Unless stated otherwise, medium A and defined medium B contained 25 mM glucose as the carbon source. All cultures were grown in anaerobic tubes under a gas phase of 100% CO_2, at 39°C unless stated otherwise, and without agitation.

Stock cultures were maintained on 0.1 g of wheat straw (milled to pass through a 1-mm dry mesh screen) in 10 ml of defined medium B. Cultures were inoculated with 1 ml of culture supernatant (containing zoospores), and subcultures were made every 7 days. Experimental cultures were grown in 10 ml of medium A or 10 ml of defined medium B which was inoculated with 1 ml of zoospore suspension obtained

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from cultures grown for 3 days on defined medium B; these were in the late exponential phase of growth and typically contained 10^3 zoospores ml^{-1}. For fermentation tests, tubes of defined medium B containing 0.4% (wt/vol) carbon source were inoculated and incubated as described above, and growth was determined visually.

Growth of fungus. The biomass produced in liquid medium was determined from dry weight measurements. Whatman no. 1 filter papers (5.5-cm diameter) were washed with 100 ml of distilled water and dried to constant weight. Cultures were harvested on these filter papers, washed with 100 ml of distilled water, and dried at 80°C to constant weight. Samples of culture supernatants were stored at −20°C for subsequent analyses of reducing sugars and fermentation products. Molar growth yields (grams of biomass dry weight produced per mole of substrate consumed) were determined from the fungal dry weight formed in batch cultures. Values are the means of at least four replicates and have been corrected for control fermentations incubated without added substrate.

Effect of temperature on growth. The effect of temperature on growth of R1 was determined by incubating roll tube cultures at different temperatures for various lengths of time. Roll tubes were prepared by adding 1 ml of a zoospore suspension to 2.5 ml of molten, defined medium B containing 1.8% (wt/vol) agar (Oxoid Ltd., London, United Kingdom). By inoculating roll tubes with a zoospore suspension and immediately incubating them at different temperatures, the effect of temperature on zoospore development was determined. To study the effect of temperature on plant development, roll tubes were inoculated and incubated at 39°C for 10 h to allow the zoospores to develop into immature plants; these were then incubated at different temperatures. An inverted microscope was used at regular intervals throughout the experiments to determine the maximum number of zoospores or immature plants which completed their life cycle (as assessed by liberation of zoospores from mature zoosporangia). Measurements were made with a traveling micrometer eyepiece (C. Baker, London, United Kingdom).

End product analysis. Formate was determined by the method of Hopner and Knappe (14). Acetate and ethanol were measured with a Pye Unicam series 104 chromatograph as described by Gottschal and Morris (9). The column (6 ft [ca. 1.83 m; length] by 4 mm [internal diameter]) was packed with Chromosorb 101 (Phase Separations Ltd.). The column oven temperature was 195°C, and the detector temperature was 250°C. The carrier gas was nitrogen at a flow rate of 20 ml min^{-1}. The gas-liquid chromatograph was linked to a Hewlett-Packard 3390 integrator calibrated with internal (6 mM pentane-3-one [Aldrich Chemical Co., Inc., Gillingham, Dorset, United Kingdom] in 20% (vol/vol) phosphoric acid) and external (acetate, ethanol, butanol, and butyrate) standards.

Lactate was determined by the method of Brooks and Moore (6) with a Pye Unicam series 104 chromatograph. The column (6 ft [length] by 4 mm [internal diameter]) was packed with 10% diethylene glycol adipate–2% phosphoric acid-coated packing material (Pye Unicam). The detector temperature was 300°C, and the oven temperature was 146°C. The carrier gas was nitrogen with a flow rate of 30 ml min^{-1}. Quantitation of lactate was made directly from calculation of peak area on a chart output. Calibration of the assay was performed by analyzing a series of standards. Samples for lactate analysis were derivatized by adding 0.25 ml of boron trifluoride in methanol (14%) plus 25 μl of concentrated HCl to 0.25 ml of sample. The tubes were incubated overnight at 37°C, 0.25 ml of chloroform was added, and the contents were mixed; after the tube was allowed to stand, 2 μl was withdrawn from the lower layer and loaded onto the column. L-(+)-Lactate was determined by the method of Gutmann and Wahlefeld (12).

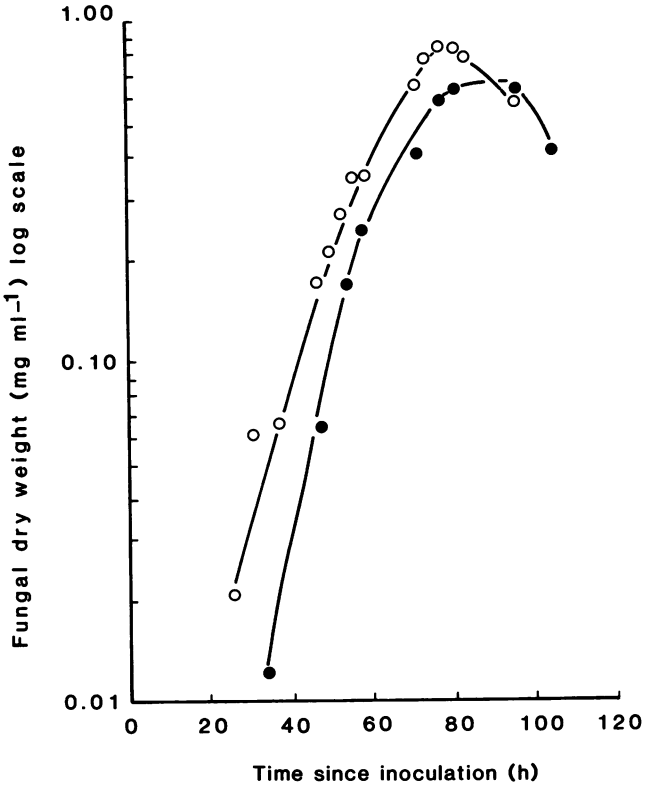

FIG. 1. Comparison of growth of the R1 strain in medium A (containing rumen fluid) and defined medium B; the carbon source in both media was 25 mM glucose. The contents of three tubes, each containing 10 ml of medium and inoculated with a 1-ml zoospore suspension, were pooled for each dry weight determination. Symbols: ○, fungal dry weight on medium A; ●, fungal dry weight on defined medium B.

The culture supernatant was analyzed for reducing sugar with dinitrosalicylic acid (21); the ratio of sample to reagent was 1:2, the tubes were placed in boiling water for 15 min before being cooled in iced water, and A_{640}s were read. Glucose was determined with glucose oxidase-peroxidase with 2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate as the chromogen (5).

Carbon, hydrogen, and nitrogen analyses were carried out on cultures grown on defined medium B; the biomass was harvested, freeze-dried, and analyzed with a Carlo Erba C, H, and N analyzer.

RESULTS

Growth on glucose in medium A and defined medium B. Growth of R1 on a medium containing rumen fluid (medium A) and on defined medium B is shown in Fig. 1. The organism had a doubling time of 7.43 h on medium A, and the glucose was exhausted by 81 h after inoculation; this coincided with maximum biomass formation (0.84 mg ml^{-1}). The organism had a doubling time of 5.56 h on defined medium B and entered the stationary phase 81 h after inoculation;

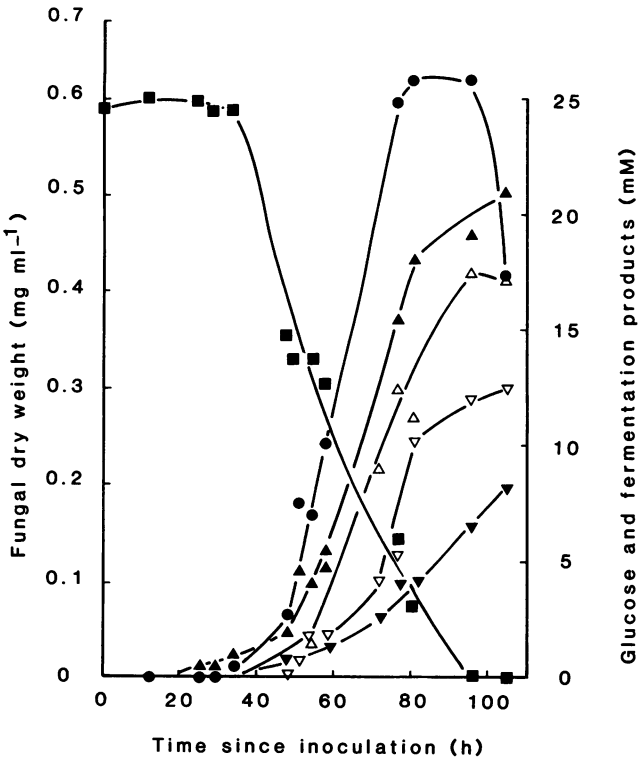

FIG. 2. Growth of the R1 strain in defined medium B containing 25 mM glucose. See the legend to Fig. 1 for experimental procedures and dry weight determinations. All other analyses were made with culture supernatants from individual tubes (i.e., three replicate tubes per determination). Symbols: ●, fungal dry weight; ■, glucose; ▲, formate; △, acetate; ▽, D-(−)-lactate; ▼, ethanol.

glucose was exhausted 96 h after inoculation. The maximum dry weight obtained on this medium was 0.62 mg ml^{-1}. Autolysis occurred in both types of culture media after glucose exhaustion.

Growth on a variety of carbon sources. The ability of R1 to use a range of carbon sources in defined medium B was investigated. Growth occurred on inulin, pullulan, starch, and xylan but not on carboxymethyl cellulose (CMC) or pectin. The organism also grew on raffinose and was able to use all of the disaccharides tested (cellobiose, lactose, maltose, and sucrose), although growth was poor on lactose. Molar growth yields of 69.1, 73.9, and 75.0 were obtained for cellobiose, maltose, and sucrose, respectively. Of the eight monosaccharides tested, only fructose, glucose, and xylose supported growth, and the molar growth yields on these sugars were 34.0, 29.2, and 29.2, respectively. The monosaccharides which did not support growth included arabinose, galactose, mannose, rhamnose, and ribose.

Fermentation end products. Growth of the R1 strain on defined medium B containing glucose (Fig. 2) or xylose (Fig. 3) was studied. The fermentation end products from cultures grown on glucose were formate, acetate, D-(−)-lactate, and ethanol (Fig. 2; see also Table 1). L-(+)-Lactate was not detected with lactate dehydrogenase. Formate was the major end product in these cultures, reaching 21.0 mM by 110 h after inoculation. During the same period, acetate increased to 17.0 mM, and lactate and ethanol were present at 12.4 and 8.2 mM, respectively. The organism entered a short stationary phase 81 h after inoculation, before the exhaustion of glucose, and autolysis occurred rapidly after the glucose was exhausted. During exponential growth of R1 on defined medium B, there was a significant correlation ($r = 0.98$; $y = 0.039x + 0.004$, where y = formate concentration and x = fungal dry weight) between formate and fungal dry weight concentrations. During the stationary and autolytic phases, the correlation no longer applied, as dry weight decreased while formate production continued (Fig. 2). Elemental analysis of the biomass from a culture grown for 83 h on defined medium B (late exponential phase of growth) showed that it consisted of 42.7% carbon, 6.8% hydrogen, and 6.4% nitrogen.

Growth and product formation of the R1 strain on defined medium B containing xylose are shown in Fig. 3 (see also Table 1). These cultures had a doubling time of 6.67 h and achieved a biomass concentration of 0.55 mg ml^{-1} after 77 h of incubation; the maximum biomass produced on glucose during the same incubation period was 0.62 mg ml^{-1}. As with cultures grown on glucose, autolysis occurred toward the end of fermentation. The major end products were acetate (21.4 mM), formate (16.4 mM), D-(−)-lactate (7.3 mM), and ethanol (5.0 mM).

A carbon balance was constructed to compare the growth of R1 on defined medium B containing glucose or xylose (Table 1). Values for CO_2 and H_2 concentrations were estimated from established stoichiometry (Table 1, footnotes *c* and *d*). For both substrates, fermentation resulted in production of equivalent amounts of formate and ethanol (Table 1). Cultures grown on xylose produced more acetate and less D-(−)-lactate than did cultures grown on glucose. Calculated values for CO_2 and H_2 were also higher for

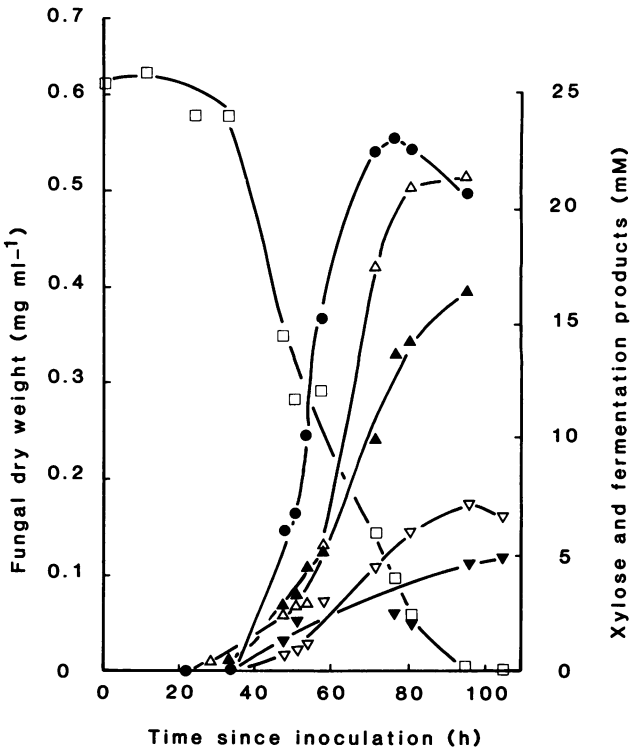

FIG. 3. Growth of the R1 strain in defined medium B containing 25 mM xylose. See the legends to Fig. 1 and 2 for experimental procedures and dry weight determinations. Symbols: ●, fungal dry weight; □, xylose; △, acetate; ▲, formate; ▽, D-(−)-lactate; ▼, ethanol.

TABLE 1. Carbon balances of the R1 strain grown on glucose or xylose in defined medium B

Substance measured	Fermentation products on glucose[a]			Fermentation products on xylose[b]		
	mol/100 mol of glucose fermented	mol of carbon/100 mol of glucose fermented	mol of carbon/100 mol of carbon fermented[c]	mol/100 mol of xylose fermented	mol of carbon/100 mol of xylose fermented	mol of carbon/100 mol of carbon fermented[c]
Carbon in cells	88.3	88.3	14.7	84.5	84.5	16.9
Formate	76.8	76.8	12.8	61.4	61.4	12.3
Acetate	69.6	139.2	23.2	90.9	181.8	36.4
D-(−)-Lactate	47.6	142.8	23.8	26.4	79.2	15.8
Ethanol	26.4	52.8	8.8	21.0	42.0	8.4
CO_2[d]	19.2	19.2	3.2	50.5	50.5	10.1
H_2[e]	62.4		10.4	120.4		24.1

[a] A 25 mM glucose concentration was used during fermentation.
[b] A 23.1 mM xylose concentration was used during fermentation.
[c] These values are normalized to 100 mol of carbon fermented to allow direct comparison between glucose and xylose data.
[d] CO_2 = (acetate + ethanol) − formate.
[e] H_2 = ([acetate + ethanol] × 2) − (formate + [ethanol × 2]).

cultures grown on xylose than for cultures grown on glucose. Carbon recoveries of 86.5% (for cultures grown on glucose) and 100.1% (for cultures grown on xylose) showed that the major fermentation end products had been detected.

Batch culture with glucose and xylose. Growth of R1 was monitored in defined medium B containing both xylose (10 mM) and glucose (10 mM). After a lag of 30 h, fungal dry weight increased (Fig. 4), and 70 h after inoculation the dry weight attained 0.66 mg ml^{-1} (the maximum value observed). The doubling time of R1 during the exponential phase of growth was 8.70 h, and diauxic growth was not observed since glucose and xylose were used simultaneously (although glucose was exhausted first). Like fermentations on glucose or xylose, exhaustion of the monosaccharide mixture was followed by autolysis of fungal biomass.

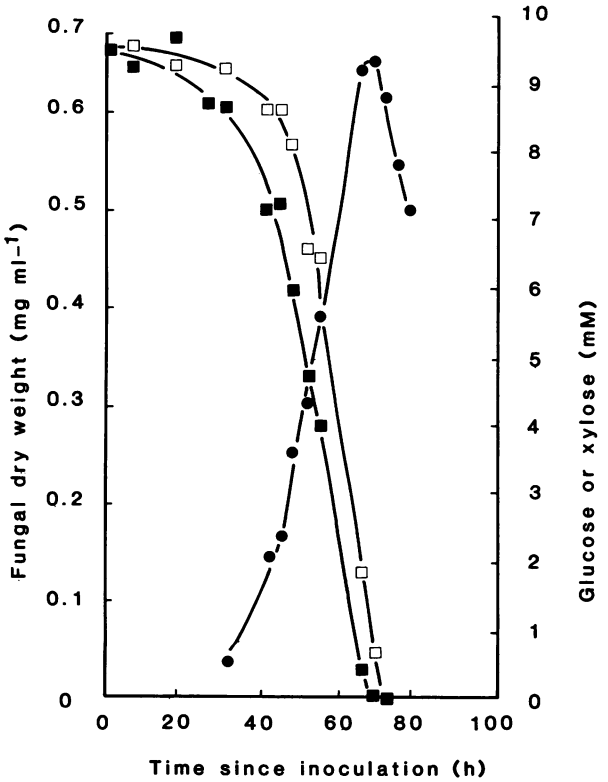

FIG. 4. Growth of the R1 strain in defined medium B containing 10 mM glucose and 10 mM xylose. See the legends to Fig. 1 and 2 for experimental procedures and dry weight determinations. Symbols: ●, fungal dry weight; ■, glucose; □, xylose.

Effect of temperature on growth. The life cycle of R1 consists of two phases in which the zoospores alternate with the vegetative phase, which consists of a rhizomycelium supporting a single zoosporangium (the plant). The effect of temperature on the growth and development of both of these stages was investigated.

Zoospores did not encyst or germinate at temperatures of 30°C or lower or above 41°C, and the optimum temperature for zoospore development was 39°C (Fig. 5a). After incubation of zoospores at 39°C for 10 h, zoosporangia of the immature plants which developed had a mean diameter of 28 μm. After 40 h of incubation at 41°C, 96.0% of these zoosporangia had matured and liberated their zoospores, a result which was similar to that observed for cultures incubated continuously at 39°C (Fig. 5b). Development of plants at lower temperatures was considerably slower. After 20 h at 36°C, there was no increase in the mean size (24.2 μm) of immature zoosporangia, but 61 h later 71.3% of the zoosporangia had matured and produced zoospores. Incubation of immature plants at 33°C severely affected their subsequent development; only 22.3% had completed their life cycle after 83 h at 33°C. The zoosporangia of immature plants incubated at 30°C increased in size to 58.0 μm after 33 h but did not develop further with continued incubation. Immature plants transferred to 20 or 25°C did not increase in size or liberate zoospores.

DISCUSSION

The R1 strain was able to grow on medium A (containing rumen fluid) and defined medium B, and similar growth curves were obtained on both media (Fig. 1). However, R1 had a longer doubling time on medium A than on defined medium B, possibly because of the the presence of inhibitory compounds in the rumen fluid, and produced a higher yield of biomass on medium A than on defined medium B, suggesting that rumen fluid contains carbon compounds which can be used for growth.

When grown on defined medium B, the R1 strain was able to utilize all of the polysaccharides tested except pectin and CMC; similar results have been reported for *N. patriciarum* (30). Although production of CMCase by R1 (S. E. Lowe, M. K. Theodorou, and A. P. J. Trinci, submitted for publi-

cation) and cellulases by *N. patriciarum* (27) has been demonstrated, neither organism utilizes CMC as a carbon source, possibly because methylation of CMC prevents its uptake. The disaccharides which supported growth of the R1 strain also support the growth of *N. frontalis* (23) and *N. patriciarum* (28, 30). Growth of R1 on monosaccharides was more restricted, and of the eight tested only fructose, glucose, and xylose supported growth. Similar results have been reported for *N. frontalis* (23) and *N. patriciarum* (28, 30), although the latter organism can also utilize galactose.

Growth on a mixture of glucose and xylose in defined medium B produced a doubling time longer than that observed on either of the individual sugars, suggesting that the substrates were in some way antagonistic. However, a full explanation warrants an analysis of the sugar uptake and fermentation pathways. Diauxic growth (32) was not apparent, and the sugars were used simultaneously. These results differ from those of Mountfort and Asher (23), who found that *N. frontalis* did not start to utilize xylose until almost all of the glucose had been fermented.

The carbon/hydrogen/nitrogen ratio for the biomass of R1 are typical of values reported for other fungi, for example, *Aspergillus nidulans* (1) and *Paecilomyces fumosoroseus* (17). The end products formed during fermentation of glucose or xylose were formate, acetate, lactate, and ethanol (assays for CO_2 and H_2 were not made during these experiments, but our unpublished data showed that significant quantities of hydrogen were produced). *N. frontalis* (4) produces a similar spectrum of fermentation product when grown on cellulose. The fermentation profile of R1 on glucose is typical of the mixed-acid fermentation observed with bacteria belonging to the *Enterobacteriaceae* (10). Under anaerobic conditions, *Escherichia coli* produces end products similar to those of R1; however, formate does not attain high concentrations in *E. coli* since it is metabolized to H_2 and CO_2 via the formate-hydrogen lyase pathway (11). The observation that high concentrations of formate are present in culture supernatants of R1 and persist during the stationary phase suggests that this organism does not possess formate-hydrogen lyase activity.

Other workers have used H_2 formation as an indicator of growth of anaerobic fungi (23, 24, 33). With the R1 strain, formate was also a good indicator of fungal biomass, but only during exponential growth since formate production continued, even during autolysis, after the growth of R1 had ceased (Fig. 2 and 3).

Fermentation of xylose resulted in the same end products as observed for growth on glucose, although in the former the major end product was acetate while in the latter it was formate (Fig. 2 and 3; Table 1). Although an alteration in the ratio of end products occurred, a 2-3 cleavage of xylose was not implicated since the increase in acetate production of xylose occurred without a concomitant increase in lactate production. Other fungi which are able to ferment xylose, for example, *Saccharomyces* sp. and *Candida* sp. (8), form xylitol as a major end product. The presence of this product was not implicated with the R1 strain since the carbon balance obtained from growth on xylose indicated that the main end products had been identified (Table 1). A basic difference seems to exist between procaryotes and eucaryotes in the initial metabolism of xylose. Bacteria generally use an isomerase to convert xylose to xylulose, whereas yeasts and fungi carry out the same conversion through a two-step reduction and oxidation (18). With the R1 strain, however, the former system was implicated since xylitol was not produced as an end product. The absence of this compound clearly indicates that R1 does not encounter the redox problems experienced by some yeasts (34).

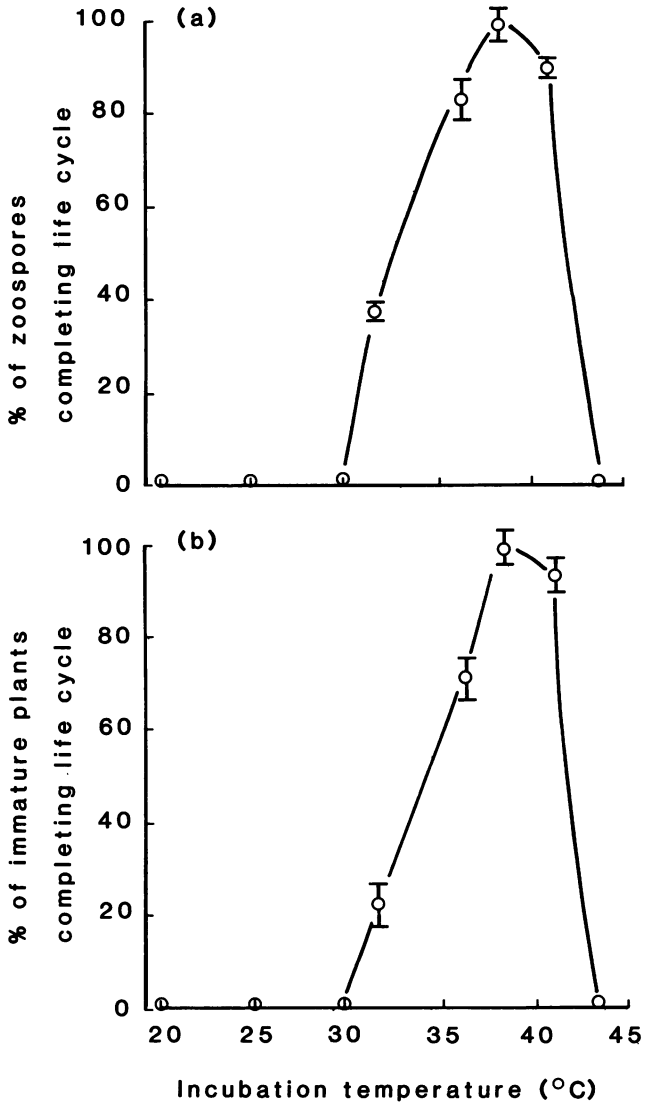

FIG. 5. Effect of temperature on the growth and development of zoospores and immature plants of the R1 strain. Roll tubes containing 2.5 ml of molten, defined medium B agar were inoculated with 1 ml of zoospore suspension. Inoculated tubes were incubated either (a) immediately at different temperatures or (b) at 39°C for 10 h before incubation at different temperatures. Microscopic observations were made throughout the experiment to determine the percentage of zoospores or immature plants completing the life cycle. Bars indicate the standard errors of the mean.

Rumen temperature is usually between 39 and 40.5°C but can fall to 37°C after feed ingestion and rise to 41°C during active fermentation (15). The effect of temperature on the growth and development of zoospores and immature plants of the R1 strain showed that the optimum temperature for growth was 39°C, although significant growth occurred between 36 and 41°C. These results are similar to those obtained by Orpin (26) and Orpin and Greenwood (29), who found that the optimum temperature for growth of *N. patriciarum* was 40°C, although some growth occurred at 45°C. Some fungi of the *Chytridiomycetes*, for example, *Blastocladiella emersonii*, produce resistant spores which

can survive adverse conditions such as low temperatures (19); however, these resting stages were not observed in cultures of R1.

The present results demonstrate that the R1 strain undergoes mixed-acid fermentation and can utilize a wide range of polysaccharides and disaccharides but comparatively few monosaccharides. Physiological and morphological data indicate that R1 is a member of the genus *Neocallimastix* but is probably not *N. frontalis* or *N. patriciarum* (this paper; Lowe et al., submitted).

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