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GENUS *HELICOBACTER*: AN EXPANDING TAXONOMIC GROUP, WITH ZOONOTIC CHARACTERISTICS

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MINI REVIEW

ABSTRACT

In the last years, the genus *Helicobacter* experienced a great expansion in the number of recognized species and in the spectrum of animal hosts where the new species can be found. Some species are associated with the gastric mucosa and others with the intestinal epithelium. Some of them are considered zoonotic agents and able to infect humans and animals simultaneously. This paper presents a review of this genus, with special reference to *Helicobacter* species with zoonotical features.

Key words: *Helicobacter*, zoonosis, gastritis, epidemiology, taxonomy.

INTRODUCTION

With the isolation and description of *Helicobacter pylori* (*H. pylori*) in the early 1980s (41), the relationships between gastric bacteriology and pathological processes grew in prominence, confirming the infectious theory of gastric ulcers proposed by Lebert and Cohn (67) more than 100 years ago. The isolation of other species of the genus from the gastric mucosa of mammalians (23) confirmed the description of bacteria with similar characteristics made at end of the last century (40).

The association between presence of *H. pylori* and development of gastritis and peptic ulcer in humans lead to propose a new approach to the ethiopathogenesis of these diseases. However, this approach was initially controversial and surrounded by skepticism, because the acceptance of the ethiological role of an unknown bacterium represented the modification of the traditional conception of the pathophysiology of these clinical entities (25) and of the accumulated knowledge on the ecology and distribution of the gastrointestinal microbiota (8).

Bearing in mind the high interest of clinicians, bacteriologists, pathologists, veterinarians and epidemiologists on this bacterial group, as well as the great expansion experienced by the genus *Helicobacter* in the number of described species, much of them with capacity to infect humans and animals simultaneously, and the host spectra where they are found, we present here a review of this genus, with special reference to *Helicobacter* species with zoonotical features.

TAXONOMICAL POSITION

The genus *Helicobacter* (from greek: *helix*, helicoidal; *bacter*: bacteria) belongs to the superfamily V of the class *Proteobacteria*, division

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Gracillicutes and comprises curved or helicoidal Gram negative rods that are 0.3 to 1 μm wide and 1.5 to 5 μm long. In old cultures, they may form spherical or coccoid bodies and are motiles by lophotric or monotric sheated flagella. They are microaerophilic with a respiratory type of metabolism and unable to oxidize or ferment carbohydrates. Their optimal growth occurs usually at 37°C. Due to the morphological similarities, they were initially considered members of the genus *Campylobacter*. However, molecular taxonomic studies using rRNA sequencing and DNA-rRNA hybridization, as well as their ultrastructural characteristics, fatty acids and respiratory menaquinones composition and enzymatic capacities, showed that their phylogenetic position was different to that of *Campylobacter* species, thus emerging the genus *Helicobacter* in 1989 (23,33,70,71). In the last years, new species have been incorporated to the genus that currently comprises 19 species: *H. pylori*, *H. cinaedi*, *H. fennelliae*, *H. acynonix*, *H. bilis*, *H. bizzozeroni*, *H. canis*, *H. colecystus*, *H. felis*, *H. hepaticus*, *H. muridarum*, *H. mustelae*, *H. nemestrinae*, *H. pametensis*, *H. pullorum*, *H. trogontum*, *H. heilmannii* (formerly *Gastrospirillum hominis*), *Gastrospirillum suis* and *Flexispira rappini*. The last two species conserve their original nomenclature, despite taxonomic studies at molecular level allowed their adscription to the genus *Helicobacter*.

From the 19 species described, only one (*H. fenneliae*) has been isolated exclusively from human beings. Eleven species were isolated from different mamammalian species and seven species are able to infect both humans and animals.

With the exception of *H. heilmannii* and *G. suis*, all these bacteria can be cultured *in vitro*. According to their habitat or to the initial colonization site, they were divided in two groups (57):

1 - species that colonize the gastric mucosa and
2 - species having the intestinal mucosa as their preferential habitat.

ZOONOTIC *HELICOBACTER* SPECIES THAT COLONIZE THE GASTRIC MUCOSA

Helicobacter pylori. In 1982, Marshal and Warren, in Australia, isolated for the first time from human gastric mucosa a Gram negative curved rod that was originally denominated "GCLO" (gastric *Campylobacter* like organism) and later *Campylobacter pyloridis*, *C. pyloricus* and *C. pylori*. In 1989, with the creation of the genus *Helicobacter*, this bacterium was included in this taxonomic group as *H. pylori* (14). Since the first isolations, the presence of *H. pylori* in the gastric mucosa was correlated with the development of gastritis and gastric and duodenal ulcers. Initially the association of *H. pylori* with these pathologies was controversial but at present great amount of information substantiate the pathogenical capabilities of *H. pylori* (1,2,35,57,61).

Many of the epidemiological aspects of the infection by *H. pylori* were clearly established. Despite being isolated only from human beings, the existence of animal reservoirs was suspected (24,65,68). Recently, it was demonstrated that in natural conditions *H. pylori* can be isolated from primates, pigs and cats (9,10,13,16,27,30,49). These findings and the isolation of the bacterium from saliva and feces of human beings (46), water (34), vegetables (31) and flies (26) allowed to postulate that besides the iatrogenic transmission (24), oral-oral and fecal-oral transmission also occurs. Animals identified as reservoirs may have an important role in the zoonotical and environmental transmission of *H. pylori* (16).

Helicobacter felis. This species was first isolated in 1988 from the gastric epithelium of cats (37). This bacterium presents periplasmic fibres as their distinctive ultrastructural morphological characteristic (72). Some studies (12,17) have also shown the presence of *H. felis* in dogs and cheetahs and others have demonstrated that this bacterium is not excreted with the feces, suggesting an oral-oral transmission route (36,38).

Through 16S rRNA sequencing, it was demonstrated that *H. felis* is taxonomically and phylogenetically very close to *H. Pylori* (50). This species is also found in the gastric epithelium of patients with gastritis that showed seroconversion with a stronger immunological response against *H. felis* than *H. pylori* (37,48,72). These observations suggest that *H. felis* is an agent with zoonotical dissemination (48).

Helicobacter heilmannii. The systematic search for *H. pylori* in gastric biopses highlighted the presence of *Gastrospirillum hominis*, a spiral bacterium that presents genotypical characteristics also present in members of the *Helicobacter* genus. *G. hominis* phylogenetically very close to *H. felis* and currently denominated *H. heilmannii* (43,59,60). This microorganism is not culturable *in vitro* but can be maintained *in vivo* by intragastric inoculation of mouse with contaminated gastric mucus or

epithelium (7). *H. heilmannii* was been isolated from dogs, cats, cheetahs and primates (12,22,27,32,48,72) and the transmission resembles that of *H. felis* and seems to be oral-oral since experimentally infected mice are unable to transmit the infection. Furthermore, the infection occurs when the animals are inocculated with contaminated mucus or gastric biopses (7) but not when inocculated with naturally contaminated cat feces (36). Despite the low frequency (0.1%) of human infection due to *H. heilmannii*, this bacterium seems to be cosmopolitan, associated to chronic active gastritis that improves with the elimination of the bacterial agent by means of an adequate antimicrobial therapy (29,42,64). *H. heilmannii* is also observed in healthy individuals (42) and the zoonotical transmission was proposed mainly based on the high frequency of infection observed in domestic animals and on the strong epidemiological relationships between these animals and the patients (29,42,64). In Germany, 70.3% of the patients referred to having had contact with domestic animals (64).

ZOONOTIC *HELICOBACTER* SPECIES THAT COLONIZE THE INTESTINAL MUCOSA

Helicobacter canis. This species seems to recognize the intestinal tract of dogs as its natural habitat without a significant association with canine diarrhea (63). In humans, *H. canis* was isolated along with rotavirus from a boy with diarrhea by Burnens et al., who concluded stated that dogs could be an infectious source for humans (4).

Helicobacter cinaedi. This bacterium, formerly known as *C. cinaedi*, was isolated in 1984 (66), being later transferred to the genus *Helicobacter* (70). *H. cineaedi* can cause enteritis, proctocolitis, bacteremia and asymptomatic infection in homosexual men and AIDS patients (15,52,55,66). *H. cinaedi* was also isolated from blood and feces of immunocompetent children and adult females and from blood and cerebrospinal fluid of a newborn (47,69). The hamster was recognized as a natural reservoir (21) and may be a source of contamination for human beings as indirectly demonstrated in a case of neonatal infection (47) where the mother had been in contact with this type of animal along the first six months of pregnancy.

Helicobacter pullorum. This species, characterized by the presence of nacked flagella, was isolated from the caecal content of healthy broilers indicating that the intestinal tract of poultry cold be the natural habitat. However, *H. pullorum* was also isolated from the liver of hens presenting lesions similar to those observed in cases of hepatic vibriosis. *H. pullorom* was also isolated from immunocompetent children and adults presenting gastroenteritis and from a patient with AIDS (3,61). The transmission pathways of *H. pullorum* are not known but Stanley et al. provided evidences of a possible zoonotic dissemination (3).

Flexispira rappini. Taxonomic studies at molecular level have demonstrated that this species belongs to the genus *Helicobacter* being phylogenetically very close to *H. muridarum* (39,50,70). It was isolated from ovine abortion and from the intestinal tract of mice, suggesting to be part of their intestinal microbiota (5,56). *F. rappini* was also isolated from human patients with chronic gastroenteritis as well as from fecal material of dogs (54,58). Schauer et al. stated that mice could be the infectious source for humans and other animal species (56).

OTHER *HELICOBACTER* SPECIES

Helicobacter fenneliae. This bacterium, formerly *C. fenneliae*, has been isolated from proctitis, proctocolitis and bacteremia in homosexual, bisexual and AIDS patients (15,66,70). The natural reservoir remains still unknown. However, there is some evidence that *H. fenneliae* could be associated to the intestinal tract of dogs (4).

Helicobacter mustelae. This bacterium was isolated in 1989 from the gastric epithelium of ferrets and it can produce gastritis and ulceration of the mucosa (23,50).

Helicobacter nemestrinae. This bacterium was isolated in 1991 from the gastric mucosa of primates (*Macacca nemestrina*) without gastric pathological alterations (58).

Helicobacter muridarum. Probably isolated in 1983, this bacterium was described and included in the genus *Helicobacter* in 1992. It colonizes the gastric epithelium of rats and mice (39).

Helicobacter acinonyx. This bacterium was isolated from the gastric mucosa of cheetahs with gastritis and included in the genus *Helicobacter* in 1993 (11,12).

Helicobacter bilis. Isolated from the intestine, bile and liver of rats where this bacterium can cause hepatitis (19). It is possible that *H. bilis* is transmitted to fowls and can cause liver lesions similar to those observed in rats (61).

Helicobacter bizzozeroni. Described in 1996, this bacterium was isolated from gastric biopses obtained from the body and antral regions of the stomach of

dogs, also associated to the mucus and gastric glands and inside the parietal cells as well (28).

Helicobacter cholecystus. This species, phylogenetically related to *H. pametensis*, was isolated from the gall blader of hamsters with colangiofibrosis and centrolobular pancreatitis. Despite having not been isolated from fecal material, an association between this bacterium and the intestinal mucosa as reported for *H. hepaticus*, *H. bilis* and *H. pullorum* was proposed (20).

Helicobacter hepaticus. This microorganism is recognized as agent of chronic hepatitis and hepatocellular neoplasia in laboratory rats. In these animals, *H. hepaticus* seems to be part of the intestinal microbiota. Experimental evidences suggest that the hepatic lesions are associated to the production of a cytotoxin (18,53).

Helicobacter pametensis. This bacterium, isolated from the intestinal content from wild birds, particularlly seagulls, and from pigs, was described and included in the genus *Helicobacter* in 1994 (6).

Helicobacter trogontum. This species, described in 1996, was isolated from the colonic mucosa of laboratory rats. Phylogenetically this species is very close to *H. hepaticus* (44).

Gastrospirillum suis. This is a non-culturable bacteria, first observed in the gastric mucosa of pigs with ulcer (45). Later, rRNA sequencing studies showed that *G. hominis* is very close to *H. heilmannii* and the change of its nomenclature to *H. heilmannii* type 1 was proposed (51). However, it is possible that these strains correspond to two very close taxa (59).

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RESUMO

Gênero *Helicobacter*: um grupo taxonômico em expansão, com características zoonóticas

O gênero *Helicobacter* é um grupo taxonômico que tem experimentado uma notável expansão, tanto do ponto de vista das espécies que fazem parte deste gênero como dos animais reservatórios nos quais estas espécies têm sido encontradas. Algumas destas espécies podem colonizar a mucosa gástrica e outras, a mucosa intestinal. Muitas têm sido consideradas como agentes zoonóticos, podendo infectar humanos e animais simultaneamente. Este trabalho apresenta uma revisão do gênero *Helicobacter*, com especial referência às espécies com características zoonóticas.

Palavras-chave: *Helicobacter*, zoonoses, gastrite, epidemiologia, taxonomia

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GROWTH OF *SPIRULINA MAXIMA* USING RICE STRAW ASHES AS CULTURE MEDIUM

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ABSTRACT

Comparative studies were carried out on the growth of *Spirulina maxima* in the standard bicarbonate medium (control) and in an alternative medium consisting of rice straw ashes (semi-natural) in periods under continuous light and intermittent light (light/dark cycles of 12/12 h), with light intensity of 2.4 Klux. The experiment was carried out under laboratory conditions, in a photobioreactor with 2.2 liters of culture, temperature of 32 ± 1°C and air agitation for 192 hours. The cultures under continuous light presented higher results in productivity, growth rate and specific growth rate. The maximum specific growth rates were between 0.541 and 0.523 (d^{-1}) for the cultures under continuous light and between 0.469 and 0.415 (d^{-1}) for the ones under intermittent light. The control medium presented the best growth result in both culture systems. The protein content of *Spirulina maxima* biomasses varied between 45 and 68% in dry weight.

Key words: *Spirulina maxima*, culture medium, blue-green alga

INTRODUCTION

Several factors have contributed for the worldwide food crisis: the great population increase, absence or inefficiency of agricultural policies and climatic problems, among others. The production of food from non-conventional sources presents an alternative to the decreasing food supply (5). The microalgae constitute an extremely efficient system on protein synthesis. Therefore, their culture to obtain proteins for human and animal consumption has been studied in large scale in recent years (3). With this purpose, *Spirulina* has been considered the favorite microalga due to its nutritional value, good digestibility, easy cultivation, and a long history of consumption by the Aztecs and the Kanembous (5). The microalgae also play an important role as purifying agents for urban and industrial wastewater. In the case of industrial wastewater, they act as decontaminating and disintoxicating agents, removing toxic ions that are harmful to the macro and microscopic inhabitants of the natural waters to which they would flow (9). Due to its high cost, the production of Spirulina in large scale is more difficult than the production of other traditional protein sources, when considering only the nutritional aspects. One of the factors that contributes to the rise of the production cost is the composition of the culture medium currently used. The utilization of residues as nutrient sources in the cultivation of *Spirulina* or other microorganisms may solve two important problems: the reduction of costs in biomass production and the decrease of environmental problems originated by the disposal of such residues in soil or water. Several studies have sought alternative media for *Spirulina* culture, such as the

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solution of rice straw ashes containing 100 g ashes/l water with the following substances (in mg/ml): Copper 1.15; Zinc 0.20; Lead 0.20; Magnesium 11.00; Manganese 0.10; Iron 6.00; Potassium 5,209.00; Sodium 2,460.00; Phosphorus 2.40 and Nitrogen 49.00 (13). This study aims at evaluating the growth of *Spirulina maxima* in rice straw ashes under laboratory condition.

MATERIALS AND METHODS

The study utilized *Spirulina maxima*, 4 Mx, from the culture collection of the "Centro di Studio dei Microrganismi Autotrofi del CNR" - University of Firenze, Italy. The semi-natural culture medium was developed from rice straw ashes, dried at 105°C till constant weight. The ashes were ground together with distilled water (75 g ashes/liter) and let standstill for 48 hours. The supernatant was filtered and let standstill for 48 hours. Rest and filtration were repeated three times for clarification of this solution. The solution was enriched with 2.57 g/l of KNO_3 as a nitrogen source because of the deficiency of this nutrient in the rice straw ashes, and with 15.15 g/l of $NaHCO_3$ which acts as a buffer in the medium to prevent a rapid decrease of pH during growth. The pH (8.5) was corrected to 9.0 adding 1.5 ml of KOH 30% per litre of medium. After a rest of 24 hours, the medium was filtered and set under U.V. irradiation for 24 hours. The standard bicarbonate medium of Paoletti et al. was used as control (11). This medium contained (g/l): $NaHCO_3$ 15.15; Na_2CO_3 8.89; NaCl 0.92; Na_2SO_4 1.88; KNO_3 2.57; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.25 and $CaCl_2.2H_2O$ 0.05; Fe-EDTA complex (mg/l: EDTA-Na_2 29.8 and $FeSO_4.7H_2O$ 24.9) and microelements solution (mg/l: H_3BO_4 2.86; $MnCl_2.4H_2O$ 1.81; $ZnSO_4.7H_2O$ 0.22; $Na_2MoO_4.2H_2O$ 0.39; $CuSO_4.5H_2O$ 0.079 and $Co(NO_3)_2.H_2O$ 0.049).

The experiments were carried out in photobioreactor (DURAN), in two culture systems: continuous light (CL) and intermittent light (IL) - light/dark cicles of 12/12 h. The culture conditions were as follows: culture volume 2.2 liters; inoculum 10% (v/v) corresponding to about 50 mg/l of dry biomass; temperature 32 ± 1°C; continuous agitation with air (0.2 vvm or l/l/min); light intensity 2.4 Klux and culture duration of 192 hours. The experimental outline was done by chance at the factorial scheme, with three repetitions. Two culture media and two culture systems were utilized. Multifactor Analysis of Variance and Tukey's test were used for statistical analysis of the results.

The culture growth was estimated by absorbance reading in 560 nm, according to LeDuy and Therien (7). The culture growth was measured with a spectrophotometer (BAUSCH & LOMB, model Spectronic 20), and the determination of the cell dry weigth was done with Millipore filter membranes (0.45 μm). It was then washed twice with distilled water and dried at 105°C till constant weight. The absorbance data and the respective dried weight supplied the estimated regression equation: absorbance = 1.85236 x cell dry weight + 0.00664, with square correlation coefficient (R^2) = 0.995 to control medium, and absorbance = 1.94755 x cell dry weight + 0.0402696, with square correlation coefficient (R^2) = 0.988 to semi-natural medium. The growth rate (dx/dt) and the specific growth rate (μ) were calculated according to the method proposed by LeDuy and Zajic (8), which establishes the derivative from the straight line tangent to the considered point, assuming that there exists an arc of circle y (x) passing through any of three points A (x_a, y_a), B (x_b, y_b) and C (x_c, y_c). The reference curve for calculation of this derivative is the growth curve of *Spirulina maxima*, obtained from the daily measurements of the average of biomass concentration. The inoculum was concentrated in a centrifuge (FANEN, model Excelsa 3) at 2000 rpm during 7 minutes, diluted in 200 ml of culture medium and inoculated in 2000 ml of medium, to supply an initial concentration estimated at about 50 mg dry wt/l. The culture temperature was controlled by a heat exchanger, a 0.5 mm glass coil, immersed in the culture. The circulating liquid (distilled water) was supplied by two baths with centrifuge pumps: a warm bath at 32°C (VEB MLW PRÜFGERÄTE, model U4) and a cold one at 14°C, which was kept cold by a cooling unit (FANEM, model 114 B). The culture stirring was done by bubble air (0.2 vvm) as recommended by Paoletti et al. (12) using a glass tube submersed in the culture.

The light was supplied by two fluorescent lamps (Phillips TL day light 65 W) set over the photobioreactor, and three lamps (Phillips pholha 65 W) surrounding the photobioreactor. These were arranged to give out light intensity of 2.4 Klux on the photobioreactor external surface. Light intensity was measured with a light meter (ICEL, model LD-500). According to the manufacturer, the pholha lamps (Phillips) emit red and blue light. On the other hand, the fluorescent day light lamps emit blue, red and green light, which are required for *Spirulina*'s culture (6).

Absorbance readings (three times) and pH measurements (pocket pHmeter, CORNING, model PS-15) were conducted in 20 ml samples, collected from the culture every 24 hours. The cultivation was centrifuged (JANETZKI, model S 60) at 3000 rpm for 10 minutes to collect *S. maxima* biomass. The sediment was washed with distilled water and centrifuged for 5 minutes at 3000 rpm. After repetition of this procedure, the sediment (paste of *Spirulina*) was transferred, with distilled water, to a porcelain capsule and set to dry in a stove. The biomass protein grade from the *S. maxima* was measured according to Williams (18) technique nº 2057 (Kjeldahl method with conversion factor 6.25).

RESULTS AND DISCUSSION

The results obtained in the different treatments concerning cell concentration, culture pH, growth rate and specific growth rate, are shown in Tables 1 and 2. The pH of the cultures varied from 9.0 to 10.2, which is considered excellent by Richmond (14). The maximum specific growth rates, 0.541 d^{-1} and 0.469 d^{-1}, obtained in the control culture medium with continuous light (CL) and intermittent light (IL) respectively, were higher than those obtained in the semi-natural culture medium, 0.523 d^{-1} and 0.415 d^{-1} (Tables 1 and 2). These rates were higher than the rate found by Faintuch et al. (0.4 d^{-1} in CL systems) (2) and even higher than 0.3 d^{-1}, which was considered high by Santillan (16).

In the control medium with continuous light, the peak of growth rate was 59.465 mg/l.day, with cell concentration of 275.300 mg/l, obtained between the third and the fifth day of culture. When the culture was done at intermittent light, the higher growth rate was 43.612 mg/l.day, for cell concentration of 92.990 mg/l in the first day of culture (Table 1).

The biomass protein contents of the culture of *S. maxima* in control medium were 63.66 wt % and

Table 1. Cell concentration* (X), growth rate (dx/dt), specific growth rate (μ) and pH, of *Spirulina maxima* in the time function, cultured in control medium, for continuous light and intermittent light (12/12 h) systems.

	Continuous light				Intermittent light			
Time (d)	X (mg/l)	dx/dt (mg/l.d)	μ (d)	pH	X (mg/l)	dx/dt (mg/l.d)	μ (d)	pH
0	48.910	-	-	9.4	51.070	-	-	9.5
1	102.760	55.593	0.541	9.5	92.990	43.612	0.469	9.6
2	160.300	56.426	0.352	9.6	138.600	41.580	0.300	9.7
3	215.750	57.390	0.266	9.7	177.390	35.478	0.200	9.8
4	275.300	59.465	0.216	9.7	210.400	33.033	0.157	9.9
5	334.500	56.865	0.170	9.8	243.300	29.683	0.122	10.0
6	389.500	49.467	0.127	9.9	270.850	27.898	0.103	10.0
7	435.300	47.707	0.105	9.9	298.890	29.291	0.098	10.1
8	480.840	-	-	10.0	329.380	-	-	10.1

(-) null or negative values.
* average of three replicates.

Table 2. Cell concentration* (X), growth rate (dx/dt), specific growth rate (μ) and pH, of *Spirulina maxima* in the time function, cultured in semi-natural medium, for continuous light and intermittent light (12/12 h) systems.

	Continuous light				Intermittent light			
time (d)	X (mg/l)	dx/dt (mg/l.d)	μ (d)	pH	X (mg/l)	dx/dt (mg/l.d)	μ (d)	pH
0	51.000	-	-	9.0	51.000	-	-	9.0
1	105.370	55.108	0.523	9.5	83.910	34.823	0.415	9.5
2	161.340	57.921	0.359	9.8	121.080	38.625	0.319	9.8
3	221.360	65.301	0.295	9.8	161.400	39.704	0.246	9.9
4	294.570	73.053	0.248	9.9	200.380	34.065	0.170	10.0
5	367.630	63.600	0.173	10.0	231.460	24.998	0.108	10.0
6	425.240	30.192	0.071	10.0	253.350	8.614	0.034	10.0
7	450.230	-	-	10.1	260.540	5.211	0.200	10.1
8	450.290	-	-	10.1	265.310	-	-	10.2

(-) null or negative values.
* average of three replicates.

63.56 wt % (dry weight) for culture systems with continuous light and intermittent light, respectively.

In the semi-natural medium (Table 2), with continuous light, the highest growth rate of 73.053 mg/l.day for cell concentration of 294.5570 mg/l was observed between the third and the fifth day of culture. The stationary phase began between the sixth and the seventh day of culture, which was probably caused by the shortage of nutrients, especially nitrogen, due to the low protein content found in the biomass. The biomass produced in this medium presented a protein content (dry wt) of 45.05 wt % when cultured under continuous light and 67.51 wt % when cultured under intermittent light.

The highest biomass productivity was obtained in the control medium under continuous light, 53.99 mg/l/day of dry biomass, in opposition to 34.79 mg/l/day when cultured under intermittent light. The highest productivities rates obtained in the semi-natural medium were 49.91 and 26.79 mg/l/day for continuous and intermittent light, respectively.

As can be noticed in Fig. 1, the growth of *S. maxima* began at the exponential phase, bypassing the latent phase. According to Schlegel (17), this fact occurs when inoculum is originated in pre-cultures at the exponential phase. The influence of the light system in the growth of *S. maxima* can be observed in Fig. 1. The cultures under continuous light presented higher results when compared to cultures under 12/12 h intermittent light.

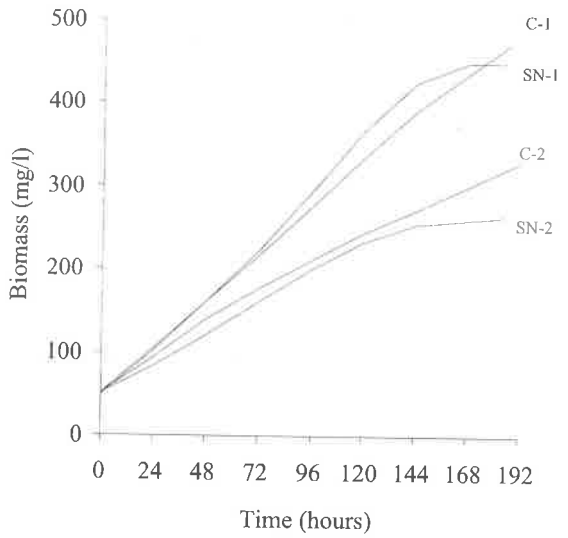

Figure 1 - Growth curves of *Spirulina maxima* in control medium (C) and semi-natural medium (SN), for continuous light (1) and intermittent light (2) systems.

The biomass protein content (63.56 - 67.51 wt %), except for the semi-natural medium in continuous light system (45.05 wt %), was higher than the contents found by Becker and Venkataraman (62.5 wt %) (1), and by Nguyen et al. (61.3 wt %) (10), but it was lower than the content found by Florenzano (68-77 wt %) (4). The protein contents found range from 60 to 65 wt %, which is considered by Richmond (15) as a good range.

In the continuous light system, the final biomass concentration average was 465.5667 mg/l, and in the intermittent light system it was 297.3450 mg/l. They differ from each other statistically, according to Tukey's test, with a probability of 1%.

The results indicate that in both light systems (continuous and intermittent), the control medium presented the best results.

CONCLUSIONS

The culture medium tested has demonstrated that the growth of *Spirulina maxima* can be viable in the conditions set in this experiment. The culture systems in continuous light presented higher *Spirulina maxima* biomass productivity results than the intermittent light system (light/dark cycles of 12/12 h). The control medium presented the best results in both light systems. However, in 144 hours of culture, the highest *Spirulina maxima* biomass concentration occured in the semi-natural medium in continuous light system. In spite of the unfavorable statistical analysis regarding the less expensive culture medium, further studies with experiments in a pilot-scale and cost/production analysis might be important for the production of *Spirulina maxima* in large scale. In addition, nutritional and toxicological studies of the biomass produced in these media can be suggested.

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RESUMO

Crescimento de *Spirulina maxima* em meio constituído de cinzas de palha de arroz

Foram realizados estudos comparativos do crescimento de *Spirulina maxima*, em meio sintético (controle) e em meio constituído de cinzas de palha

de arroz (semi-natural), em períodos de iluminação contínua e iluminação intermitente de 12/12 h, com intensidade de luz de 2,4 Klux. O experimento foi conduzido em condições de laboratório, em fotobioreator com volume de trabalho de 2.200 ml, temperatura de 32 ± 1°C, agitação com ar durante 192 horas. Os cultivos sob iluminação contínua apresentaram resultados superiores em termos de produtividade, velocidade de crescimento e velocidade específica de crescimento. As velocidades específicas máximas de crescimento ficaram entre 0,541 e 0,523 (d^{-1}) para os cultivos sob iluminação contínua, e entre 0,469 e 0,415 (d^{-1}) para aqueles sob iluminação intermitente. O meio controle obteve a melhor resposta de crescimento nos dois sistemas de cultivo. O teor protéico das biomassas de *S. maxima* apresentou uma variação de 45-68% em peso seco.

Palavras-chave: *Spirulina maxima*, meio de cultura, alga verde-azulada.

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DEGRADATION OF CYANO-METAL COMPLEXES AND NITRILES BY AN *ESCHERICHIA COLI* STRAIN

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ABSTRACT

An *Escherichia coli* BCN6 strain isolated from gold mine effluent has been previously reported to degrade the glucose-cyanide complex. This strain was able to use cyanide complexed with copper, iron or zinc and acetonitrile, isobutyronitrile, methacrylnitrite, propionitrile and acetamide as nitrogen source for growth, but did not take up copper or iron ions. The strain grew on acetonitrile at concentrations up to 69 nM. The utilization of acetonitrile and acetamide by this bacterial strain probably involves hydrolysis in a two-step reaction mediated by both inducible and intracellular nitrile hydratase and amidase.

Key words: Cyanide, *Escherichia coli*, metal-cyano complexes, nitriles

INTRODUCTION

Cyanide compounds are commonly used in various industries for chemical intermediate production and synthesis of methacrylates, dyes, synthetic fibers and agricultural products (4, 18). Gold mining and electroplating industries are responsible for large amounts of cyanide released into the environment (12). Many plants, like cassava, peach, cherry and bamboo trees, are known for their cyanogenic glycoside and cyanolipid production (14). Due to its high toxicity, cyanide-containing waste water must be treated before release, in order to meet regulatory requirements. Many chemical processes have been proposed to decompose cyanide, the most important being alkaline chlorination, copper-catalysed hydrogen peroxide oxidation and the INCO process (19). Since some processes require special equipment and, in many cases, do not degrade all cyanide complexes, biological treatment has been investigated as possible process for achieving high degradation efficiency at low cost. A full-scale biological treatment facility is currently being used to treat cyanide waste waters at Homestake Mining Co. (Lead, SD, USA); cyanide compounds and thiocyanate are oxidised to ammonia and carbonate by a microbial consortium including the bacterium *Pseudomonas paucimobilis* especially acclimated to elevated cyanide concentrations, and immobilised in a rotating biological contactor (RBC) reactor (16, 20, 19). The process reduces 95-98% of the initial cyanide and metal concentrations in a daily discharge of 4 million gallons of waste water (20). The bacterial metabolism of cyanide has been the subject of various studies since the last decade, with special interest in the proposition of new biodegradation systems, most of them using *Pseudomonas, Acinetobacter, Bacillus* and *Alcaligenes* strains (11, 8, 15, 9).

Nitrile compounds are cyanide-substituted carboxylic acids which are produced naturally and synthetically. The organic nitrile compounds and their derivatives are used extensively in many industrial operations. Indiscriminate use of these compounds could cause deleterious effects since most of them are highly toxic, and some are

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mutagenic, carcinogenic and teratogenic (10). Microorganisms such as *Arthrobacter*, *Brevibacterium*, *Nocardia rhodochrous*, *Pseudomonas* and *P. putida* have been reported to metabolise nitrile compounds and their respective carboxylic acids and ammonia (2, 5, 17).

The enzymatic hydrolysis of nitriles to their corresponding acids and metabolically available ammonia by a wide variety of both Gram-positive and Gram-negative bacteria has been well documented. The microbial metabolism of nitriles proceeds through two different pathways. Nitrilase (EC 3.5.5.1) participates in the direct conversion of nitriles to their carboxylic acids and ammonia (13). Asano et al. (2) proposed a second pathway involving a nitrile hydratase (EC 4.2.1.84) that mediates the conversion of nitriles to their amide, and an amidase (EC 3.5.1.4) that converts the amides to their corresponding carboxylic acids and ammonia.

Figueira et al. (7) isolated an *Escherichia coli* strain from gold extraction circuit fluids in Brazil which degraded cyanide only when this was present as a glucose-cyanide complex. In the present study we report the ability of *Escherichia coli* BNC6 strain to degrade metal-cyano complexes and low-molecular-weight organic nitriles as the sole source of nitrogen.

MATERIALS AND METHODS

Microorganism and culture conditions: *Escherichia coli* BCN6 strain was isolated from gold mining effluents and reported to be a cyanide-degrading bacterium (7). The strain was grown in buffered minimal medium (pH 7.0) containing potassium cyanide (50 mg.l^{-1} CN^-) as the sole source of nitrogen (72 hours, 30°C, with shaking). The *E. coli* BCN6 was stored in the same medium with glycerol at -70°C.

Growth of *Escherichia coli* BCN6 at different metal-cyano complex concentrations: The buffered minimal medium consisted of 0.03 g $MgSO_4.7H_2O$, 0.3g NaCl, and 1.0 g glucose dissolved in 100 ml of a 67 mM KH_2PO_4-K_2HPO_4 buffer, (pH 7.0). Cyanide concentrations ranging from 2.0 to 100 CN^- mg.l^{-1}, present as copper, zinc or iron, were used as the sole nitrogen source. A 0.5 ml aliquot of the bacterial growth (A_{540} = 1.0 or 0.044 mg of dry weight/ml) in KCN was used as the inoculum for 25.0 ml of medium containing different cyanometallic complexes. Cultures were incubated at 30°C in a rotatory shaker at 250 rev/min for 72 hours. Bacterial growth was estimated by measuring absorbance at 540 nm. Controls of cyanide stripping and metal precipitation due to the solution pH (7.0) were run by incubating the media in the absence of the microorganism. Total cyanide (1) and the metals Cu, Fe and Zn were determined in the supernatant. Metal uptake was determined by the difference in metal concentration in the media incubated in the absence and in the presence of the bacterium. Analyses of metals were performed using a Varian atomic absorption spectrophotometer with an air-acetylene flame, model AA-475, by reference to appropriate standard metal solutions according to the APHA (1).

Growth of *Escherichia coli* BCN6 on different nitriles and acetamide: The organic nitriles acetonitrile, isobutyronitrile, methacrylnitrile and propionitrile were obtained from Merck and acetamide from Aldrich. *E. coli* was grown in 25 ml of buffered minimal medium (pH 7.0) containing different nitriles and acetamide at increasing concentrations from 5 to 46 mM as the sole nitrogen source. Cultures were incubated at 30°C with shaking for 72 hours. In all experiments, *E. coli* culture grown in the presence of potassium cyanide (50 mg.l^{-1} CN^-) was used as the inoculum (0.5 ml, A_{540} = 1.0). In each experiment, the growth was monitored by measuring absorbance at 540 nm, and the ammonia concentration in the supernatant was measured colorimetrically by the method of Fawcett and Scott (6). Furthermore, the *E. coli* strain was acclimatized by culturing in buffered minimal medium with increasing acetonitrile concentrations from 23 to 92 mM.

Enzymatic assay: After growth of the microorganism on 35 mM acetonitrile for 72 hours, the cells were centrifuged to obtain both supernatant and cell mass. The supernatant and washed (intact) cells served as the crude enzyme source.

The supernatant was assayed at 30°C for a maximum of 30 min in tubes containing 1.0 ml of 67 mM acetonitrile or acetamide in 25 mM-sodium phosphate buffer, pH 7.0, and 1.0 ml of supernatant. Enzymatic activity was assayed by measuring the production of ammonia (6). Before this experiment the presence of ammonia was measured in the supernatant as a control. Alternatively, the centrifuged cells were washed twice in 25 ml phosphate buffer, pH 7.0, and then resuspended in 25 ml of the same buffer. For the enzymatic assay, 1.0 ml of resuspend washed (intact) cells was added to 1.0 ml of 67 mM acetonitrile or acetamide in 25 mM-sodium phosphate buffer, pH 7.0, at 30°C, for

a maximum of 30 min. The bacterial cells were removed by centrifugation and ammonia was measured colorimetrically and expressed as µmol ammonia per minute at 30°C, pH 7.0.

RESULTS AND DISCUSSION

Escherichia coli BC6 was able to grow in the presence of different cyano-metallic complexes as the sole source of nitrogen (Fig. 1). However, a different bacterial behaviour was observed in each system. Bacterial growth in iron cyanide solutions was nearly constant for concentrations up to 25.0 mg CN^-/l (corresponding to 8.9 mg/l of Fe), and higher concentrations led to a gradual decrease in bacterial growth. When the bacterium was incubated in zinc cyanide solution the microorganism showed a progressively increasing growth for cyanide concentrations up to 10.0 mg/l (corresponding to 6.3 mg/l). Since the *E. coli* BCN6 is able to grow in the presence of cyanide concentrations higher than 10.0 mg/l (7), it is assumed that such zinc concentration could be toxic enough to result in partial reduction of microbial metabolism. *E. coli* was able to grow at copper cyanide concentrations up to 5.0 mg/l CN^-/l, but with limited production of biomass. This may be explained by the high toxicity level of Cu to organisms in general, even at low concentrations (such as 4.1mg/l, when [CN^-] = 5.0 mg/l). These results are similar to those obtained by Finnegan et al. (8) in a study in which *Acinobacter* sp showed the ability to assimilate ions when optimal concentrations of zinc, copper and iron were 5.0, 5.0 and 100 mg/l respectively. However, the authors observed precipitate formation as a result of degradation of these cyanide salts, a phenomenon not observed in the present study.

The reduction of cyanide concentrations complexed with copper, iron and zinc, in which a greater bacterial growth was observed, was measured in culture after 24 hours (Table 1). The results showed that in flasks with *E. coli*, CN^- disappeared during the first 24 hours, probably because of bacterial utilisation, formation of ammonia and/or release of HCN into the environment. However, very little reduction of cyanide was observed in the control flasks. Thus, we can state that the *E.coli* strain under study was able to utilise cyanide as cyanide salts. The ability of fungi, algae and bacteria to take up metal ions is well documented (3). We investigated whether the *E. coli* strain was able to accumulate cyano complexed copper, iron and zinc. Our experiments

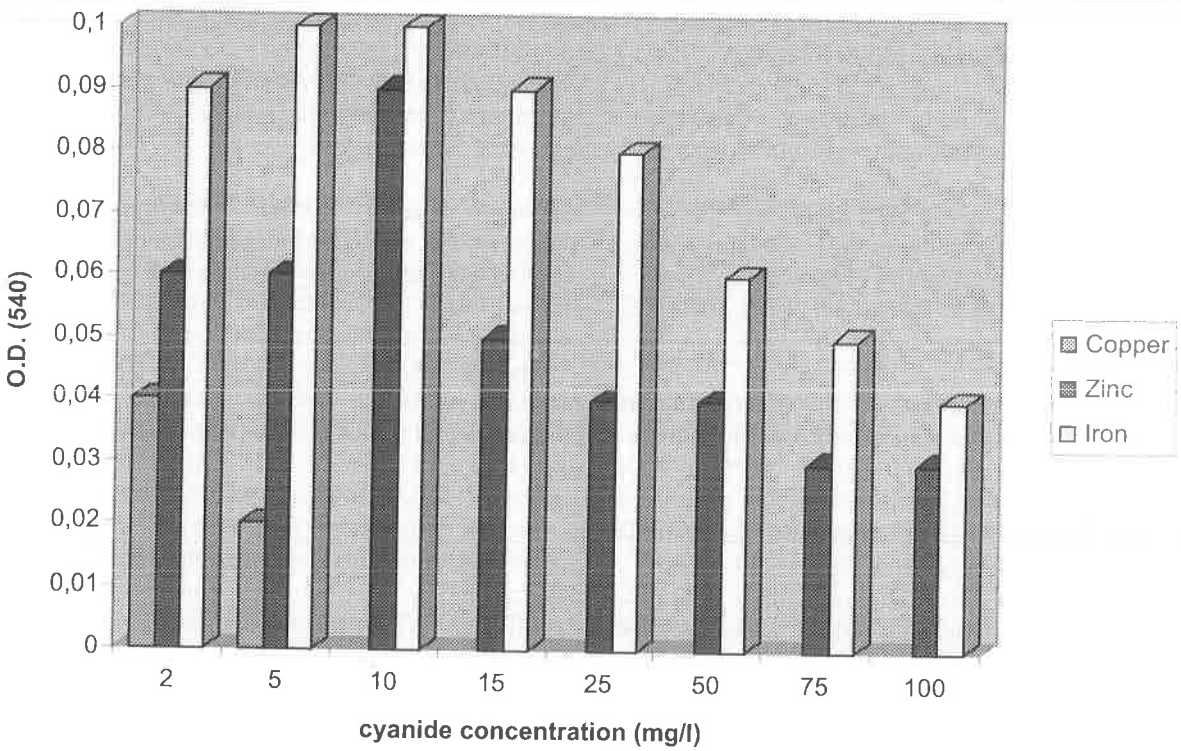

Figure 1. Growth of *Escherichia coli* BCN6 on $Cu(CN)^{-2}_3$, $Fe(CN)^{-3}_6$ and $Zn(CN)^{-2}_4$ after 72 hours of incubation.

Table 1. Total cyanide determination in buffered minimal media with glucose and cyanide salts incubated in the presence (1) and absence (2) of the *Escherichia coli* strain under study.

Cyanide salts [#]	Total cyanide (mg/l)	
	1	2
$Na_2Cu(CN)_3$	0	2.0
$Na_3Fe(CN)_6$	0.8	20.0
$Na_2Zn(CN)_4$	0	9.5

[#] Initial concentration: 5mg/l CN $[Cu(CN)^{2}_{3}]$; 10 mg/l CN $[Zn(CN)^{2}_{4}]$ and 25mg/ CN $[Fe(CN)^{3}_{6}]$
Determined after 24 hours.

showed that Zn ion was accumulated, but not copper or iron even after 96 hours of incubation (data not shown).

Table 2 shows the effect of various nitriles and acetamide on bacterial growth. The best growth were obtained when acetonitrile and propionitrile were used as the sole source of nitrogen in concentrations from 5 to 35 mM. For acetamide the growth were constant in all concentrations tested. The bacterium grew on isobutyronitrile and methacrylnitrile, but failed to utilise benzonitrile. *E. coli* BCN6 growth on increasing concentrations of acetonitrile is presented in Fig. 2, which shows that the best growth was achieved at a concentration of 69 mM. At higher concentrations, the growth started to decrease. The utilization of acetonitrile and its corresponding amide as nitrogen sources by *E.coli* suggests that the hydrolysis of nitriles might be effected in a two-step reaction mediated by nitrile hydratase and subsequent hydrolysis of the resulting amides by amidase (2). It was observed that the cell mass of *E.coli* contains intracellular enzymes that catalyse the production of ammonia from acetonitrile and acetamide (Fig. 3). The production of ammonia was not observed when the supernatant was used as a source of extracellular crude enzymes. Enzymatic activity was not observed when washed (intact) cells growing on acetonitrile were replaced with washed (intact) cells growing on ammonium sulphate. These

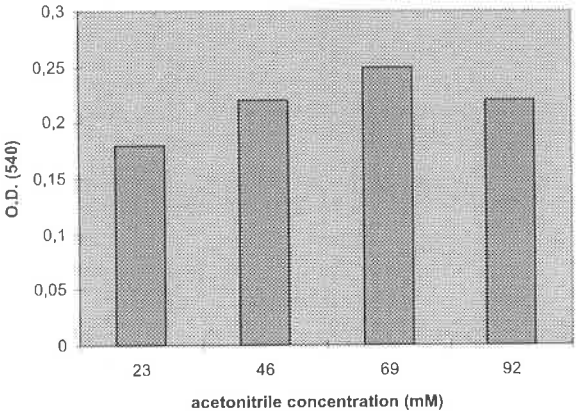

Figure 2. Growth of *Escherichia coli* BCN6 on increasing concentrations of acetonitrile after 72 hours of incubation.

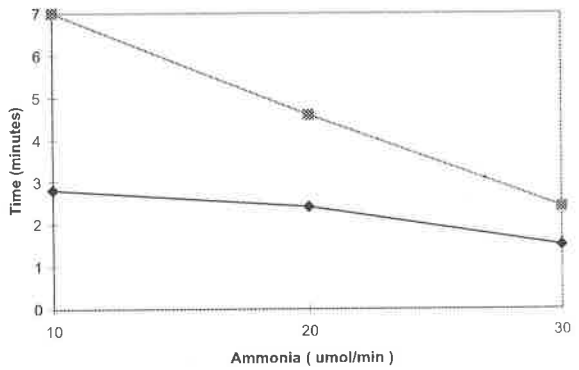

Figure 3. Production of ammonia by washed (intact) cells on acetonitrile (■) and acetamide (◆)

results suggests that both enzymes, nitrile hydratase and amidase, are intracellular and inducible.

ACKNOWLEDGEMENTS

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Table 2. Growth of the *Escherichia coli* strain on nitriles and acetamide

Concentration mM	Acetonitrile	Isobutyronitrile	Methacrylnitrile O.D (A_{540})[#]	Propionitrile	Acetamide
5	0.10	0.06	0.07	0.05	0.09
12	0.10	0.09	0.09	0.10	0.10
23	0.12	0.10	0.10	0.10	0.10
35	0.14	0.09	0.08	0.11	0.11
46	0.11	0.08	0.07	0.08	0.12

[#] Growth (A_{540}) was determined after 72 hours of incubation

RESUMO

Degradação de ciano complexos metálicos e nitrilas por uma linhagem de *Escherichia coli*

Uma linhagem de *Escherichia coli* BNC6, isolada de efluente de mineração de ouro, capaz de degradar o cianeto complexado com glicose, também utiliza o cianeto complexado com zinco, ferro e cobre e acetonitrila, isobutironitrila, metacrilonitrila, propionitrila e acetamida, como fonte de nitrogênio para o seu crescimento. A referida bactéria não biossorveu os ions cobre e ferro complexados com o cianeto. A linhagem *E. coli* BCN6 cresceu em acetonitrila em concentrações de até 69nM. A utilização de acetonitrila e acetamida pela bactéria provavelmente envolve a participação da nitrila hidratase e amidase, ambas indutíveis e intracelulares.

Palavras-chave: Cianeto; cianometais; *Escherichia coli; nitrilas*

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EFFECT OF OXYGEN SATURATION ON MEMBRANE CYTOCHROMES OF *BACILLUS STEAROTHERMOPHILUS* IN CHEMOSTAT CULTURE

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ABSTRACT

Studies of the cytochrome content and composition of membranes of *Bacillus stearothermophilus*, when grown in either a carbon-limited or a carbon-sufficient chemostat culture, showed that the availability of oxygen and the nature of the specific growth limitation exerted considerable influence on cytochrome synthesis. When this organism was grown with full and low aeration and was limited in its growth by the availability of potassium, phosphorus or sulphate, it synthesized cytochromes *c*, *d* and *o*. Under conditions of magnesium, ammonia and glucose limitation and low aeration, cyt. aa_3 and cyt. *b* were synthesized, whereas at high aeration cyt. *d* and cyt. *o* were synthesized. In addition, it was observed that the reduction of the availability of oxygen increased the synthesis of the cytochromes that were measured.

Key words: Oxygen limitation, *Bacillus stearothermophilus*, cytochromes synthesis, chemostat cultures

INTRODUCTION

The behaviour of the organisms can be observed to vary widely in response to the level of dissolved oxygen tension (d. o. t.) in microbial cultures (7, 9, 10, 20). The respiratory enzymes of most organisms for example, are saturated above a dissolved oxygen level equivalent to 8 - 10 mmHg, which corresponds to only 5% air saturation value of 160 mmHg (6). Also the quantities of the individual cytochromes vary, particularly with the growth conditions (16) and there is apparently no requirement for any specific cytochrome type (19). During studies of the growth of *Bacillus stearothermophilus* var *non-diastaticus* in glucose chemostat cultures it was noticed that the respiration rate declined as growth became overtly oxygen-limited.To investigate whether the respiratory energy conservation was altered when the dissolved oxygen level was decreased from 50 to 5% air saturation, the cytochrome of membrane fractions of *B. stearothermophilus* grown in carbon-limited and carbon-sufficient chemostat cultures were measured.

MATERIALS AND METHODS

Organism and culture conditions. The organism used in this study was a near-prototrophic strain of *Bacillus stearothermophilus* that apart from the carbon and energy source, required only biotin as an organic supplement. It was obtained from the Laboratory of Microbiology, University of Amsterdam, and named *Bacillus stearothermophilus* Amsterdam. In all its caracteristics it appeared to be identical with the strain *B. stearothermophilus* var. *non-diastaticus* described by Epstein and Grossowicz (3).

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Organisms were routinely grown in chemostats (LH Fermentation 500 series, 1 litre growth vessel with a 700 ml working volume) in defined simple salts media (5) at 55°C and pH 7.0. This media was modified according to the growth limitation to be imposed upon the culture, in the following manner: 1. Glucose-limited growth: 27.2 gl^{-1} of glucose were replaced by 4.7 gl^{-1}; 2. Ammonia-limited growth: 5.35 gl^{-1} of NH_4Cl were replaced by 1.605 gl^{-1}; 3. Phosphate-limited growth: 1.56 gl^{-1} of $NaH_2PO_4.2H_2O$ were replaced by 0.312 gl^{-1}; 4. Potassium-limited growth: 0.745 gl^{-1} of KCl were replaced by 0.149 gl^{-1}; 5. Magnesium-limited growth: 0.42 gl^{-1} of Citric Acid and 0.250 gl^{-1} $MgCl_2.6H_2O$ were replaced by 0.21 and 0.05 gl^{-1} respectively; 6. Sulphate-limited growth: 0.644 gl^{-1} of $Na_2SO_4.10H_2O$ were replaced by 0.129 gl^{-1}. Glucose was provided as the carbon and energy source and, in general, all non-limiting nutrients were present in at least a 3-fold excess. The basal medium (excluding glucose) was prepared in 20 l batches and sterilized by autoclaving at 121°C for 30 min. The required amount of glucose was made up as a 50% (w/v) solution (slightly acidified with HCl), autoclaved at 121°C for 30 min and added aseptically to the bulk medium after cooling.

The temperature and pH values of the cultures were controlled automatically and foaming was suppressed by the addition of a silicone-based antifoaming agent, on demand, as sensed by a foam probe. Dissolved oxygen was monitored by means of a galvanic oxygen electrode (Uniprobe Instruments) and its concentration adjusted and maintained at the desired degree of saturation by varying the stirrer speed.

Procedure. Organisms were grown at a specific rate of 0.2 h^{-1} (i.e. 0.15 μ_{max}) under fixed steady-state conditions (55°C, pH 7.0) in five different glucose-sufficient media (growth limiting with respect to ammonia, sulphate, phosphate, magnesium and potassium) as well as in a glucose-limiting medium. The d.o.t. was first set, and controlled, at about 50% saturation, then subsequently lowered to 5% air saturation. After equilibration at each d.o.t. for 1-2d, samples of culture (2 l) were collected from the chemostat, harvested by centrifugation (MSE, Mistral 6 l) at 6,300 x g for 15 min, washed once with 50 mM sodium phosphate buffer (pH 7.0) and stored at -20°C until required.

Cytochrome spectra. The assay was based on the method of Ball and Edwards (1). Cell suspensions were prepared containing about 1 g cells (wet weight) per 4 ml buffer (50 mM phosphate buffer pH 7.2). The sonification vessel was placed in a beaker containing an ice-water mixture. The cells were disrupted using an ultrasonic desintegrator (MSE, Model 60 w), for 4 x 1 min (8 microns peak to peak, low power, 1.0 cm probe diameter), interspersed with 1 min cooling intervals. Whole cells were removed by centrifugation at 10,000 x g for 10 min, and the resultant supernatant was centrifuged at 100,000 x g (Beckman L55OB) for 1 h at 4°C. Membrane pellets were resuspended in phosphate buffer and used immediately. Reduced minus oxidized difference spectra of membranes, were produced by simultaneously scanning samples contained in 2 x 1 ml quartz cuvetts, using a Pye Unicam SP 1800 double beam spectrophotometer coupled to a Pye Unicam AR 25 linear recorder. The reference sample, was reduced by adding few grains of sodium dithionite. The contents of the other cuvett (sample), were oxidized by the addition of a few grains of potassium ferricyanide.

RESULTS AND DISCUSSION

The cytochromes in reduced minus oxidized spectra were determined in membrane fractions of *B. stearothermophilus* grown in glucose-limited chemostat cultures at a dissolved oxygen tension of 50% and 5% air saturation. The results obtained (Fig. 1) revealed that at a d.o.t. of 50% air saturation, the cytochrome *d* (622 nm) and *o* (574 nm) were present. However, when the dissolved oxygen level was lowered to 5% air saturation, different peaks were observed that appeared to be cytochrome *b* (558 nm) and aa_3 (602 nm). Since the functional ligant-binding haem of cytochrome *o* is a *b*-type, its reduced minus oxidized spectrum in unpurified preparations is generally masked by other cytochromes *b* (14). Similar analysis done by Scott and Poole (17), revealed two bands in membranes from oxygen-limited cells at 555.5nm and 559 nm, which was attributed to *b*-type cytochromes. When *B. stearothermophilus* was grown in glucose-sufficient cultures that were limited by the availability of either potassium, sulphate or phosphate and examined spectrophotometrically, peaks were observed at 538 nm, 618 nm and 574 nm at a d.o.t. of 50% air saturation and also at a d.o.t. of 5% air saturation (Figs. 2, 3 and 5). This suggested the presence of cytochromes *c*, *d* and *o* respectively. Cytochrome *d* is often found in the same organism as cytochrome *o*. Its synthesis is favoured by conditions such as

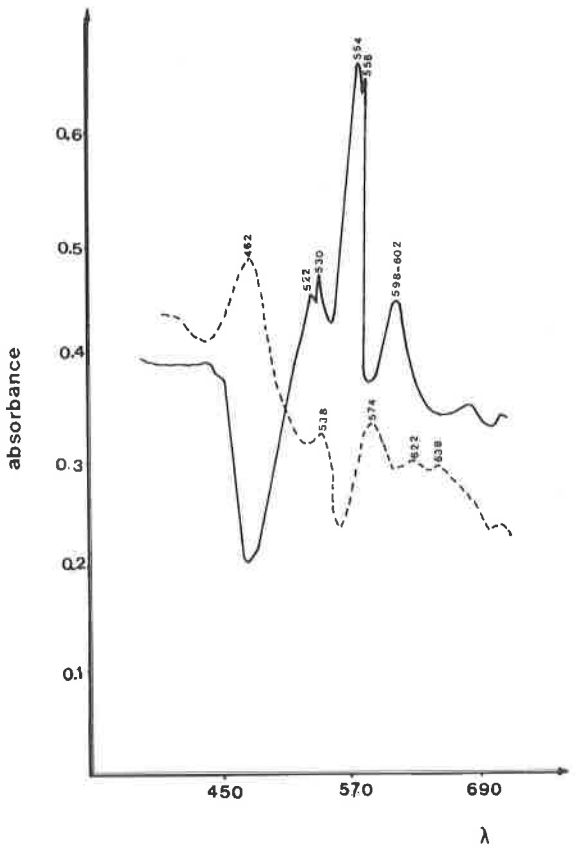

Fig.1. Reduced minus oxidized difference spectra of *Bacillus stearothermophilus* grown in glucose-limited cultures at a d.o.t. of 50% air saturation (----) and 5% air saturation (—).

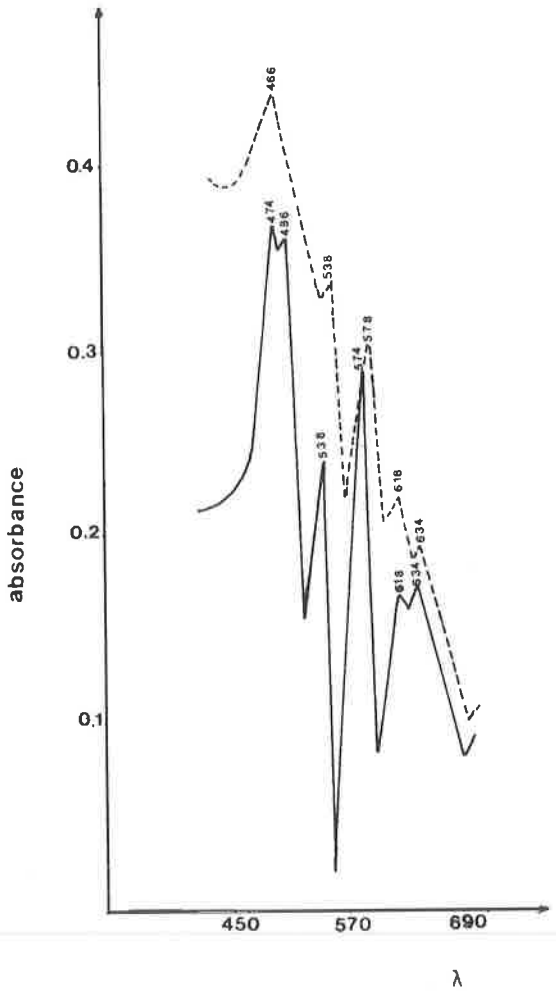

Fig.2. Reduced minus oxidized difference spectra of *Bacillus stearothermophilus* grown in glucose sufficient cultures limited by potassium at a d.o.t. of 50% air saturation (----) and 5% air saturation (—).

aerobic growth with glucose, anaerobic growth, growth under sulphate-limited conditions (17). A cytochrome *c* (552) was also found in *Escherichia coli* growing anaerobically (8) and in addition Barret and Sinclair (2) reported the presence of this same cytochrome in aerobic and microaerobic cultures.

The concentration of each of these cytochromes (*c*, *d* and *o*), subsequently were calculated using extinction coefficients given by Smith (18). It was found that when the level of the available oxygen decreased, the synthesis of these cytochromes was increased (Table 1), showing that the availability of oxygen and the nature of the specific growth limitation exerced considerable influence on cytochrome synthesis. This suggest that in this organism carbon status may be at least as important as oxygen status. Similar results were found by Wimpenny and Necklen (21), who investigated the behaviour of *Escherichia coli* and *Klebsiella aerogenes* during the transition from anaerobiosis to aerobiosis using continuous culture techniques. They

Table. 1. Influence of the dissolved oxygen level and the nature of the specific growth limitation on the cytochrome content of *Bacillus stearothermophilus* growing on glucose in chemostat culture. The values are expressed in nmoles min (mg protein) .

Limitation	Oxygen (% Sat.)	cytochromes			
		b	*c*	*d*	*e*
Glucose	50	-	-	0,050	-
	1	0,262	-	-	-
Ammonia	50	-	0,120	0,089	-
	1	0,360	-	0,080	-
Magnesium	50	-	-	0,075	-
	1	0,260	-	-	-
Potassium	50	-	0,034	0,042	0,145
	1	-	0,045	0,071	0,300
Sulphate	50	-	0,058	0,084	0,034
	1	-	0,300	0,138	0,149
Phosphate	50	-	0,114	0,042	0,060
	1	-	0,168	0,079	0,090

found that under conditions of limited aeration (about +100 mv) the cytochrome levels were maximal. There have been many other reports of changes in cytochrome levels of microorganisms in response to dissolved oxygen tension which showed that cytochrome levels were highest under low oxygen conditions (14,17,15,4). Previous studies of the metabolic response of *B. stearothermophilus* chemostat cultures to a secondary oxygen limitation, showed that except for glucose-limited, the lowest respiration rates ocurred in organisms grown under oxygen limited conditions (11,12). However, it is shown in this work that under these conditions the cytochromes levels were relatively high and this suggests, that in this case, the cytochromes do not appear to be limiting for respiration rate.

With membrane fractions from *B. stearothermophilus* cells that had being grown in magnesium-limited cultures at a d.o.t. of 50% air saturation a peak at a 538 nm (cyt. *c*) and at a 618 nm (cyt. *d*) was detected (Fig. 4). When the level of oxygen was decreased to 5% air saturation a different peaks were observed and was attributed to cyt. *b* (554 nm) and cyt. aa_3 (602 nm). These results are similar to that found to membrane fractions of *B. stearothermophilus* grown in glucose-limited chemostat cultures at a dissolved oxygen tension of 50% and 5% air saturation.

With *B. stearothermophilus* growing under conditions of an ammonium ion limitation, it was observed that cytochrome aa_3 (602 nm) was synthesized at a d.o.t. 50% air saturation and cytochrome *o (578 nm)* at a d.o.t. 5% air saturation (Fig. 6). In *Methylophilus methylotrophus* cytochrome aa_3 is produced under oxygen-excess

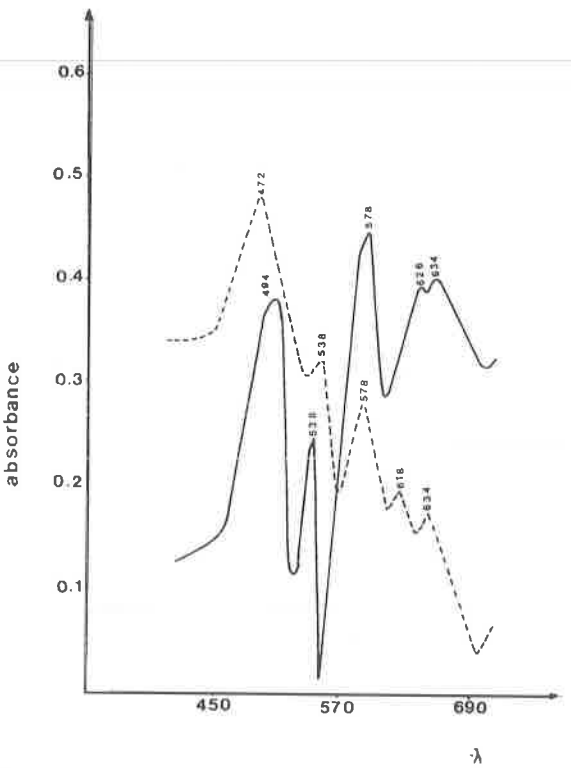

Fig. 3. Reduced minus oxidized difference spectra of *Bacillus stearothermophilus* grown in glucose sufficient cultures limited by sulphate at a d.o.t. of 50% air saturation (----) and 5% air saturation (—).

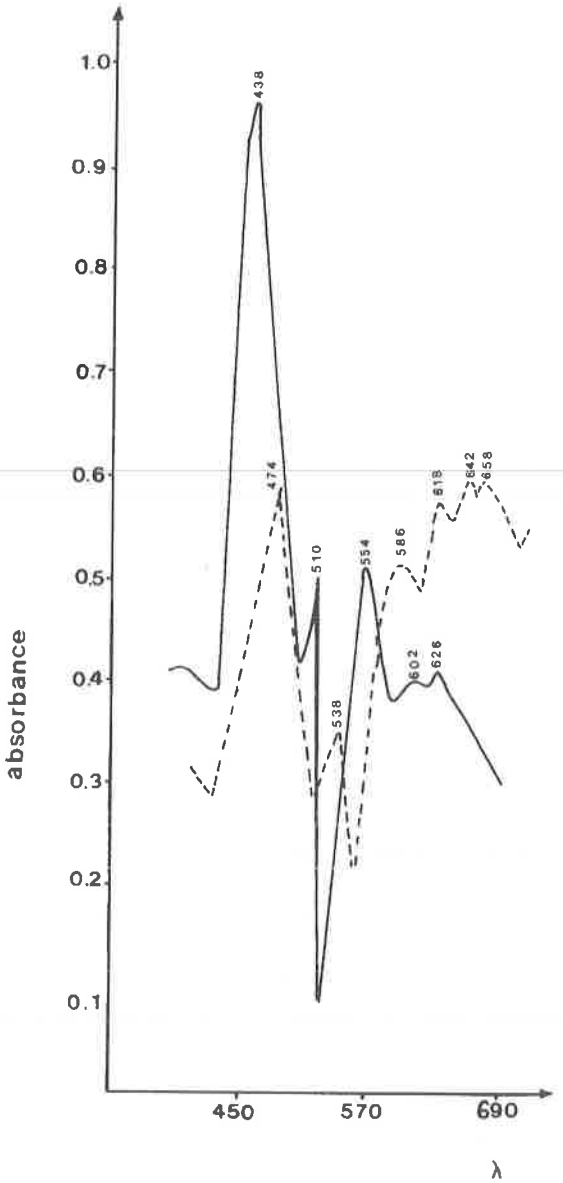

Fig. 4. Reduced minus oxidized difference spectra of *Bacillus stearothermophilus* grown in glucose sufficient cultures limited by magnesium at a d.o.t. of 50% air saturation (----) and 5% air saturation (—).

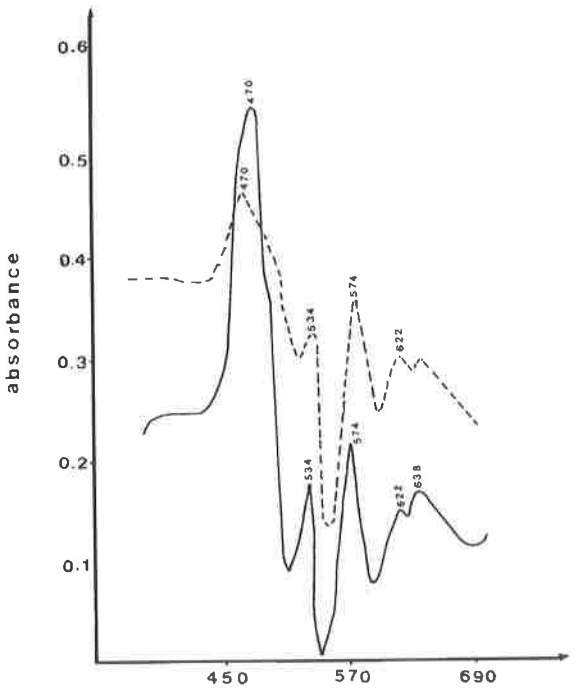

Fig. 5. Reduced minus oxidized difference spectra of *Bacillus stearothermophilus* grown in glucose sufficient cultures limited by phosphorus at a d.o.t. of 50% air saturation (----) and 5% air saturation (——).

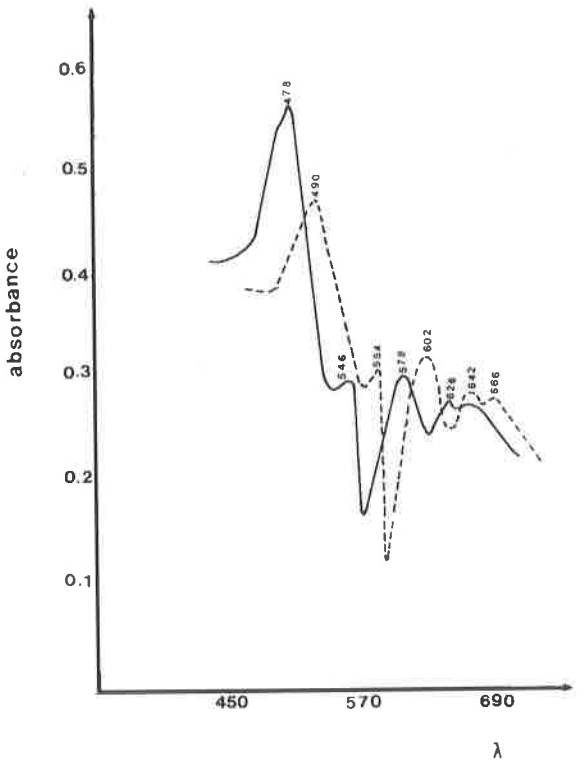

Fig. 6. Reduced minus oxidized difference spectra of *Bacillus stearothermophilus* grown in glucose sufficient cultures limited by ammonia at a d.o.t. of 50% air saturation (----) and 5% air saturation (——).

conditions and being replaced by cytochrome *o* under conditions of oxygen limitation. However, in this same organism, cytochrome aa_3 is replaced by cytochrome *o* under conditions of nitrogen limitation (i.e. carbon or oxygen excess conditions) (13), suggesting that oxygen may not be the most important regulator of oxidase synthesis (4).

RESUMO

Efeito da saturação de oxigênio nos citocromos de membrana de *Bacillus stearothermophilus*

Estudos sobre o conteúdo e a composição dos citocromos de membranas de *B. stearothermophilus* crescido em condições de limitação e excesso de carbono em uma cultura quimiostática, mostrou que a disponibilidade de oxigênio e a natureza da específica limitação do crescimento, exerceu considerável influência na síntese dos citocromos. Quando este organismo cresceu, com excesso e limitação de oxigênio e foi limitado em seu crescimento pela disponibilidade de potássio, fósforo ou sulfato, ele sintetizou citocromos *c*, *d* e *o*. Em condições de limitação de magnésio, amonia e glicose e em reduzida aeração, citocromo aa_3 e citocromo *b* foram sintetizados. Entretanto, sob alta aeração citocromo *d* e citocromo *o* foram sintetizados. Em adição foi observado que a redução da disponibilidade de oxigênio aumentou a síntese dos citocromos que foram analisados.

Palavras-chave: Limitação de oxigênio, *Bacillus stearothermophilus,* citocromos, culturas quimiostáticas.

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CORRELATION BETWEEN PELLET SIZE AND GLUCOAMYLASE PRODUCTION IN SUBMERGED CULTURES OF *ASPERGILLUS AWAMORI*

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ABSTRACT

The influence of the spore concentration on pellet size and glucoamylase synthesis by *Aspergillus awamori* in shaken flasks was investigated. A series of runs were performed, varying spore concentration in the flasks from 9.5×10^3 spores/ml to 9.5×10^5 spores/ml. The flasks were maintained in a shaker, at 35°C and 200 rpm, for 24 hours. After this cultivation period, a cell suspension composed primarily by pellet was obtained. Glucoamylase production was found to vary considerably depending on the pellet size. The growth in the form of small pellets (between 0.59 mm and 0.74 mm) favored glucoamylase production (between 442 U/l and 490 U/l), while larger pellets (1.6 mm) led to a lower glucoamylase activity (70 U/l), after 24-hour cultivation. A mathematical correlation between the pellet size and glucoamylase activity was proposed.

Key words: *Aspergillus awamori,* glucoamylase, pellet size.

INTRODUCTION

Submerged cultures of filamentous fungi may grow either as free mycelium dispersed through the culture medium (filamentous form) or as pellets, which are spherical agglomerates of hyphaes. In the filamentous form, hyphal entanglement can cause the suspension to be highly viscous and pseudoplastic. The growth in the form of pellets leads to a lower broth viscosity, which enhances mixing and mass transfer properties of the suspension. Factors which affect growth morphology are: strain characteristics, inoculum size, medium composition, pH and shear forces (10).

Fungal fermentations generally require different growth morphologies for optimum product yield. Filamentous form is recommended for glucoamylase production (6), while the growth in the form of pellets is preferred for citric acid production (3) and for aflatoxin production (9). Spore concentration in inoculum is recognized to influence the characteristics of pellets. Sharma and Padwal-Desai (9) have studied the relationship between spore concentration in inoculum, pellet size and aflatoxin production. The spore concentration was found to have an inverse relationship with the aflatoxin yield, in stationary cultures. In addition, in shaker cultures, the aflatoxin yield was dependent on pellet size.

Aspergillus awamori cultivations aiming the production of glucoamylase are usually carried out in aerated fermenters, using a mycelium suspension as inoculum, which is pre-cultivated in a rotatory shaker. Depending on culture conditions, this shaker cultivation can lead to the formation of a pellet suspension, with almost no disperse filamentous growth in the broth (4).

This paper proposes a mathematical correlation between pellet size and glucoamylase production

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considering 24-hour shaker cultures, employing different initial spore concentrations.

MATERIALS AND METHODS

Strain. Spores of *Aspergillus awamori* NRRL 3112 stored in test tubes containing Czapek agar medium (5) were used. A spore suspension was prepared in Tween-40 (2) and the spore concentration in this suspension was quantified using a Neubauer counting chamber (1). This spore suspension was used to inoculate the flasks.

Culture medium. The medium for the shaker cultivation contained cassava flour syrup as the main carbon source (2). The initial total reducing sugar concentration was 20 g/l. The medium was complemented by the following nutrients: $(NH_4)_2SO_4$ (5.0 g/l); $Na_2HPO_4.12H_2O$ (3.78 g/l); KH_2PO_4 (3.50 g/l); $MgSO_4.7H_2O$ (0.50 g/l) and yeast extract (0.1 g/l). The pH of the medium was adjusted to 5.0.

Culture conditions: Several 1 L flasks containing 200 ml of the culture medium were inoculated with the spore suspension, varying the spore concentration in the flasks (C_{spore}): 9.5×10^3 spores/ml (runs S-1 and S-2), 8.8×10^4 spores/ml (run S-3), 9.5×10^4 spores/ml (runs S-4 and S-5), 9.5×10^5 spores/ml (runs S-6, S-7 and S-8). The flasks were incubated in a rotatory shaker for 24 hours, at 35°C and 200 rpm.

Analytical techniques: Samples collected from the 24-hour cultures were evaluated for: dry cell mass (X), total reducing sugar (TRS) (7), glucoamylase activity (A) (8), pH and pellet size (D) (4). One glucoamylase activity unit (U) was defined as the quantity of enzyme that releases 1 g of glucose per hour from a 4% (w/v) soluble starch solution, at pH 4.2 and 60°C.

RESULTS AND DISCUSSION

Cell suspensions, originated from the 24-hour shaker cultivations, presented different characteristics regarding cell concentration, glucoamylase activity and pellet size. These characteristics were found to be dependent on the spore concentration in the flasks (C_{spore}). Table 1 presents the results obtained in the experimental runs.

Regarding cell concentration, it can be observed from Table 1 that the use of a spore concentration of 9.5×10^3 spores/ml led to a cell concentration of about 1.4 g/l, after 24 hours of shaker cultivation. The increase in spore concentration led to a higher cell concentration. Spore concentration in the range from 8.8×10^4 to 9.5×10^4 spores/ml conducted to a cell concentration of about 3.0 g/l and a spore concentration of 9.5×10^5 spores/ml led to a cell concentration of about 5.0 g/l.

Coherently, the consumption of the carbon source was higher in the runs with higher spore concentration, due to the higher cell concentration obtained in these runs. After 24 hours of shaker cultivation, there was a residual substrate concentration of 11.0 gTRS/l, 8.6 gTRS/l and 9.4 gTRS/l, in the runs S-6, S-7 and S-8 respectively, performed with a spore concentration of 9.5×10^5 spores/ml. On the other hand, there was about 16.0 gTRS/l in the runs S-1 and S-2, performed with lower spore concentration (9.5×10^3 spores/ml).

Table 1 shows that a spore concentration of 9.5×10^3 spores/ml produced a growth in the form of pellets with an average diameter of 1.6 mm, while higher spore concentration (9.5×10^5 spores/ml) led to the formation of smaller pellets (0.74 mm in run S-6, 0.80 mm in run S-7 and 0.59 mm in run S-8). These facts can be visualised in Fig. 1. In Fig. 1C,

Table 1. Results of the runs, obtained from 24-hour shaker cultivations of *Aspergillus awamori*, using different spore concentrations.

Parameter	Experimental run							
C_{spore} (spores/ml)	9.5×10^3		8.8×10^4	9.5×10^4		9.5×10^5		
	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8
X (g/l)	1.20	1.60	2.31	3.22	2.9	4.48	5.09	5.00
pH	2.87	2.80	2.70	2.65	2.50	2.05	2.20	2.00
TRS (g/l)	15.8	16.0	16.2	12.4	14.2	11.0	8.6	9.4
A (U/l)	6.6	76	182	173	186	442	326	490
D (mm)	1.60	1.60	0.95	0.96	1.14	0.74	0.80	0.59

C_{spore}: Spore concentration in the flasks (spores/ml);
X: Cell concentration (g/l);
TRS: Total reducing sugar (g/l);
A: Glucoamylase activity (U/l);
D: Pellet diameter (mm).

with a spore concentration of 9.5 x 10^5 spores/ml, it can be observed that there was a fraction of the biomass in the filamentous form and another fraction in the pellet form.

The glucoamylase activity was also found to be dependent on the pellet size. The growth in the form of small pellets (between 0.59 mm and 0.80 mm) conducted to higher glucoamylase production (between 300 U/l and 500 U/l) than the growth in the form of larger pellets (1.6 mm), which led to a glucoamylase production of about 70 U/l, as shown in Table 1. Thus, spore concentration of 9.5 x 10^5 spores/ml led to the formation of smaller pellets and higher glucoamylase production than lower spore concentrations.

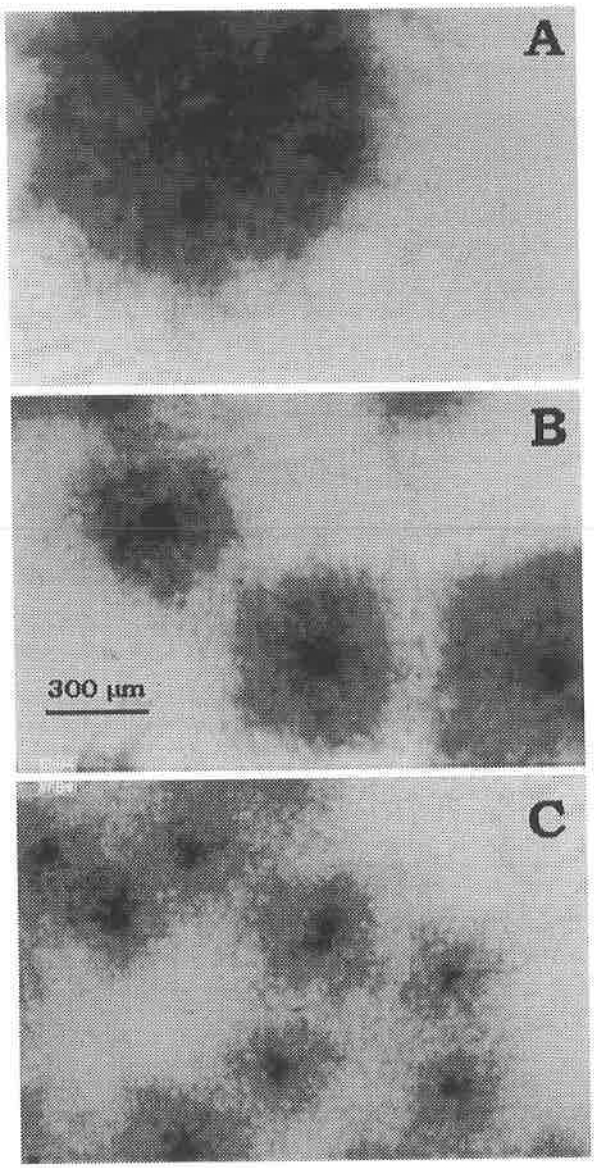

Figure 1. Pellets obtained in 24-hour shaker cultivations of *Aspergillus awamori*: (A) Spore concentration of 9.5 x 10^3 spores/ml. (B) Spore concentration of 9.5 x 10^4 spores/ml. (C) Spore concentration of 9.5 x 10^5 spores/ml.

From these results, it can be proposed a correlation between pellet size and glucomylase production in submerged cultures of *Aspergillus awamori*, after 24 hours of cultivation in shaker, as expressed below:

$$A = Ao \exp(-K.D) \quad (I)$$

The parameter Ao represents the theoretical value of the glucoamylase activity when the pellet diameter is zero (exclusively disperse filamentous form). It is a theoretical parameter since equation (I) is not valid in this limit condition. The parameter K is a decay constant.

Linearization of equation (I) leads to:

$$\ln A = \ln Ao - K D \quad (II)$$

Experimental results from Table 1 can be fitted to equation (II), obtaining the following parameters:

Ao = 1432 U/l; K = 1.9 mm^{-1}; correlation coefficient (r) = 0.97.

Fig. 2 shows the fit of equation (II) to the experimental data and Fig. 3 shows the relationship between glucoamylase activity and pellet size, after 24 hours of cultivation in shaker.

Thus, glucoamylase production was benefited by the growth in the form of pellets with a diameter from 0.59 mm to 0.74 mm. Pellets with a larger diameter (1.6 mm) decreased glucoamylase synthesis. It must be emphasized that the core (nucleus) of the pellet is formed mainly by non-viable cells because nutrient and oxygen transfer into the pellet is mainly due to molecular diffusion, which becomes more difficult with the increase in pellet size. Thus, pellet cores often become anaerobic and consequently fungal growth becomes confined to the external region of the pellet. So, the biomass viability in a 0.59 mm

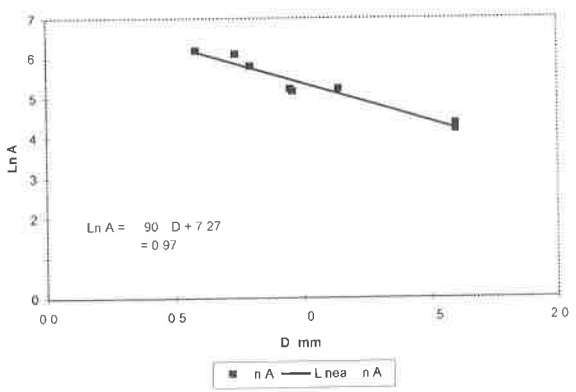

Figure 2. Fit of equation (II) to the experimental data, obtained from the 24-hour shaker cultivations of *Aspergillus awamori*.

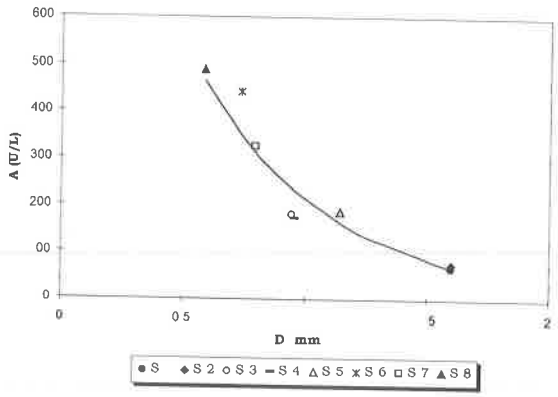

Figure 3. Relationship between glucoamylase activity and pellet size, after 24 hours of *Aspergillus awamori* cultivation in shaker.

pellet is expected to be higher than that one in a 1.6 mm pellet. The results indicate that, as a primary metabolite, glucoamylase is better produced by a culture with high cell viability and high specific growth rate, as reported in Ruohang and Webb (6).

In *Aspergillus awamori* cultivations, fungal morphology plays an importante role in glucoamylase synthesis. In particular, in shaker cultures, glucoamylase activity is dependent on pellet size. Higher glucoamylase production is reached if the growth occurs in the form of small pellets (between 0.59 mm and 0.74 mm). Larger pellets (1.6 mm) decrease glucoamylase synthesis. A mathematical correlation between glucoamylase production and pellet size could be proposed.

RESUMO

Correlação entre o diâmetro de "pellets" e a produção de glicoamilase por *Aspergillus awamori* em cultivo submerso

Neste trabalho, foi estudada a influência da concentração de esporos sobre o diâmetro de "pellets" e sobre a produção de glicoamilase por *Aspergillus awamori*. Foram realizados ensaios em incubador rotativo variando-se a concentração de esporos utilizada na inoculação dos frascos (entre 9,5 x 10^3 esporos/ml e 9,5 x 10^5 esporos/ml). Os frascos foram mantidos em incubador rotativo a 35°C e 200 rpm, por 24 horas, obtendo-se uma suspensão de células composta predominantemente por "pellets". A produção de glicoamilase nos frascos agitados mostrou-se dependente do tamanho dos "pellets". O crescimento na forma de "pellets" pequenos (entre 0,59 mm e 0,74 mm) favoreceu a produção de glicoamilase (entre 442 U/l e 490 U/l), enquanto "pellets" maiores (1,6 mm) levaram a uma atividade enzimática menor (cerca de 70 U/l), após 24 horas de cultivo. Foi proposta uma correlação matemática entre a atividade da glicoamilase e o diâmetro médio dos "pellets" presentes na suspensão.

Palavras chave: *Aspergillus awamori,* glicoamilase, diâmetro de "pellet".

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INDUCED FLOCCULATION INTENSITY OF A STRAIN OF *PICHIA STIPITIS*

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ABSTRACT

A procedure to induce flocculation intensity in the yeast *Pichia stipitis* is described. It basically consisted on successive transfers of the yeast cells retained within a column reactor to shake cultures, and reuse the cells that grew into aggregate form as an inoculum for another continuous run. Free cells culture were discharged. This selective methodology allowed to isolate a flocculating mutant, designated *P. stipitis* SP 10, which showed to be better retained within the reactor under continuous operation. The results reported here suggest good prospects to enhance fermentation rates from xylose substrates.

Key words: *Pichia stipitis*, xylose, induction, flocculation

INTRODUCTION

Flocculation is a widespread phenomenon in the microbial world and frequently used as an alternative scheme to obtain high cell density fermentation system (2, 3, 12). The most common application of flocculating microorganisms can be found in the production of ethanol for beverages and non beverages uses, employing several reactor configurations (2, 9).

Contrary to hexoses fermenting yeasts (i.e., typically *Saccharomyces cerevisiae*) which is a reasonably well understood system (3, 12), flocculation in pentoses fermenting yeasts is a very recent model of study (4, 6, 10). In these yeasts, flocculation has been reported in cells of *Pachysolen tannophilus* (4) and *Pichia stipitis* (6, 7, 10). However, the feasibility of using such kind of yeasts under continuous runs is still limited by their poor stability and weak flocculation tendencies (4, 6). If floc retention inside the reactor could be increased, then the phenomenon would have a great technological importance for ethanol production from hemicellulosic wastes.

In this work, an experimental protocol to induce flocculation intensity in the yeast *P.stipitis*, the most suitable yeast for bioconversion of xylose to ethanol, is described. This methodology has been already tested with satisfactory performance for several yeasts (4, 6) and bacteria (1) and therefore, it is expected that similar performance could also be achieved with *P. stipitis* in order to turn out this yeast strain potentially suitable for continuous fermentation.

MATERIALS AND METHODS

Microorganism and inoculum

P. stipitis CBS 5773 was employed. Stock culture was maintained at 4°C on malt agar slants. Yeasts were grown at 28°C for 24 hours in Erlenmeyer flasks containing basic YMP (yeast extract, 3.0 g/l; malt extract, 3.0 g/l; peptone, 5.0 g/l) with 10 g/l of xylose.

Media and Fermentation Conditions

The medium presented the following composition (in g/l): D-xylose, 50; $(NH_4)_2\ SO_4$, 5; K_2HPO_4, 1.0; $Mg\ SO_4.7H_2O$, 1.5; $CaCl_2$, 0.5 and yeast extract, 0.5. The fermentation runs were carried out in a simple

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column reactor fitted with a separate settling device and working volume of 0.25 l. The fermentation system utilized appropriate sensors to control the temperature and pH. Detailed specifications of the reactor are given elsewhere (9). All experiments were carried out at 30°C in a pH range of 4.8-5.0 (11).

Isolation of flocculate yeast strain

Actively growing yeast cells were obtained by incubation of YMP medium with 10 g/l xylose for 24 h at 28°C. After incubation, yeast cells were harvested by centrifugation and then transferred into a column reactor. Freshly prepared medium with 50 g/l xylose was then pumped into the column at linear flow rate of 20 ml/h to induce the formation of cell aggregates. Yeast aggregates were then removed from the reactor, centrifuged and inoculated into liquid YMP medium with 10 g/l xylose and incubated on a rotatory shaker for 24 - 48h. After this period, the cells were centrifuged and reused to start up another continuous run. This procedure was repeated several times, up to a point where significant change in the flocculation intensity was observed. Strain *P. stipitis*, codified as PS 10, was selected for a more detailed study.

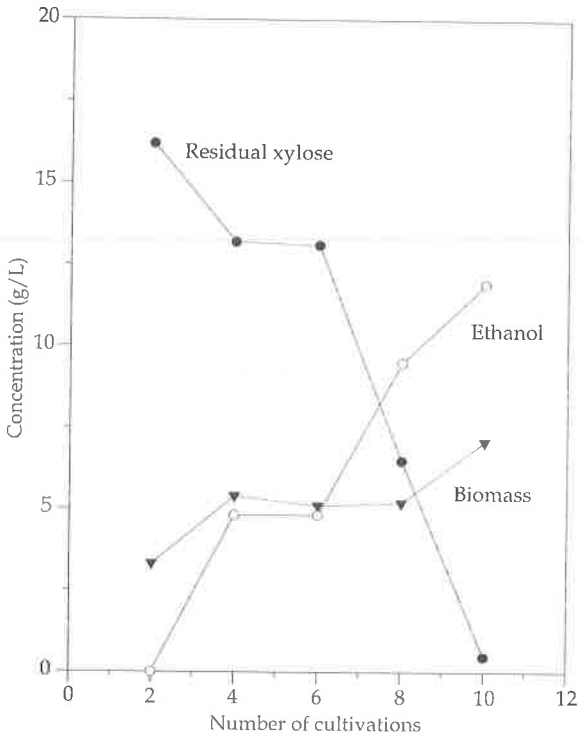

Figure 1. Concentration profile of xylose, ethanol and biomass as a function of transfer numbers carried out under continuous runs.

Analytical Methods

The cell concentrations in the overflow were determined based on the calibration curve correlating the optical density (600 nm) with celular biomass. Cell density in the reactor was determined directly by centrifuging 10 ml of culture, resuspending the cells in distilled water, recentrifuging, and then drying at 105°C to constant weight. The degree of flocculation, expressed as sedimentation rate, was determined by measuring the height of cells sediment in a milimetrically graduated cylinder as it settled at intervals of 1 minute (10). Xylose was assayed by DNS method (8). The ethanol concentration was measured by gas chromatography (CG model 3537) equipped with a FFAP 20% Chromosorb W column and flame ionization detector with an injection temperature of 110°C (11).

RESULTS AND DISCUSSION

Isolation of the variant strain

The concentrations of xylose, biomass and product, during cultivations under continuous mode, are shown in Fig. 1. Under batch cultivations, no ethanol was formed due to the low xylose levels used (data not shown).

The ratio between cell concentrations in the reactor (C) and that in the overflow (Co) was defined by Deverell and Clark (4) as the cell concentration factor (C_F) and will be used here to measure the flocculation intensity of *P. stipitis* cells during the continuous runs. As shown in Fig. 2, in the first continuous run, the concentration of cells in the overflow liquid was higher than in the mixed region (C_F = 0.6). By carrying out the successive culture transfers, as described in the methodology, there was a noticeable increase in the yeast settling accompanied by a build-up of wall growth and an increase in the amount of yeast deposited at the fermentor bottom. Therefore, a gradual increase in the flocculation intensity (C_F) was achieved, resulting in a cell concentration factor of about 12, after ten alternated cultivations under batch and continuous mode. A sample of the flocculating culture, taken from the reactor, was incubated on agar slants. No attempt was made to explain the mechanism by which cell aggregation occurred. However, the flocculation tendencies of the isolate type was tested by running a set of standard flocculation tests and the results were compared to those attained by the original strain, as shown in

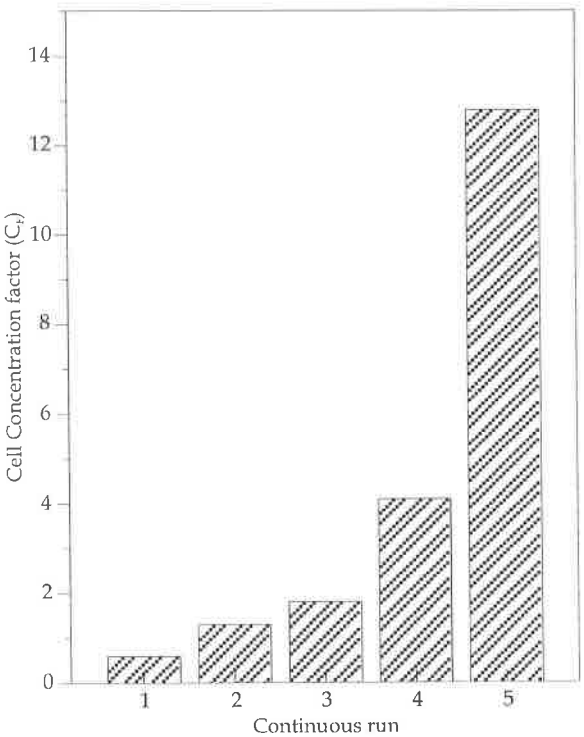

Figure 2. Modification of the flocculation intensity of *P.stipitis* during continuous runs using D-xylose. Cell concentration factor (C_F) is defined as the ratio between cell density inside the reactor and cell density lost in the effluent, as proposed by Deverell and Clark (4).

Table 1. Strain SP 10 exhibited much higher flocculation tendencies than the original strain. A marked increase in the settling values was also observed. Furthermore, the properties of the aggregated cells were similar to those of other induced yeast strains (4, 6); the aggregates were very stable in the presence of water and sodium acetate but could be dispersed by addition of sodium citrate in the presence and absence of magnesium (Table 2).

Behavior of *P. stipitis* SP 10 under continuous runs

To test the flocculating ability under continuous runs, a reactor incorporating cell recycle was inoculated with strain SP 10 cell suspension and fed with D-xylose (50 g/l). Performance data for the reactor, obtained at the working limits for a stable operation, are summarized in Table 3, along with the data attained by the original strain. The results show that with *P. stipitis* SP 10 an almost complete conversion of the substrate was possible at an overall dilution rate of 0.03 h^{-1}. Due to the low dilution rates needed for a complete conversion, the specific ethanol production rate was low: maximal 0.26 g/l.h.

On the other hand, with the original type of *P. stipitis*, the limitations were more severe. At a similar dilution rate, substrate conversion was 67% and the amount of yeast inside the reactor tended to decrease rapidly. Therefore, no cell aggregation occurred and the intrinsic instability of the system became the dominant feature.

Nevertheless, the utilization of the strain SP 10 attained cell concentrations in the range of 8-10 g/l in the fermentation system, which compares favorably with other reports (4, 6). This can be used as a starting point for the establishment of appropriate conditions for the reactor operation using xylose as the carbon source. These results also suggest that the main problem of the process is directly associated with the characteristics of the yeast aggregates. In this sense, the strategy of isolating a variant strain, according to the experimental methodology described in this work, can be considered a feasible solution to overcome the limitations in the reactor operation.

Table 1. Sedimentation properties of strains of *P. stipitis* CBS 5773 and SP10.

Characteristics	*Pichia stipitis* strains	
	CBS 5773	SP10
Flocculation velocity (cm/s)	0.03	1.56
Specific activity of flocculation (mg/ml/min)	0.008	0.02

Table 2. Effect of chemicals on the suspension of yeast aggregates by *P. stipitis* SP 10.

Chemicals	Aspect of yeast cells
Water	Flocs
Sodium acetate (0.1 M, pH 7.0)	Flocs
Sodium citrate + $MgCl_2$ (0.1M)	Dispersed
Sodium citrate (0.1M, pH 7.0)	Dispersed

Table 3. Comparison of the reactor performance using original and isolate *P. stipitis* strains in xylose.

Parameters	Strains of *P. stipitis*	
	CBS 5773	SP10
Residence time (h)	35.7	33.0
Xylose (g/l)	48.6	46.2
Residual xylose (g/l)	16.1	1.5
Biomass (g/l)	3.3	8.8
Ethanol (g/l)	4.8	8.70
Substrate conversion (%)	66.8	96.7
Volumetric productivity (g EtOH/l.h)	0.13	0.26

CONCLUSIONS

The procedure adopted in this work resulted in the isolation of a self-aggregating variant of *P. stipitis* SP 10, which showed better performance than its original strain under continuous process. Due to its flocculation intensity, the concentration of the yeast cells retained in the reactor was up to 12 times greater than in the overflow. This property resulted in a maximum volumetric productivity of 0.26 gEtOH/l.h. However, further improvements are still needed in order to increase the flocs retention inside the reactor up to the level attained by yeasts of the genus *Saccharomyces*, i.e, concentrations up to 90 g/l.

RESUMO

Indução da intensidade de floculação de uma linhagem de *Pichia stipitis*

A intensidade de floculação da levedura *Pichia stipitis* foi induzida através de seu crescimento alternado em regime de batelada e contínuo, empregando D-xilose como fonte de carbono. Foi isolada uma linhagem variante, codificada como *P. stipitis* SP 10, com propriedades floculantes mais intensas que as observadas na linhagem original. A avaliação dos resultados sugere que existem boas perspectivas para aumentar a velocidade de fermentação a partir de substratos de xilose.

Palavras-chave: *Pichia stipitis*, xilose, indução, floculação.

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OCCURRENCE OF SIALIC ACID IN *MAGNAPORTHE GRISEA*

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ABSTRACT

Cell surface of *Magnaporthe grisea* conidial forms were analyzed by binding assay with fluorescein isothiocyanate-labelled (FITC) lectins and enzymatic treatment. Flow cytofluorimetric analysis probed with lectins was also used. Our results showed that sialic acid and/or galNAc, gluNAc and galactose residues were present on the fungus surface, recognized by *Limulus polyphemus* (LPA), Wheat germ (WGA) and Peanut agglutinin (PNA) lectins, respectively. In addition, it was shown that sialic acids are glycosidically linked to galactopyranosil units as suggested by the increased reactivity of neuraminidase-treated conidial forms with PNA lectin. The presence of sialic acid on surface structures of *M. grisea* conidial forms may be an important function in the first stages of plant infection.

Key words: *Magnaporthe grisea*, sialic acid, conidia

INTRODUCTION

Magnaporthe grisea is the perfect state of what were formerly known to be *Pyricularia orysea*, pathogen of rice (*Oryza sativa*), and *Pyricularia grisea*, pathogen of grasses other than rice (2, 19, 20). It is a filamentous ascomycete involved in diseases of economically important crops such as barley, wheat, rice and millet, and is best known as the causal agent of rice blast disease and it is a problem in most rice-growing regions of the world (11).

In plant-fungus interaction, the cell surface of the fungal partner plays an important role, however, few molecular entities have been isolated from fungal phytopathogens (3, 4, 5, 7, 12). Sialic acid residues are constituents of glycoconjugates in some viruses, bacteria, protozoa, and a wide range of higher animals. They represent a family of about 30 derivatives of N-acetyl or N-glycolyl neuraminic acids (16). These components are acidic sugars and the major ionogenic component responsible for the negative charge on the surface observed in many cell types (14).

In pathogenic fungi, there are few reports suggesting the occurrence of sialic acid. The first description was in the hyphomycete *Sporothrix schenckii*, agent of sporotrichosis (1). It has also been found in *Fonsecaea pedrosoi*, one of the agents of chromoblastomycosis (18), and in the basidiomycetous yeast *Cryptococcus neoformans*, agent of cryptococcosis (8). In *Sporothrix schenkii* it was shown that sialic acids are important in the interaction of the yeast forms and mouse peritoneal macrophages (10). We reported previously that both yeast and mycelial forms of *Paracoccidioides brasiliensis* express sialic acids on their surface (17). The present investigation reports the presence of sialic acid on the cellular surface of the plant-pathogenic fungus, *M. grisea*.

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MATERIALS AND METHODS

Microorganism. *M. grisea* strain P_2/ 86 was obtained from Dr. Vanda Malavolta (Instituto Agronômico de Campinas, S.P., Brazil) and was maintained on potato dextrose agar medium (13). Conidial forms were obtained after cultivation for 48 h with shaking condition at ambient temperature (26 ± 3°C).

Enzymatic treatment. Cells were washed twice in PBS (0.01 M phosphate buffer 0.15 M NaCl), pH 7.0 and incubated for 30 min at 37°C in the presence of 0.4 $U.ml^{-1}$ neuraminidase from *Clostridium perfringens* (Sigma Chem Co., St. Louis, M.O., USA, type X).

Lectin binding. Neuraminidase-treated and untreated cells were fixed in 4% paraformaldehyde in PBS, pH 7.2, for 1h, rinsed with PBS, pre-incubated sequentially for 30 min in PBS containing 150mM NH_4Cl and then in PBS with 1% of bovine serum albumin (PBS-BSA) for 1h. Then, the cells were rinsed with PBS pH 7.2 and incubated with the lectins for 1 h in the presence of 1.2 $\mu g.ml^{-1}$ of one of the following FITC-conjugated lectins (Sigma): 1) LPA (*Limulus polyphemus*), 2) PNA (Peanut agglutinin), and 3) WGA (Wheat germ agglutinin). After incubation, the cells were washed three times in PBS and observed in flow cytometry analysis, using an EPICS ELITE flow cytometer (Coulter Electronics, Hieleah, FL), equipped with a 15 mW argon laser emitting at 488 nm. The system measures the fluorescence and laser light scattered from cells passing through a laser beam. The forward light scatter correlates with the particle size, and side scatter correlates with the granularity. The FITC fluorescence was measured between 510-540 nm, and both forward angel light scatter and side scatter were measured at 488 nm. The data obtained were run using listmode, which makes further analysis possible. Control cells were first analyzed in order to determine their autofluorescence, relative size and granularity.

The lectins FITC-labelled WGA, LPA and PNA were also used for determination of agglutinant titers of the conidial forms. Cells (1.5 x $10^5 ml^{-1}$) were suspended in PBS, pH 7.2 and fixed on slides with 5% formaldehyde in methanol for 30 min. Slides were washed 3 times with PBS and incubated with each of the lectins-FITC conjugates, at an initial concentration of 1.2 $\mu g.ml^{-1}$ in PBS for 30 min at 23°C. After incubation, the slides were washed three times in PBS, covered with 10% glycerol in PBS pH 7.2 and examined in a Zeiss epifluorescence microscope (Axioplan).

RESULTS

The agglutination titers of conidial forms of *M. grisea* with FITC-LPA, WGA and PNA are shown in Table 1. Treatment of this fungus with neuraminidase significantly decreased its binding to LPA and WGA. However, in the case of PNA, neuraminidase treatment significantly increased the recognition of the cells by this lectin, in relation to untreated-cells.

The flow cytofluorimetric analysis of fluorescent reaction of FITC-LPA, FITC-WGA and FITC-PNA lectins with *M. grisea* conidial forms, neuraminidade-treated and untreated, is shown in Fig. 1 and Table 2. Significant fluorescence was observed after incubation of the cells with FITC-labelled lectins LPA and WGA. Neuraminidase

Table 1. Lectin[a] binding to the *Magnaporthe grisea* conidial forms by fluorescence reactions with fixed cells, for determination of minimal concentration for fluorescent reaction.

Treatment of cells	LPA	WGA	PNA
	Minimal concentration (ng.ml) for fluorescent reaction[b]		
None	37.5	9.3	1200.0
Neuraminidase	150.0	75.0	9.3

[a]FITC-labeled lectins specificities: LPA (NeuNAc, GlcNAc and GalNAc), WGA ($GlcNAc_2$ and NeuNAc) PNA (ß-Gal(1-3)GalNAc).
[b]Concentration tested were 1/2 dilutions of a starting lectin solution at 1.200 ng.ml

Table 2. Percentage of fluorescent (% F) cells and mean of relative fluorescence (MRF) intensity determined by flow cytofluorimetry of neuraminidase-treated and untreated *M. grisea* conidial forms incubated in the presence of FITC-labelled lectins.

Treatment of cells	Control	LPA	WGA	PNA	LPA	WGA	PNA
		(%F)			MRF (+ SD)		
None		26.4	34.75	0.44	0.98±0.03	0.14±0.01	0.05±0.01
Neuraminidase		4.95	0.34	22.4	0.71±0.01	0.24±0.01	0.16±0.06

The values obtained were calculated and refered to the control and to the enzyme-treatment values, whithout binding of lectin. Data are the means ± standard deviation for one representative experiment (n=3).

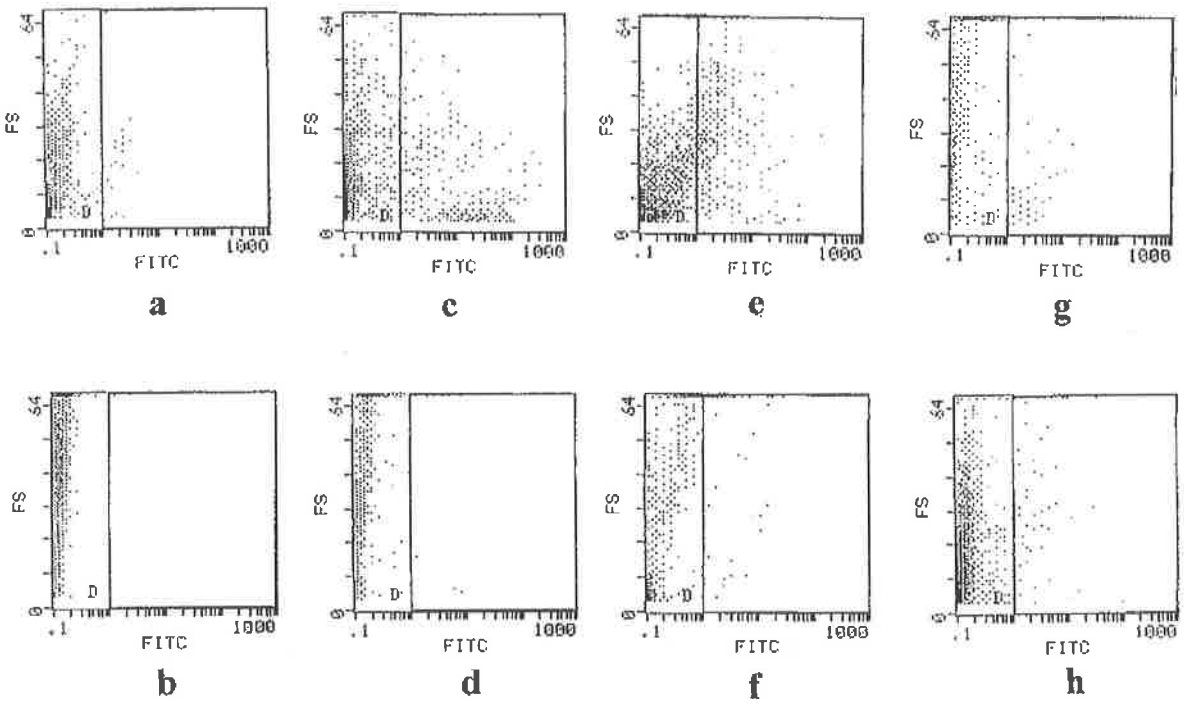

Figure 1. Representative flow cytometric results of *M. grisea* conidial forms neuraminidase-untreated or treated incubated in the presence of FITC-labelled lectins. **a**- Forward scattered light and log of the green fluorescence of untreated *M. grisea*, showing the autofluorescence inherent to this cell. **b**- Forward scattered light and log of the green fluorescence of neuraminidase-treated *M. grisea* showing the autofluorescence inherent to this cell. **c**- Distribution of *M. grisea* labelled with FITC-LPA. **d**- Distribution of neuraminidase-treated *M. grisea* labelled with FITC-LPA. **e**- Distribution of *M. grisea* labelled with FITC-WGA. **f**- Distribution of *M. grisea* neuraminidase-treated labelled with FITC-WGA. **g**- Distribution of *M. grisea* labelled with FITC-PNA. **h**- Distribution of neuraminidase-treated *M. grisea* labelled with FITC-PNA.

treatment of conidial forms before interaction with FITC-LPA and FITC-WGA resulted in a decrease in number of fluorescent cells. Whereas with FITC-PNA showed a significant increase in the number of fluorescent cells takes place.

Experiments carried out with fluorescence microscopy confirmed the results obtained by cytometry analysis. Labelling with FITC-WGA (Fig. 2c) was slightly more intense than FITC-LPA (Fig. 2a), probably because of chitin presence in these cells. FITC-PNA lectin (Fig. 2f) labelling showed that in this fungus, sialic acid is linked to galactose unit.

DISCUSSION

Lectins are useful tools for identification of carbohydrate residues exposed on cell surfaces (6,9). Sialic acids comprise several derivates of neuraminic acid including N-glycolyl, N-acetyl and O-acyl derivates (14). In *S. schenckii* N-glycolylneuraminic acid was the main derivate, and it could be isolated from the polar glycolipid fraction of the yeast form (1). In *F. pedrosoi,* N-acetyl neuraminic acid predominated in the mycelium, whereas N-glycolylneuraminic acid was mainly expressed in conidia (18). The differential expression of N-acetyl and N-glycolylneuraminic acids in *F. pedrosoi* may be related to the morphogenesis, or may reflect a response to different cultural conditions. Shaken, and therefore more aerated cultures, induce the formation of conidia which in turn synthesize mainly the N-glycolyl derivate.

The role of sialic acids in fungal pathogenicity in still uncertain. In *S. schenkii*, sialic acids have a protective effect against phagocytosis, before an efficient immune response takes place (10). Also, these molecules are essential components of receptors for peptide hormones, toxins, viruses and *Mycoplasma* species. Infection of cells by these bacterial species is exclusively dependent on the presence of sialic acids on the cell membranes (15). On the other hand, the expression of sialic acids by fungal species which cause local infections with little tendency to spread argues against a role of sialic acid in fungal invasiveness (1).

The results obtained *M.grisea* showed that sialic acids are distributed all over the cell surface when binding is done with FITC-LPA and -WGA lectins. WGA recognize GluNAc and NeuNAc, and also chitin. Neuraminidase treatment significantly reduced the binding of both LPA and WGA, indicating the presence of neuraminidase-sensitive sialic acid residues on the cell surface of this fungus.

Flow cytometer measurement results have shown that binding with WGA and LPA was 3.9 and 3.5, respectively, of the cells showing fluorescence, when compared with untreated neuraminidase cells. When neuraminidase treated cells were bound with FITC-PNA lectin, the cells showed an increase in fluorescence of about 22.4%. This suggests that sialic acid residues are preceded by galactose, and as on the cell surface, probably in the sialoglycoconjugates (14).

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RESUMO

Ocorrência de ácido ciálico em *Magnaporthe grisea*

A superfície celular de formas conidiais de *Magnaporthe grisea* foi analisada através de ensaio com lectinas marcadas com isotiocianato de fluoresceína (FITC), tratamento enzimático e citometria de fluxo. Foi observada a presença de

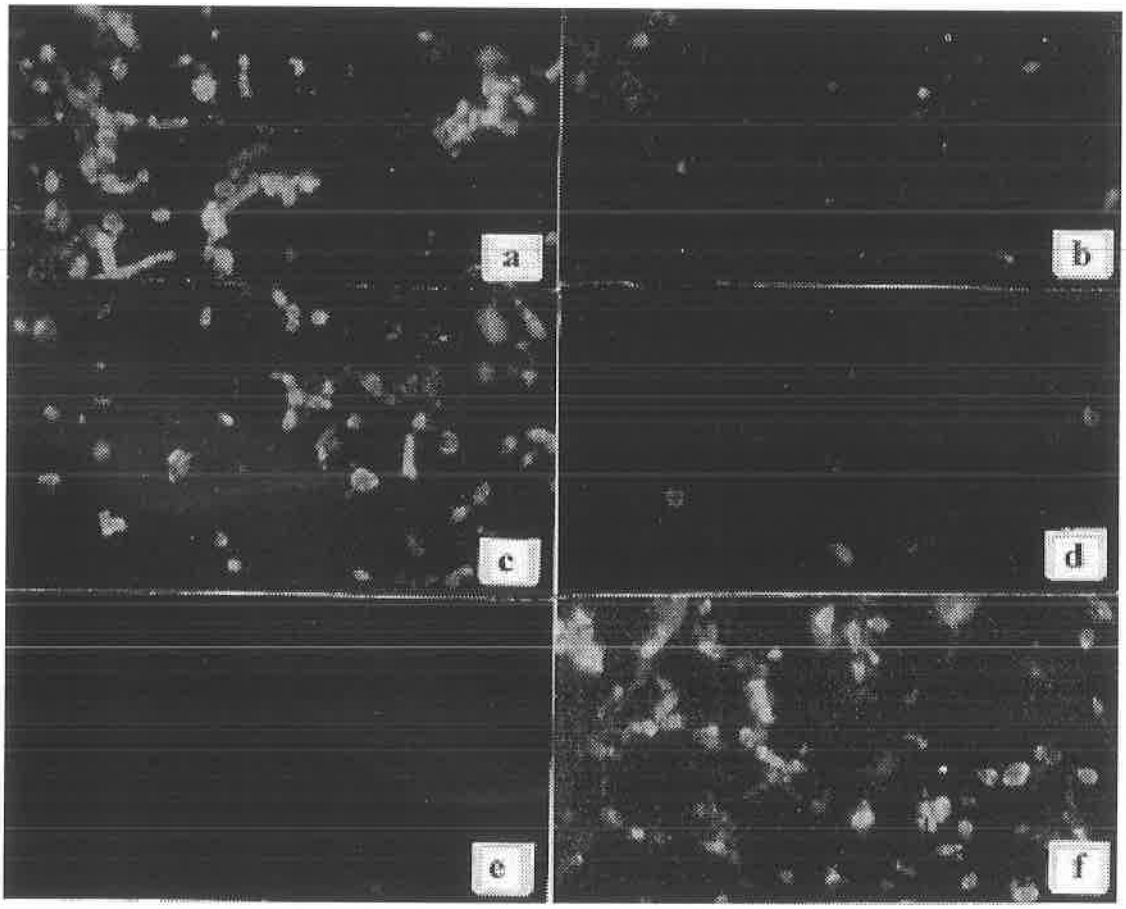

Figure 2. Fluorescence image of neuraminidase-untreated or treated *M. grisea* conidious forms and incubated in the presence of FITC-labelled lectins. **a-** *M. grisea* labelled with FITC-LPA showing strong fluorescence all over the cell. **b-** neuraminidase-treated *M. grisea* cells labelled with FITC-LPA showing fluorescence was markedly reduced. **c-** *M. grisea* labelled with FITC-WGA showing intense fluorescence all over the cell. **d-** neuraminidase-treated *M. grisea* cells labelled with FMITC-WGA showing fluorescence was markedly reduced. **e-** *M. grisea* labelled with FITC-PNA showing no fluorescence was observed. **f-** Neuraminidase-treated *M. grisea* cells labelled with FITC-PNA.

ácido siálico e/ou GalNAc, GluNAc, resíduos de galactose dispostos na superfície do fungo e reconhecidos pelas lectinas *Limulus polyphemus* (LPA), Wheat germ (WGA) e Peanut *a*gllutinin (PNA), respectivamente. Em adição, os ácidos siálicos estariam glicosidicamnte ligados à unidades de galactopiranosil, como sugerido pelo aumento da reatividade das formas conidiais após tratamento enzimático e ensaio com PNA. A presença de ácido siálico na superfície das formas de conídio de *M. grisea* pode desempenhar importantes funções nos primeiros estágios de infecção na planta.

Palavras chave: *Magnaporthe grisea*, ácido ciático, conídios

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CYANIDE PRODUCTION BY BRAZILIAN STRAINS OF *AZOSPIRILLUM*

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SHORT COMMUNICATION

ABSTRACT

Azospirillum strains were screened for cyanide production using picric acid impregnated paper strips and 73 of 110 were found positive. Confirmation by a colorimetric method in 12 of 20 randomly selected strains confirmed HCN production by *Azospirillum*.

Key words: *Azospirillum*, cyanide production, nitrogen fixer, bacterial metabolites

The metabolism of cyanide by microorganisms has been investigated with respect to its production and degradation (7,11,13). Cyanide is a secondary metabolite of microorganisms (4). It can be produced directly from some aminoacids like glycine and methionine, from cyanogenic glycosides and from benzonitrile derivative herbicides (11,15,19). Although cyanide production is widespread in fungi (7), certain pseudomonads (*Pseudomonas aeruginosa, P. fluorescens, P. clororaphis and P. auerofaciens)*, *Chromobacterium violaceum* and *Flavobacterium* sp are the only bacteria known to produce this substance (1,2,11,19).

Little is known about cyanogenesis in the rhizosphere. The substrates for cyanogenesis, glycine and cyanogenic glycosides, have been demonstrated in root exudates (15). Some investigations have provided evidence for the occurrence of non-parasitic cyanide producing bacteria with a potential to suppress root plant disease (6,15,20). *Azospirillum* are Gram-negative soil bacteria that fix nitrogen under microaerophilic conditions. Five species described occur as free-living forms in soil or in association with roots of many important cereal crops, grasses and tuber plants (12). They are considered to be plant growth promoting rhizobacteria (PGPR) (3). Indirect plant growth promoting activity by PGPR may occur when these bacteria displace or antagonize native deleterious microorganisms. There is an increasing interest in the active substances produced by azospirilla that could participate in the process of root colonization and biological control mechanisms. It is already established that some azospirilla strains produce bacteriocins (17), antibiotics (10) and siderophores (18), but *Azospirillum* has not been reported to be cyanogenic. The purpose of this work was to investigate cyanide production by azospirilla, especially strains isolated from maize roots.

Thirty seven strains of *A.lipoferum* and 73 strains of *A. brasilense,* were studied, 46 of which were isolated recently from maize roots growing in podzolic red-yellow soil (from EMBRAPA fields, Seropédica, RJ), and 64 came from the laboratory culture collection. The bacteria were maintained at 4°C on semi-solid potato agar (12) and transfered every two months. The bacteria used for the

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experiments were obtained from 24 hour cultures grown on the semi-solid potato agar at 32°C.

The bacteria were grown on slants of casaminoacids peptone-glucose agar-CPG medium (5), peptone agar (4), tryptic soy agar supplemented with 0.5% yeast extract (8), glutamic acid-glycine agar (4) succinic acid-glycine agar (21) and nutrient agar-NA (17). A piece of filter paper impregnated with 0.5% picric acid and 2% sodium carbonate was inserted into the tube without touching the medium and held in place with a screw-cap. The tubes were sealed with parafilm and incubated at 37°C for five days. A color change, after incubation, of the filter paper from yellow to orange-brown or to orange, indicated microbial production of cyanide (2,4).

To ascertain if the picric acid color change was actually due to cyanide production, 20 strains positive for the picric acid test were reexamined using the isonicotinic acid-barbituric acid method for the determination of cyanide (16).

The strains were grown on succinic acid-glycine agar containing 0.1M K H_2PO_4, 0.02M NH_4 Cl, 0.001M Mg SO_4 7 H_2O, 0.02M succinic acid, 15g agar per liter, and adjusted to pH 7.2 (21). After autoclaving, the medium was enriched with 10 ml of a separately-autoclaved solution containing 2 mM glycine, 0.02 M Fe Cl_3 6 H_2O, and 0.1 M Na_4 P_2O_7, distributed into steril tubes and inoculated. Filter papers impregnated with 1.0 M NaOH were placed in the tubes (2). The cultures were incubated at 32°C for 4 days. Cyanide trapped in the filter paper was detected by using the method of Nagashima and Ozawa (16). The solid-Nfb medium supplemented with 0.5% NH_4Cl and 2 mM glycine was also examined using these strains (12).

The effect of temperature (28°C, 32°C and 37°C), incubation time (24h, 48h, 96h), and phosphate on cyanide production by *A. brasilense* RZ 38, a rhizosphere maize isolate, was also examined on succinic acid-glycine agar using the same procedure described above.

Cyanide production in a plant-bacteria system was studied *in vitro*. Maize seeds surface sterilized by Hg Cl_2 were pregerminated in Petri dishes with humid paper. After incubation in darkness at room temperature, four- day seedlings were transferred to the surface of Hoogland nutrient solution solidified with 0.5% of agar, distributed in 200 x 20 mm tubes (9). The seedlings were inoculated with $1x10^8$ colony forming units (cfu) of *A. brasilense* strain RZ 38, which had been grown in 50 ml of CPG broth at 32°C for 24h. Control plants were treated with water. The plants were incubated in a greenhouse with natural light, at room temperature. Bacterial production of HCN was determined as previously described using the isonicotinic acid-barbituric acid method, after 7 days incubation in the Hoogland nutrient solution, supernatant was obtained by the freeze-drawn method (14) and in filter paper strips impregnated with 1.0 M NaOH, put into the tubes at the inoculation time.

Of the 110 strains examined, 17 strains of *A. lipoferum* and 56 strains of *A. brasilense* were cyanogenic at least in one medium, with variable production. The results obtained showed that the capacity to produce this compound was influenced by the medium composition (Table 1). The percentage of producer strains ranged from 2.7% on glycine-L-methionine glutamic acid medium, to 55% on casaminoacids-peptone glucose agar (CPG medium). The peptone agar and CPG medium were more suitable to screen for cyanide production (Table 1). There were no differences observed in cyanide production between the strains recently isolated from maize roots and those maintained in the laboratory collection for more than 10 years (data not shown).

Table 1 - Cyanide production by *Azospirillum*

Medium*	N° of Strains	N° of Producer Strains**			% of Producers
		+++	++	+	
1- CPG	109	7	20	34	55.9
2- NA	109	8	20	20	44.0
3- PEP	109	12	24	23	54.0
4- TSA + YE	105	4	23	20	44.7
5- GGA	110	0	0	3	2.7
6- SGA	110	3	13	30	41.8

* CPG = casaminoacids peptone-glucose agar; NA = nutrient agar; Pep = peptone agar; TSA +YE = tryptic soy agar supplemented with 0.5% yeast extract; GGA = glutamic acid-glycine agar; SGA = succinic acid-glycine agar

** Results based on the color change of the filter paper to: intense orange-brown (+++) or orange brown (++) or weak orange (+) color.

Of 20 strains reexamined, using the succinic acid-glycine agar and the colorimetric method to detect cyanide production, 12 strains produced cyanide. These results have confirmed that HCN could be produced on a chemically-defined medium by *Azospirillum*. Solid Nfb supplemented with 0.5% NH_4Cl and 2mM glycine was also examined using these strains, but only two strains were cyanide producers in this medium.

The effect of temperature, incubation time, iron and phosphate on cyanogenesis by *A. brasilense* strain RZ38 isolated from the maize rhizosphere was studied on succnic acid-glycine agar. The maximum cyanide production occurred at 32°C after 96 h of incubation. The HCN production was not observed in this medium without phosphate. In the plant-bacteria experiment, cyanide production also was not observed.

Growth of potentially cyanogenic microorganisms on individual agar slants, with filter paper strips impregnated with a cyanide-sensitive chemical, alkaline picrate, suspended above the growing culture, demonstrated that some *Azospirillum* strains were cyanogenic. Microbial cyanide biosynthesis has been demonstrated in many species of fungi, and cyanide is probably a normal fungal metabolite, but only a few species of bacteria in the genera *Chromobacterium, Pseudomonas* and *Flavobacterium* are known to produce this substance (2,11,19). This is the first report of cyanogenesis in *Azospirillum.*

The cyanide production by *Azospirillum* follows the same pattern observed for *Pseudomonas* (21). The differrence observed in cyanide production with the media examined was probably due to nutrient composition of these media that contain different amounts of factors enhacing bacterial cyanogenesis, e.g. aminoacids like glycine and methionine (1,4). In this study, succinic acid-glycine agar (21) and the special assay developed by Nagashima and Ozawa (16) were used to confirm cyanide production by *Azospirillum*. This medium was selected since glycine is a cyanide precursor (1).

We have demonstrated that some *Azospirillum* strains are cyanide producers. Current knowledge of biocontrol mechanisms exerted by PGPR bacteria have established that bacterial metabolites involved in this phenomenon include antibiotics, siderophores and cyanide. As *Azospirillum* is also regarded as a PGPR, its cyanide production ability in association with other metabolites could contribute to the suppression of deleterious rhizosphere microorganisms.

RESUMO

Produção de cianeto por cepas brasileiras de *Azospirillum*

Cento e dez cepas de *Azospirillum* foram examinadas para a produção de cianeto usando tiras de papel de filtro impregnadas com ácido pícrico, destas 73 foram positivas. A confirmação da produção de HCN por *Azospirillum* foi obtida, através de um método colorimétrico, em 12 de 20 cepas selecionadas ao acaso.

Palavras-chave: *Azospirillum,* produção de cianeto, fixador de nitrogênio, metabólitos bacterianos.

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INFLUENCE OF THE MAINTENANCE METHOD ON THE CADMIUM BIOSORPTION CAPACITY OF *MICROCOCCUS LUTEUS*

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ABSTRACT

The influence of microbial maintenance in subculture on the cadmium biosorption capacity of *Micrococcus luteus* strain CD5, isolated from soil contaminated with heavy metals, and INCQS/CCOC strain 012 was evaluated. Storage on nutrient agar, with and without mineral oil, supplemented and without cadmium, was efficient in preserving cadmium biosorption for both strains.

Key words: Maintenance, *Micrococcus luteus*, biosorption, cadmium

Industrial activities have contributed extensively to the increased levels of heavy metal pollutants in natural waters, through the discharge of contaminated effluents from industrial plants. Some of these metals, including cadmium, can accumulate up the food chain, even if present at low levels in industrial effluents, and build up to dangerously elevated concentrations in fish and other animals eventually consumed as food by humans (1). Several studies (9, 11) have demonstrated that removal of metal pollutants by selected microorganisms is a promising approach, since they can present a high and efficient rate of metal accumulation. Furthermore, as they have a potential for biomass regeneration, they can be recycled and allow recovery of the biosorbed metal. When the technology is based on the use of microorganisms, their preservation with stable properties is an important aspect to be considered, deserving the same attention as standardization of equipment or selection of raw material. The aim of this study was to evaluate the influence of different subculture methods on preservation of the cadmium biosorption capacity of two bacterial strains (*Micrococcus luteus* CD5, isolated from soil contaminated with heavy metals, and *M. luteus* INCQS/CCOC 012 (=ATCC 14452) from the Instituto Nacional de Controle de Qualidade em Saúde culture collection) selected in previous studies (5).

The strains were maintained by subculturing on nutrient agar media supplemented as needed with 17.9 mg/l of $CdCl_2.H_2O$ and stored at 5°C, with and without a covering layer of mineral oil. Tests for evaluation of cadmium uptake were carried out in flasks containing an aqueous solution of $CdCl_2.H_2O$, pH 5.3, (metal ion concentration = 30 mg/l) and 0.5 g/l of cells (dry weight at 450 nm). The flasks were incubated with shaking (150 rpm) at 30°C for 6 hours and samples taken after 5 minutes and 6 hours. The amount of cadmium accumulated by the cells

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was calculated as the difference between the initial concentration (30 mg/l) and the residual concentrations of the metal in solution. Cadmium concentrations were determined by atomic absorption spectrophotometry (Varian Techtron Spectrometer, Model AA6). All the maintenance subculturing and biosorption experiments were conduced in triplicate.

The cadmium biosorption capacity of *M. luteus* INCQS/CCOC 012 preserved without mineral oil are shown in Fig. 1A. After 8 weeks of storage, a nearly 100% increase in uptake capacity was observed for nutrient agar with and without Cd, although a greater variability of biosorption was observed in cultures maintained in the absence of the metal. *M. luteus* INCQS/CCOC 012 comes from a culture collection and had been kept lyophilized for 9 years, a procedure known to affect some microorganisms by causing, for instance, damage to the cell membrane (3,7). Therefore, this strain may require a lag time before reaching full expression of its "dormant" ability to accumulate cadmium, which could explain the observed improvement in cadmium uptake following the initial period of maintenance (up to 8 weeks) in subcultures.

Fig. 1B shows cadmium uptake by *M. luteus* CD5 preserved with and without mineral oil. A practically constant biosorption behavior can be observed under both maintenance conditions, indicating that the characteristic of Cd uptake remained stable in this soil-borne strain during the storage period studied (24 weeks).

Results on the cadmium biosorption capacity of *M. luteus* INCQS/CCOC 012 and *M luteus* CD5 kept under mineral oil are presented in Figs. 2A and 2B, respectively. Fig. 2A shows a 50% increase in the uptake of cadmium after the 4th week of maintenance of *M. luteus* INCQS/CCOC 012 in nutrient agar only. However, starting on week 8, a total lack of cell viability was recorded at each evaluation time over the following 16 weeks of storage, and consequently evaluatin at Cd uptake was not possible. In contrast, the presence of cadmium in the maintenance medium coupled to preservation under mineral oil led at first to a progressive increase in cadmium biosorption, with maximum levels (approximately 135%) recorded on week 8. This was followed by some reduction of uptake between weeks 12 and 24 and total loss of cell viability only after 24 weeks of storage. Addition of cadmium to the maintenance medium appears to have conferred some degree of protection to the bacterium, retarding the early loss of cell viability observed in medium without cadmium. This protection may be related to the presence of metallothioneins (cysteine-rich proteins) described in several eukariotic microbes and bacterias (8), that bind heavy metals such as Cd, Zn, Cu and Hg, giving more resistance to the microorganism.

Fig. 2B shows a reduction in cadmium uptake by *M. luteus* CD5 4 weeks after storage in media with and without the metal (15% and 20%, respectively). From week 8 onwards, the levels of cadmium accumulation increased, reaching a maximum after 16 weeks (52 mg/g for both types of maintenance medium).

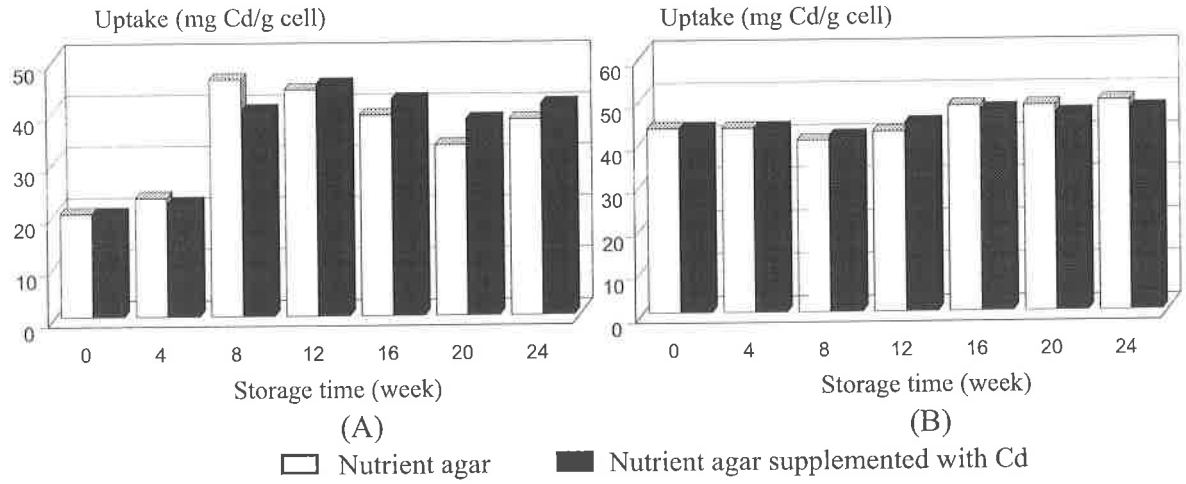

Figure 1 - Cadmium uptake profile of *Micrococcus luteus* INCQS/CCOC 012 (A) and *Micrococcus luteus* CD5 (B) maintained in nutrient agar or nutrient agar supplemented with cadmium, in the absence of mineral oil. Metal uptake evaluation was performed in an aqueous solution of $CdCl_2$ Initial cadmium concentration = 30 mg/l; *M. luteus* concentration = 0.5g cells(dry weight)/l.

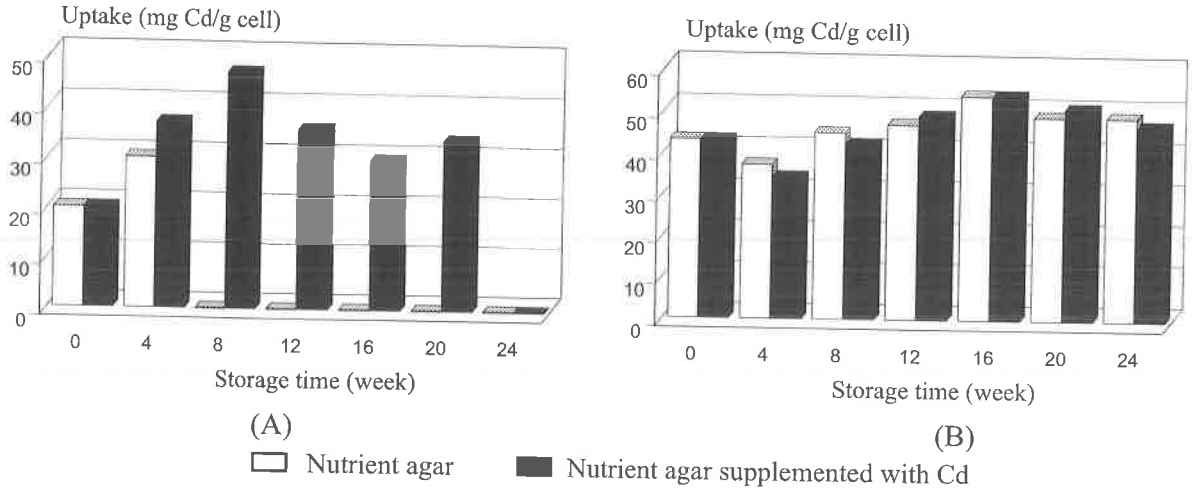

Figure 2 - Cadmium uptake profile of *Micrococcus luteus* INCQS/CCOC 012 (A) and *Micrococcus luteus* CD5 (B) maintained in nutrient agar or nutrient agar supplemented with cadmium under mineral oil. Metal uptake evaluation was performed in an aqueous solution of $CdCl_2$. Initial cadmium concentration = 30 mg/l; *M. luteus* concentration = 0.5g cells(dry weight)/l.

The effects of mineral oil on cultures of microorganisms are not very discussed in the literature (2,6) and partly because of the appearance of more efficient and sophisticated storage methods such as lyophilization and cryopreservation.

Hartsell (6) studied microbial preservation on subcultures under mineral oil and established the efficiency of the method for various genera of fungi and bacteria. These microorganisms remained viable for storage periods as long as 14 years. However, the author also reported the acquisition or loss of some characteristics, such as carbohydrate utilization and production of NH_3 and H_2S among various genera of bacteria after prolonged storage. The influence of the maintenance procedure on microbial behavior, as demonstrated by the loss of certain characteristics, was also observed by Nadirova and Zemlyakov (10). These authors described a reduced rate of carbohydrate utilization in some cultures of *Pseudomonas* sp maintained under mineral oil for 3 years. In this particular case, however, the effect was reversed by propagation of the stored culture in fresh medium.

The presence of cadmium in the maintenance medium appears to have conferred some resistance to *M. luteus* INCQS/CCOC 012, apparently retarding a lethal effect of the oil on viable cells, and the initially lower levels of heavy metal uptake exhibited by *M. luteus* CD5 were followed by a period of gradual increase yet final reduction in cadmium biosorption over 24 weeks of storage. This suggests that a longer period of preservation should be investigated. In this way, it will be possible to evaluate more comprehensively to what extent the capacity of *M. luteus* to accumulate cadmium from contaminated soil remains stable after prolonged storage in subculture under mineral oil. The method of maintenance by subculture allowed a good stability of the characteristic of cadmium biosorption of *M. luteus* CD5 in both types of nutrient agar with and without Cd preserved or not under mineral oil.

RESUMO

Influência da manutenção na capacidade de biossorção de cádmio por *Micrococcus luteus*

Neste estudo foi avaliada a influência da técnica de manutenção em subcultura na captação de cádmio por *Micrococcus luteus* linhagem CD5, isolada de solo contaminado por metais pesados, e linhagem INCQS/CCOC 012. Os resultados mostraram que as condições de estocagem em ausência e em presença de óleo mineral, em meio suplementado ou não com cádmio, foram eficientes na preservação da característica de captação para ambas as linhagens.

Palavras-chave: Manutenção, *Micrococcus luteus*, biossorção, cádmio.

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LEAD AND COPPER TOXICITY TO *NEPHROCYTIUM LUNATUM (CHLOROPHYCEAE)* AND THEIR COMPLEXATION WITH EXCRETED MATERIAL

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ABSTRACT

Cells of the freshwater Chlorophyceae *Nephrocytium lunatum* were grown in batch cultures and submitted to various copper and lead concentrations when cultures were already in the exponential phase of growth. Cultures exposed to a total copper concentration of 2.5×10^{-7} M or 1.0×10^{-6} M of lead resumed growth after a short period of adaptation, while those exposed to 2.5×10^{-6} M of copper and 1.0×10^{-4} M of lead failed resuming growth even after 10 days of incubation with the metal. Metal complexation capacity of the excreted material produced by *N. lunatum* in two different growth phases (exponential and stationary) was evaluated using ion selective electrodes. The results revealed that copper is chelated by the dissolved organic materials, while lead is not.

Key words: toxicity, *Nephrocytium lunatum*, copper, lead, complexation

INTRODUCTION

The release of heavy metals such as copper and lead into natural water systems as a result of certain industrial processes has lead to increasing concern about the effects of toxic metals in the environment. The capacity of self-purification of a healthy and natural aquatic ecosystem is dependent on the maintenance of the equilibrium on the functioning of phytoplanktonic based food webs (20). Hence, any disorder which affects these organims will also affect the natural ability of self-purification of the system.

The biological fate of toxic metals is organism-dependent and related to the life history of the organism. It is the ability of ions of both essential and toxic metals to combine with other entities that determines, to a large extent, their biological activity and speciation in aqueous solution. Toxicity occurs when an organism is unable to cope with additional metal concentration either by direct usage, storage or excretion (1). Moreover, not all chemical forms of a metal are toxic or dangerous to aquatic life. It has been shown that the most toxic is the hydrated or free ion (2, 14, 15).

Complexation generally lowers the biological availability of a given metal (16). Thus, by release of metabolites able to complex metals, algae may modify metal speciation in culture media and natural waters, controling metal availability and/or toxicity in the environment. Allen and Hansen (1) have presented a detailed assessment of how speciation is expected to affect the toxicity.

Nephrocytium lunatum (Chlorophyceae) was used in the present investigation due to the high amount of mucilaginous material produced and released into the external environment. The present study was undertaken to evaluate copper and lead toxicity to cells of the freshwater algae during exponential growth phase and also to verify copper and lead complexation by the excreted material.

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MATERIALS AND METHODS

Cultures. Axenic cultures of *Nephrocytium lunatum* (clone 065CH), isolated from Reserva Ecológica do Jataí, SP, Brazil obtained from the culture collection of the Departamento de Botânica, Universidade Federal de São Carlos. Cultures were kept in modified WC medium (7). The modification consisted of absence of EDTA and Tris when cells were cultivated to obtain the excreted material; without EDTA but with Tris (300 mg x l^{-1}) for the toxicity experiments. Growth was quantified by chlorophyll-a analysis (19). Cultures were kept at controled temperature (20 – 22°C), with a light/dark cycle of 12:12 hours and light intensity of 200 $\mu mol.m^{-2}.s^{-1}$ (photon flux density) provided by 40W fluorescent tubes. For the toxicity experiments, metal additions (copper as $CuSO_4$) or (lead as $Pb(NO_3)_2$) were done when cultures were already at the exponential phase of growth. Note that metal concentrations presented in the toxicity experiments refer to values added to the culture medium and consequently ionic concentration is lower (11). This is due to problems of adsorption onto the culture flask (glass), complexation with culture medium (inorganic nutrients and Tris) and also with the excreted material. Two replicate experiments were performed on 1000 ml glass Erlenmeyer flasks with 450 ml of culture.

Metal complexation. Filtrates from algal cultures were obtained by gentle vacuum filtration (35cm Hg) employing firstly glass fiber filters which were previously baked overnight at 400°C and then pre-washed membrane filters (Schleicher and Schüll, 5 µm and 1.2 µm). These filters are known to release metals and organic materials even after the passage of one litre of water (9), thus they were left in 1.0 M HCl for 24 hours and then rinsed with glass distilled water. Samples were filtered at both exponential and stationary growth phases.

Copper complexing properties of the filtrates were obtained by titrations monitored using ion-selective electrode (ISE). Equilibration times for ISE were dictated by the response of the electrode and free metal ion concentration. For a total copper concentration of 8 x 10^{-7} M, the first addition in the titration curve, an equilibration time of about 4 hours was required. The ISE equilibration time decreased as total copper concentration increased. Potential readings were obtained using a Digimed DMPH-2 pH meter with 0.01 mV resolution. The cupric ion-selective electrode (ORION - Model 94-29) and the lead ion-selective electrode (ANALION - Model F656) were both used in conjunction with an ANALION double junction reference electrode (Ag/AgCl). Constant temperature (21°C) was maintained throughout.

The titrations, in which copper ($CuSO_4$) or lead ($PbNO_3$) were added incrementally from 8 x 10^{-7} M to 4.4 x 10^{-5} M at constant pH and ionic strength, were buffered (pH 6.1 ± 0.1) with sodium acetate (MicroSelect, Fluka Chemie, Germany) at a concentration of 1 x 10^{-2} M and ionic strength adjusted to 2 x 10^{-2} M with $NaNO_3$ (Fluka, MicroSelect).

Data treatment. Titration data were converted from millivolts to pCu or pPb by means of the Nernst equation, in which the electrode slope was taken from calibration curves. This calculation may be found in specialized textbooks such as Evans (5) and Covington (4).

The complexation parameters conditional stability constant (K') and total ligand concentration (CL) were obtained using Scartchard Plot. This technique is based on the concentration of metal bound to the ligand (CuL) divided by the ionic metal concentration (Cu^{2+}) which is then plotted as a function of CuL. When more than one binding site is present, a concave shaped curve is obtained. As presented in Fig. 1, the plot may be divided into two (or more) straight lines. Conditional stability constants K'_1 and K'_2 are the -(slope of line 1) and -(slope of line 2) respectively. The extrapolation of the two lines to both the *y* and *x* axes provides (K'_1L_1 + K'_2L_2) and ($L_1 + L_2$) respectively. Thus, after a linear regression analysis of each line, one will have: $y = a + bx$, in which a/b = CL and b = K'. For details of the treatment and comparison to other models the reader is refereed to Scatchard et al. (12), Jardim (8) and Buffle (3).

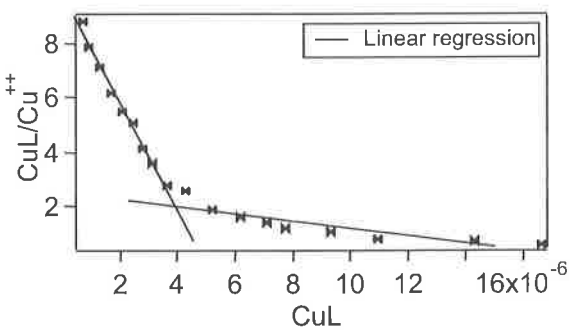

Figure 1. Scatchard plot for titration data obtained from culture filtrates of *Nephrocytium lunatum* at the exponential growth phase. I=2 x 10^{-2}M, pH=6.1.

RESULTS AND DISCUSSION

The toxic effect of copper, an algal nutrient required in diminute, but toxic in higher concentrations, was detected at 2.5 x 10^{-7} M, which is about 10 times the initial concentration present in the WC culture medium (4 x 10^{-8} M). At 2.5 x 10^{-7} M of total copper, the toxic effect was noticed by a reduction in population density (Fig. 2). After an adaptation period of 5 days, *N. lunatum* finally resumed normal growth. Figs. 2 and 3 show the effect of copper (Fig. 2) and lead (Fig. 3) on growth curve, as detected by chlorophyll-a analysis.

The mean concentration of the toxic metal required to cause a reduction of 50% in the growth yield in relation to the control value over a 96 hours period, known as LC50 (6) was 1 x 10^{-6} M for copper and 50 x 10^{-6} M for lead (Fig. 4). This indicates that copper is more toxic than lead to *N. lunatum*.

Considering copper, the LC50 corresponds to a concentration approximately 100 times higher than the original present in the culture medium. Investigating the mechanisms of copper toxicity to *Chlorella pyrenoidosa*, Stauber and Florence (13) have observed that 7.9 x 10^{-7} M (ionic copper) was required for the LC50, a value very close to the one we have obtained. The difference may be expected, since we have used different organisms and we report total copper unstead of the ionic species.

Notice that the LC50 for copper after 4 days of exposition, is close to the value that caused a total population death after 23 days of exposition (2.5 x 10^{-6} M). This fact show that interpretation of toxicity experiments involving metals and LC50 with only 96 hours of exposition are subject to errors.

Although toxicity is normally defined by acute effects, we suggest that the less readly detectable cronic effects are important in stabilishing a permissible concentration.

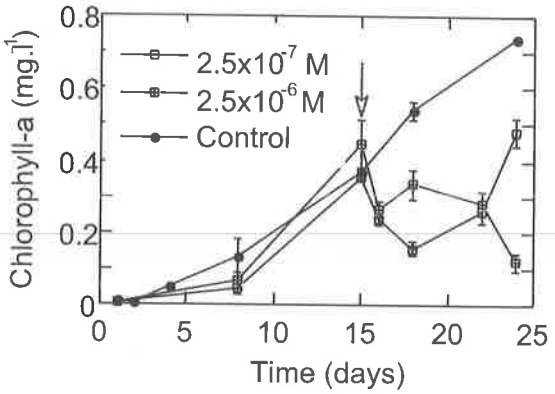

Figure 2. Growth curves represented by chlrophyll-a versus time after innoculation. Arrow indicate moment of copper addtion. Points are mean of two replicate experiments and error bars are standard deviation from the mean.

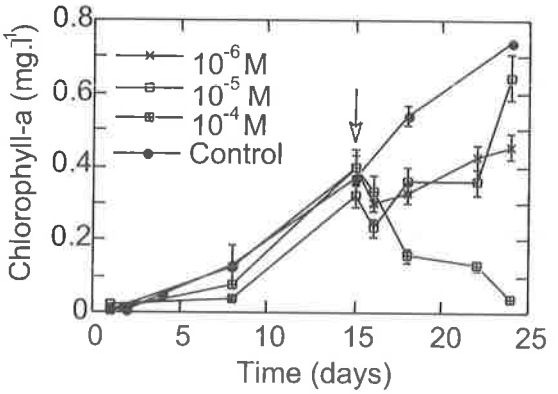

Figure 3. Growth curves represented by chlrophyll-a versus time after innoculation. Arrow indicate moment of lead additions. Points are mean of two replicate experiments and error bars are standard deviation from the mean.

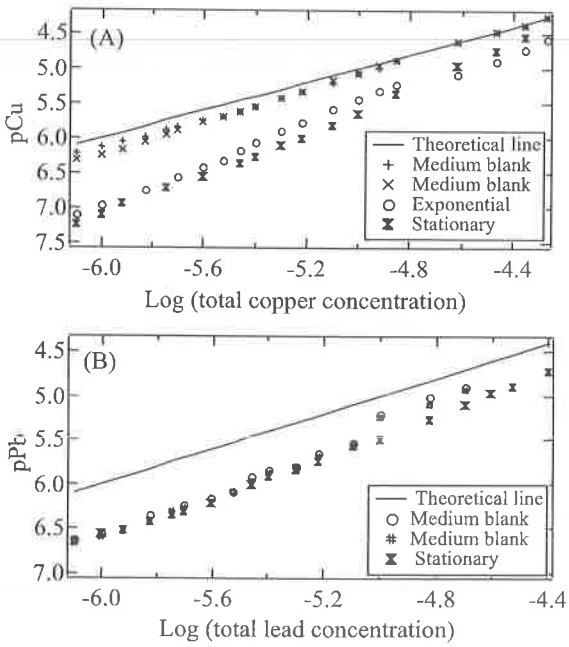

Figure 4. Titration curves for culture filtrates: (4A) copper titrations, (4B) lead titrations. Ionic strength = 0.02 M, pH = 6.1, temperature = 20°C. Theoretical line represent no complexation at all (pCu = -Log (total copper concentration); Medium blank represent the complexation of culture medium before innoculation; Exponential, and Stationary represent titration of culture filtrate obtained at exponential and stationary growth phases respectively.

Two main aspects must be considered when analysing the different behavior of the metals investigated: the first is the high affinity lead has to inorganic nutrients when compared to copper, and the second is the high affinity copper has with cell structure, once it is a required micronutrient. The high affinity between lead and inorganic nutrients of the culture medium may form inorganic complexes, which although considered labile, can cause a reduction in lead activity. Considering that the free metal is the most toxic fraction to phytoplankton (16), a reduction in this metalic species will reduce the toxic effect. On the other hand, copper has high affinity to organic acids, which is likely to constitute the major part of the excreted material produced by certain phytoplanktonic cells (10, 17). The consequence of such high affinity is the formation of inert complexes, which in turn will cause a reduction in free copper ion concentration. However, the affinity copper has with cell structure and consequent route(s) for copper uptake is quantitatively more important than copper complexes formed with the excreted material under culture conditions. There is no known route for lead uptake, and no known nutrient requirement for this metal, which may result in difficulties of lead migration into the cell.

Lead concentrations of 1×10^{-6} M e 1×10^{-5} M were only sufficient to cause a reduction in growth rate immediately after metal addition, after which cells resumed normal growth.

Complexation of copper and lead with culture filtrate were verified for two growth phases, exponential and stationary (Fig. 4), which present some important characteristics in relation to complexation studies:

Exponential phase: culture medium with high concentration of inorganic nutrients, but low concentration of excreted organic material. In this situation, lead will tend to be complexed with inorganic salts. The low amount of organic material limits the detection of complex formation by ion selective electrodes.

Stationary phase: culture medium with low concentration of inorganic nutrients, but high concentration of excreted organic material. The complex metal-organic material, if present, wil be detected by ion selective electrodes.

Fig. 4 show that the products excreted by *N. lunatum* are able to complex copper (4A), but not lead (4B), or at least, with the present experimental conditions lead complexation with the excreted material was not detected. Copper complexation parameters for cultures at the stationary phase of growth are log $K'_1 = 6.95$ and $CL_1 = 2.1 \times 10^{-6}$ M; log $K'_2 = 5.91$ and $CL_2 = 1.1 \times 10^{-5}$ M, and at the exponential phase, log $K'_1 = 6.30$ and $CL_1 = 4.8 \times 10^{-6}$ M; log $K'_2 = 5.10$ and $CL_2 = 1.8 \times 10^{-5}$ M. A reduction in copper toxicity to *Ankistrodesmus densus* has been shown by Vieira and Nascimento (18) to be due to the complexation of the metal with organic material produced by the cells under culture conditions. Hence, the copper complexation ability presented by the organic material produced and liberated by cells of *N. lunatum* in the present investigation will propably affect the toxiciy of the metal.

These results show that qualitatively, copper-complexing ligands produced by *N. lunatum* at two different growth phases are similar, as may be interpreted by log K'_1 and log K'_2 values. Also, the concentration of copper-complexing ligands were similar for either growth phase, indicating no change in their composition.

CONCLUSIONS

Copper was more toxic than lead to *N. lunatum*.

The organic material excreted by *N. lunatum* is able to complex copper in detectable concentration, but not lead.

ACKNOWLEDGMENTS

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RESUMO

Toxicidade e complexação de chumbo e cobre por excretado de *Nephrocytium lunatum (Chlorophyceae)*

Células de *Nephrocytium lunatum,* Chlorophyceae de água doce, foram mantidas em culturas tipo "batch" e, quando em fase exponencial de crescimento, foram submetidas a várias concentrações de cobre e chumbo. As culturas expostas a 2.5×10^{-7} M de cobre ou 1.0×10^{-6} M de chumbo reiniciaram crescimento após um curto período de adaptação, enquanto aquelas expostas a 2.5×10^{-6} M de cobre e 1.0×10^{-4} M de chumbo falharam em reiniciar o crescimento mesmo após um período de 10 dias. A capacidade de complexação do

material excretado em duas fase de crescimento (exponencial e estacionária) foi verificada através de titulações complexométricas utilizando-se eletrodos seletivos a íons. Os resultados revelaram que cobre é quelado pelo material dissolvido no meio de cultura, enquanto que chumbo não apresenta afinidade detectável.

Palavras-chave: toxicidade, *Nephrocytium lunatum*, cobre, chumbo, complexação

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LEUCAENA LEUCOCEPHALA AS A TRAP-HOST FOR *RHIZOBIUM TROPICI* STRAINS FROM THE BRAZILIAN "CERRADO" REGION

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ABSTRACT

Using *Leucaena leucocephala* as a trap-host, 422 strains of rhizobia were isolated from the bean (*Phaseolus vulgaris* L.) rhizosphere in the Cerrado region of Brazil. Traditional bean-producing areas with no history of rhizobial inoculation were sampled. Colony DNA hybridizations with gene-specific probes were used to identify strains that could be grouped as *R. tropici*, type I strains (*R. leguminosarum* bv. *phaseoli* or *R. etli*), or as other *Rhizobium* species. The same typing procedure was applied to 61 isolates from a large collection of strains isolated from bean plants inoculated with the same soil samples. *Rhizobium tropici* was present at all sites. The population of rhizobia isolated from bean plants was more heterogeneous than rhizobial population isolated from *Leucaena*. More than 90% of *Leucaena* isolates could be typed as *R. tropici*. Strains typed as *R. tropici* hybridized with *R. leguminosarum hup* structural genes, but not with *R. leguminosarum* bv. *phaseoli* ORF3, and the majority of them had a relative efficiency above 0.75. All the *Leucaena* strains were found to effectively nodulate beans and most of them yielded higher shoot dry weight than *R. tropici* strain CIAT899.

Key words: *Rhizobium*, genetic diversity, *Phaseolus vulgaris*, colony hybridization

INTRODUCTION

Beans (*Phaseolus vulgaris* L.) are a traditional crop in Brazilian agriculture and the main component of the Brazilian diet. Under favourable conditions, biological nitrogen fixation (BNF) by rhizobia can supply all the nitrogen required by bean plants (13). Under field conditions, BNF by the bean-rhizobia association is frequently limited by biotic and abiotic factors and by the lack of adaptation of the introduced strains (12), resulting in a wide range of responses to inoculation (7, 42).

Under controlled conditions, beans nodulate with a wide range of species of rhizobia. The first attempts to characterize populations of native bean nodulating rhizobia did not reveal large genetic differences between isolates from England and they were all typical *R. leguminosarum* bv. *phaseoli* (45). However, when tropical bean rhizobia were analyzed, two groups were identified: type I and type II (20, 21, 22). Type I strains have a narrow host range, harbour multiple copies of the *nifH* gene (30, 22) and hybridize with the *psi* gene (4), whereas type II strains exhibit a broader host range (including *Leucaena* and *Macroptilium purpureum*), possess a single copy of the *nifH* gene and do not hybridize with the *psi* gene (20, 22). Further studies have shown that type I strains belong to *R. leguminosarum* bv.

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phaseoli and to the new species *R. etli* (36, 8). Type II strains were classified as the new species *R. tropici* (25). Within *R. tropici*, two subgroups have been identified by Martínez-Romero et al. (25): type IIA represented by strain CFN299, and type IIB, represented by strain CIAT899. Recently *Rhizobium* strains isolated from beans in France were characterized and a great diversity of species, including *R. tropici*, was found. It has been suggested that this variability is determined by the acidity of the soils from which they were isolated (1).

R. tropici strains were found to be more heat-tolerant than type I strains (13, 14, Mercante, unpublished data). However, Sá et al. (32) selected both *R. tropici* and type I strains able to grow at high temperatures (38/39°C) and found no differences in symbiotic efficiency, protein pattern and *nifHDK* hybridization profiles of strains grown at normal or elevated temperatures. Nevertheless, among type I and *R. tropici* heat tolerant isolates, strain CIAT899 was the most efficient in nitrogen fixation (31, 32). *R. tropici* strain CIAT899 has been used for field inoculation in Brazil, but competitiveness with indigenous rhizobia of the Cerrado may be a limiting factor in establishing symbiosis.

Most co-inoculation experiments with *R. tropici* have used only a few strains and CIAT899 is by far the most studied (10, 24, 44). In pot experiments, it was observed that CIAT899 is more competitive under adverse conditions, such as low pH and high temperatures (28). Under soil conditions however, results with CIAT899 are variable: it was found to be a poor competitor in Hawaiian soils (38) and in an acidic Colombian soil (44), a moderate competitor in a fertile Colombian soil (44) and a good competitor in a non-fertile Brazilian sandy soil during the first crop, but not during subsequent crops (42).

We have used *Leucaena leucocephala* as a trap-host to isolate broad-host range rhizobia from bean rhizosphere soil from the Brazilian Cerrado region. These new isolates are a potential source of more tolerant, competitive, stable and efficient rhizobia for bean inoculation under tropical conditions. In addition we have used colony hybridization with specific gene probes to characterize the genetic diversity of these *Leucaena* isolates in comparison with rhizobia isolated from nodules of bean plants inoculated with the same soil samples.

MATERIALS AND METHODS

Sampling sites. The sampling sites were in eight representative bean-producing municipalities in the central-west Brazilian "Cerrado" with moderate to very acidic soils (pH 4.5-5.5) and low P availability.

Seed source and germination. Seeds used in the trap host experiment and for effectiveness trials were produced by EMBRAPA-CNPAB. Before use, bean (cvs. Carioca and Negro Argel) and *Leucaena* (*L. leucocephala* v. K72) seeds were surface-sterilized by 3 min exposure to 0.2% $HgCl_2$, followed by thorough washing in sterile water. Prior to surface sterilization, *Leucaena* seeds were scarified for 10 min in concentrated H_2SO_4. In all experiments, five seeds were planted in each jar and thinned to two within a week after emergence.

Plant growth conditions. Plants were grown in autoclaved Leonard Jars (41) in a mixture of vermiculite and sand (2:1,v:v), under green-house conditions in Rio de Janeiro. They were distributed in a randomized block design and supplied with Norris solution modified for beans (15) or *Leucaena* plants (5).

Isolation of bacteria from bean and *Leucaena* nodules. *Rhizobium* isolates were obtained from bean (cv. Negro Argel) nodules grown for 35-40 days in Leonard jars inoculated with 10g of soil samples selected from the rhizosphere of field-grown beans. After the beans were havested, the soil-substrate mixture used was kept moist, and within one month reused in jars for growth of *Leucaena* plants. *Leucaena* plants were harvested 65 days after planting, 5 nodules were detached from each jar and individually evaluated for acetylene reduction activity (ARA). The three most, and the least active nodules were selected for bacteria isolation. Bacteria were isolated as described by Vincent (41), resulting in 482 bean and 422 *Leucaena* isolates. All isolates were purified by selecting one individual colony in yeast mannitol agar (YMA) media (41). The cultures were freeze-dried for long storage periods and working cultures were maintained in YMA medium.

Cultura media and isolates growth conditions. Fresh cultures were used to inoculate YMA, TY and Luria Broth (LB) (33) plates. YMA plates were then incubated at 30 and 39°C, whereas LB and TY plates were incubated for 5 days at 30°C.

Tests for bean nodulation and effectiveness. All *Leucaena* isolates were tested for nodulation of bean plants (cv. Carioca). Duplicate Leonard jars were inoculated with 1 ml of 3-day pure cultures of rhizobia grown in YM medium (41). The 422 isolates from *Leucaena* nodules were divided into 9 experiments. Uninoculated controls, with and without combined nitrogen, and the bean efficient

rhizobial strain BR322 (CIAT899) were included in each experiment. Bean plants were harvested 35 days after planting and evaluated for nodulation and effectiveness. The nitrogen controls received a total of 70 mg of N. plant^{-1} in split weekly applications.

Nitrogenase activity and relative efficiency determinations. Nitrogenase activity was estimated by the acetylene reduction activity (ARA) assay (3). Detached *Leucaena* nodules were individually placed in 1 ml syringes and incubated under 10% acetylene for 20 min. Excised bean roots were sealed in 300 ml jars and incubated under 10% acetylene for 10 min. Acetylene and ethylene were analysed using gas chromatography (15). Evolved H_2 was estimated using an analyser constructed by the EMBRAPA instrumentation center (EMBRAPA-CNPDIA, São Carlos, SP).The relative efficiency (RE) of electron transfer to N_2 via nitrogenase was calculated as RE = 1 - (H_2 evolved/C_2H_4 produced) (35).

Colony DNA hybridization. All DNA manipulations were performed according to Ausubel et al. (2) and Sambrook et al. (33). Sterilized Hybond-N membranes were placed on the surface of solid YM medium and were inoculated by replica plating of bacterial cultures from microtiter plates. The inoculated membranes were incubated for 48 h at 30°C to permit bacterial growth. Membranes were removed and sequentially treated in the following solutions: 25% (w/v) sucrose, 20 mM Tris-HCl pH 8.0, 10 mM EDTA, lysozyme (1.5 mg/ml), for 10 min.; 10% (w/v) SDS, for 10 min.; 0.2% (w/v) Triton X-100, 0.5N NaOH, for 10 min.; 0.5N NaOH, for 5 min. and 1 M Tris-HCl pH7.4, for 5 min. After washing in 2X SSC, the membranes were air-dried over absorbent papers and sealed in transparent plastic film. DNA was fixed by 3 min.exposure of membranes to UV light.

The membranes were prehybridized at 65°C for 3-5h in hybridization solution [5X SSC, 1% (w/v) blocking reagent, 0.1% (w/v) N-lauroylsarcosine and 0.02% (w/v) SDS]. Subsequently, hybridization to heat-denatured probe DNA was performed for 12 h at 65°C and labelled with the DIG DNA Labelling Kit (Boehringer-Mannheim, Germany), in hybridization solution. After hybridization, membranes were sequentially washed in: 2X SSC, 0.1% (w/v) SDS, for 15 min. at room-temperature; and 1X SSC, 0.1% (w/v) SDS, for 15 min. at 65°C. Detection of probe, autoradiography and probe removal were performed according to Boehringer-Mannheim recommendations.

The probes used (Table 1) were internal DNA fragments of well characterized genes: *nodABC* from *Sinorhizobium meliloti*, *nodSU* from *Sinorhizobium* sp. NGR234 and *hupSL* (hydrogenase structural genes) from *R. leguminosarum* bv. *viciae*. Also included as a probe was ORF3 (a genomic sequence cloned from a *R. leguminosarum* bv. *phaseoli* strain isolated from the "Cerrado" region).

RESULTS

Effective nodulation of *Leucaena leucocephala* by indigenous rhizobia. Rhizobia nodulating *Leucaena* were present at all sites sampled in the central-west Brazilian Cerrado. Shoot dry weight of *Leucaena* inoculated with bean rhizosphere soil samples were significantly higher than the uninoculated control, with no differences between sites (Fig. 1B). Great variability was found in nodule nitrogenase activity (Fig. 1A). Although the majority of nodules presented an ARA of less than 100 nmoles C_2H_4.nodule^{-1}. h^{-1}, a few were much more efficient.

Efficiency of *Leucaena* nodulating rhizobia in symbiosis with bean. All strains isolated from *Leucaena* effectively nodulated bean plants. The majority of the rhizobia isolated from *Leucaena* promoted higher values for ARA and nodule dry weight than those obtained from plants inoculated with strain BR322. Similarly, a majority of *Leucaena* isolates resulted in higher shoot dry weight, compared to the plants in the nitrogen control (NC) or

Table 1- Genes and probes used in this study.

GENES	SOURCE OF CLONED DNA		DNA PROBE		REFERENCE
	SPECIES	STRAIN	SOURCE	SIZE	
nodABC	*S. meliloti*	AK631	pEK12	3.0Kb (*Sac*I/*Cla*I)	34
ORF3	*R. leg.* bv. *phaseoli*	CNPAF512	A9SS1	2.3Kb (*Sal*I)	26
nodSU	*Sinorhizobium* sp.	NGR234	pA16	2.5Kb (*Eco*RI/*Pst*I)	18
hupSL	*R. leg.* bv. *viciae*	128C53	pRWH3	2.4Kb (*Xho*I)	19

Size of the internal DNA fragment labelled with digoxigenin.

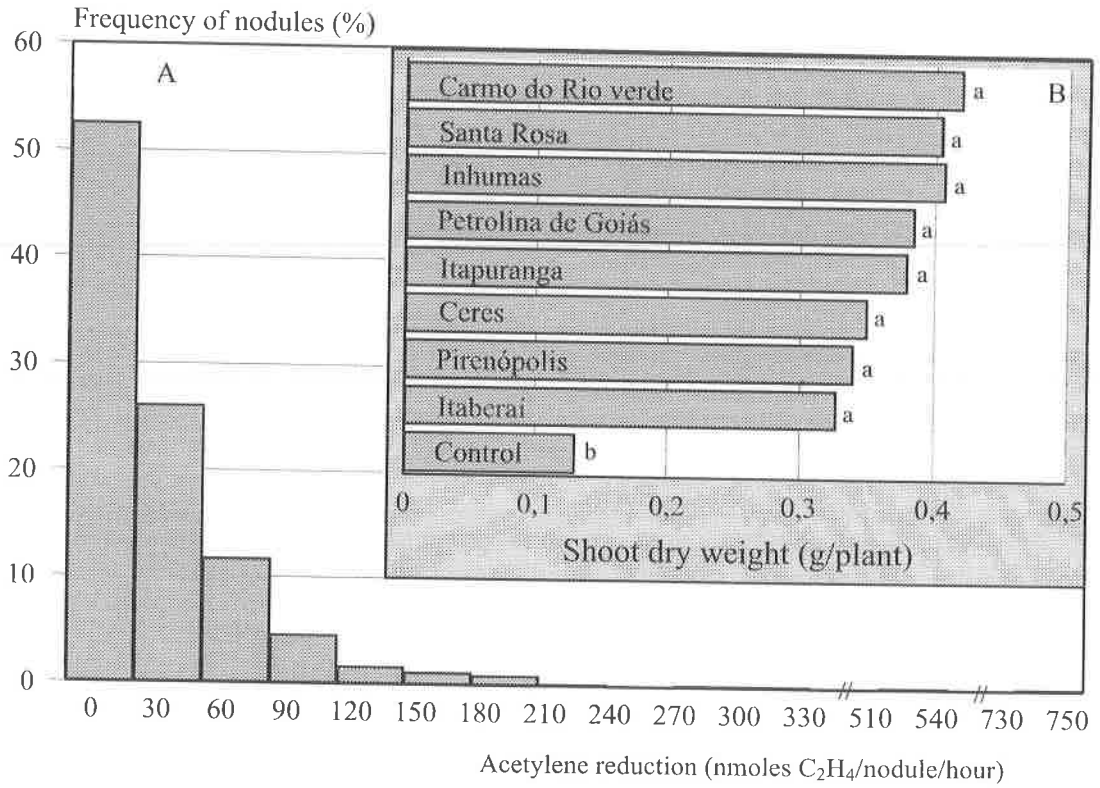

Fig. 1. Acetylene reduction activity of detached nodules (A) and shoot dry weight (B) of *Leucaena leucocephala* var. K72 plants. Plants were inoculated with 10g of bean rhizosphere soil samples, collected at 8 sites in central-west Brazilian Cerrado, and harvested 65 days after planting. Values of shoot dry weight are means of 6 plants and bars accompanied by the same letter are not significantly different (Tukey, $P<0.05$).

those inoculated with BR322. A relative efficiency higher than 0.75 was observed for most strains (Fig. 2).

Characterization of "Cerrado" indigenous rhizobia. All isolates from *Leucaena* were typical fast-growing, acid-producing rhizobia. The bean isolates were all fast-growing, but with more heterogeneous morphological characteristics on YMA plates. From the total of 326 isolates revovered from *Leucaena*, 85% were able to grow in LB medium against 60.7% of the 61 isolates recovered from *P. vulgaris*. Growth at 39°C was highly correlated with growth in LB medium.

DNA colony hybridizations with different probes were used to verify the diversity of indigenous "Cerrado" rhizobia nodulating *Leucaena* and bean (Table 2 and Fig. 3). *Sinorhizobium meliloti nodABC* hybridized with the majority of isolates (98 and 80% of *Leucaena* and bean isolates, respectively), and to all reference strains used in previous studies, under the conditions tested (Table 3). DNA homologous to *nodSU* or to *hupSL* are present in the majority of the *Leucaena* isolates, but only in half of the bean isolates (Table 2). *R. leguminosarum* bv. *phaseoli* ORF3 hybridized with DNA from all type I reference strains used in previous studies (Table 3), to 53% of the bean isolates, but only to 0.6% of the *Leucaena* isolates (Table 2). Strains hybridizing with both *nodSU* and *hupSL* are present in both populations, and these genes are present in all *R. tropici* reference strains used in previous studies (Table 3). No DNA homology to *nodSU* or *hupSL* genes was detected in any type I reference strains used in previous studies and no homology to ORF3 was detected to the *R. tropici* reference strains used in previous studies listed in Table 3.

On the basis of their hybridization with gene-specific probes, growth in LB medium and colony morphology in YMA medium, reference strains used in previous studies (Table 3) and "Cerrado" isolates (Fig. 3) were assigned to the following groups: Group I, *R. leguminosarum* bv. *phaseoli* and *R. etli* type I strains (do not grow in LB, hybridize with *nodABC* and ORF3 but not to *nodSU* and *hupSL*); group II, *R. tropici* IIA (do not grow in LB, hybridize with *nodABC*, *nodSU*, *hupSL*, but not to ORF3); group III,

R. tropici IIB (grow in LB, hybridize with *nodABC*, *nodSU*, *hupSL*, but not to ORF3); or to species not determined (SND), referring to all other combinations than those fitting in the previous groups.

DISCUSSION

The Cerrado represents around 25% of Brazilian territory and is considered to be its most promising agricultural frontier. Despite adverse soil characteristics such as low pH, Al^{+3} toxicity, and an abundance of soil actinomycetes, bean plants are satisfactory nodulated by indigenous rhizobia but grain yield is frequently low. For many years, rhizobial strains used in commercialized inoculants, in Brazil, belonged to the group of type I strains (*R.*

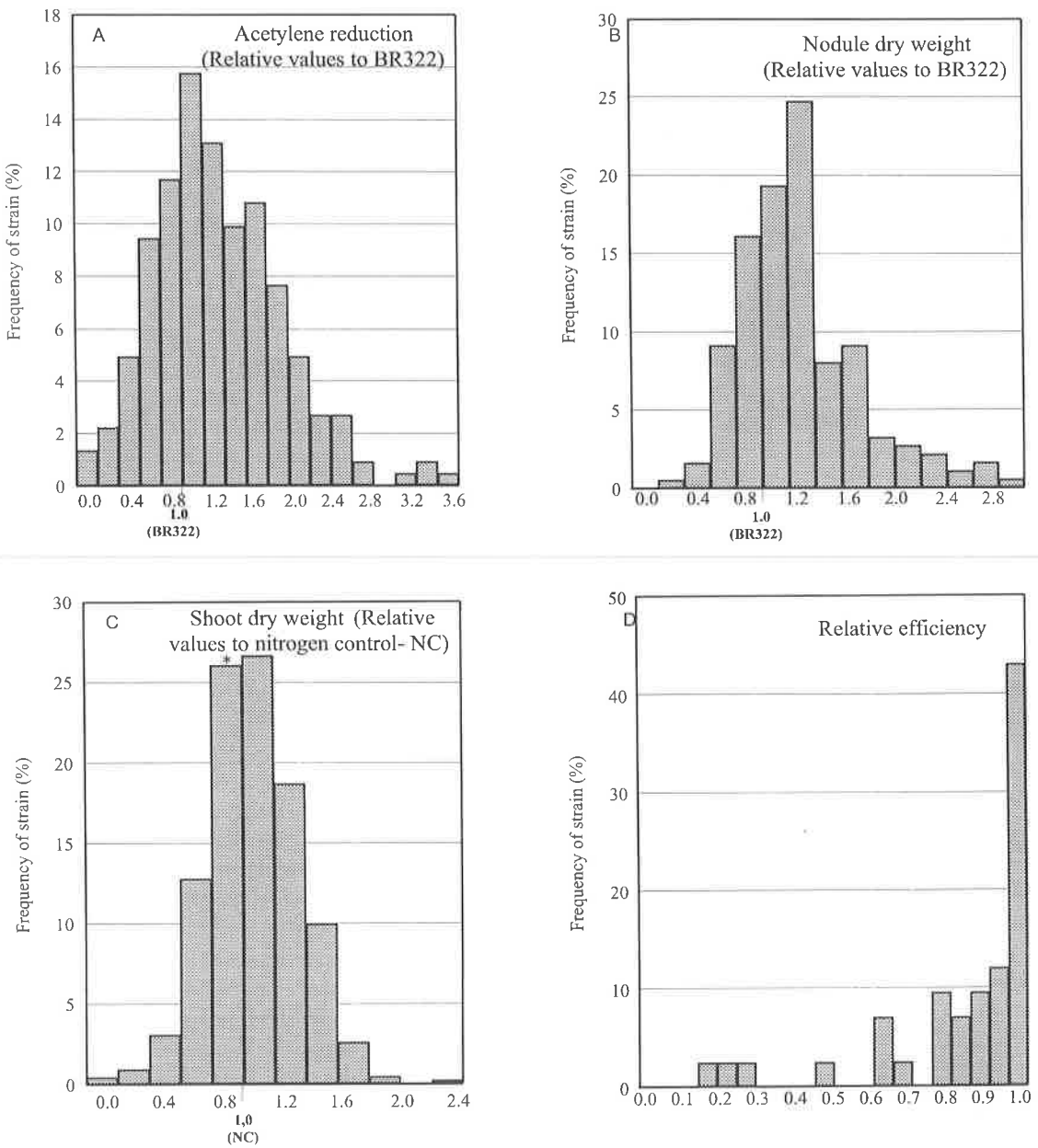

Fig. 2. Acetylene reduction (A), nodule dry weight (B), shoot dry weight (C) and relative efficiency (RE=1- H_2/C_2H_4) (D) of nodulated roots of bean plants, inoculated with 222(A), 186(B), 422(C) and 42(D) indigenous rhizobia strains, respectively. *Including strain BR322.

Table 2- Characterization of Cerrado indigenous rhizobia by DNA colony hybridization with four DNA probes.

Trap-host	N° of strains isolated	N° of strains tested	PROBES			
			nodABC	*nodSU*	ORF3	*hupSL*
			% of strains with DNA homology			
Leucaena leucocephala	422	326	98	99	0.6	93
Phaseolus vulgaris	482	61	80	56	53	43

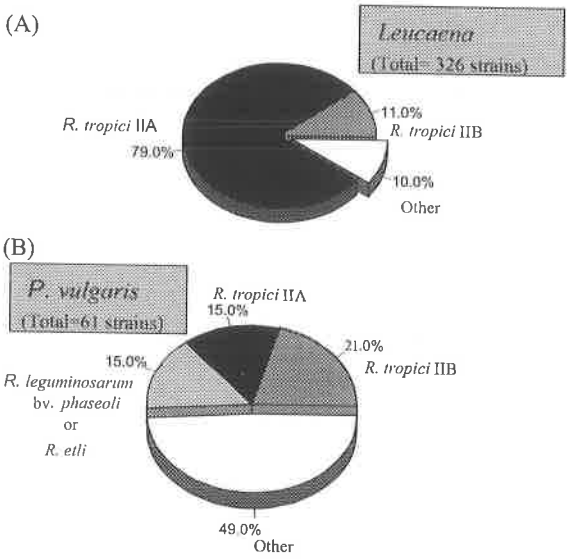

Fig. 3. Grouping of *Leucaena leucocephala* (A) and *Phaseolus vulgaris* (B) rhizobial strains isolated from Brazilian Cerrado region as: *R. leguminosarum* bv. *phaseoli* and *R. etli* (do not grow in LB, hybridize with *nodABC* and ORF3, but not with *nodSU* and *hupSL*); *R. tropici* IIA (do not grow in LB, hybridize with *nodABC*, *nodSU*, *hupSL*, but not with ORF3); *R. tropici* IIB (grows in LB, hybridize with *nodABC*, *nodSU* and *hupSL*, but not with ORF3); or as species not determined, referring to all combinations other than those fitting in the previous groups.

leguminosarum bv. *phaseoli* and *R. etli*). These strains are considered to be very unstable, with frequent loss of symbiotic efficiency and even loss of viability in stock cultures. On the other hand, *R. tropici* strains have many agronomically important characteristics such as tolerance to high temperatures, aluminum, acidity and high levels of antibiotics (25). Competition experiments also demonstrate the great potential of this group of strains (R. Straliotto, unpublished data). One strain belonging to this species, BR322 (CIAT 899), proved to be very stable, both in stock cultures and in many experiments in which its nodulation effectiveness and nitrogen fixation efficiency were tested. Indeed it has been included in commercial inoculants in Brazil in the last few years.

Nevertheless, results from field experiments have shown that many of the known factors affecting bean symbioses still limit the response to inoculation in many regions (40). Therefore, isolation and selection of more efficient and competitive indigenous strains could improve bean inoculation efficiency in Cerrado regions. To isolate a population of more tolerant indigenous rhizobial strains with greater probability of belonging to the *R. tropici* group, we have used the following strategy. Bean growers were located in central-west Brazilian Cerrado and those sites with nodulated bean plants and no history of inoculation were chosen. Bean rhizosphere soil was collected and used as inoculant in sterile Leonard jars. Bean plants were grown in the jars prior to planting *L. leucocephala*. *Leucaena* was used as a trap host because strains nodulating both plants are more tolerant to high temperatures than type I strains (14). Some type I strains have been well characterized for their genetic instability and their inability to nodulate *Leucaena* (9, 25, 37).

Isolation from *Leucaena* resulted in 422 strains, all of them nodulating bean plants. These isolates were characterized by colony hybridization with gene specific probes, colony morphology in YMA and growth in LB media. Although hybridization with gene-specific probes is not a method for studying bacterial phylogeny due to possible horizontal gene transfer, it is a practical and efficient way to identify a large number of isolates. In addition, growth of bacteria in LB medium is not sufficient to differentiate *R. tropici* subgroups and exceptions exist (39). Our results should therefore be considered as an estimation of variability and not as accurate classification of strains. After comparison with *Sinorhizobium* sp. NGR234 and reference strains of *R. etli*, *R. leguminosarum* bv. *phaseoli*, *R. tropici* and *Sinorhizobium meliloti*, around 90% of *Leucaena* isolates could be typed as *R. tropici*. These results are in agreement with those obtained by Martinez-Romero et al. (25). Their description of this species was based on 37 strains isolated during the present study and all of them were classified as *R. tropici*. The majority of *Leucaena* isolates were more

Table 3 - Strains previously studied used for comparison.

Strain (CNPAB accession nº)	Other designation(s)	Group	Reference for classification
Type I (*R. leguminosarum* bv. *phaseoli* and *R. etli*)			
BR10027	W38.12	I	25*
BR10028	W16.3SB	I	25*
BR10052^T	LMG8819^T, ATCC1482^T	I	27
R. tropici IIA			
BR266	C-05	II	25*
BR387	UMR1020, C-18	II	22
BR828	F60.5	II	25*
BR829	F114.3	II	25*
BR830	FIT.5	II	25*
BR831	F61.1	II	25*
BR833	F114.5, USDA 9001	II	25*
BR834	F99.2	II	25*
BR835	F27.4, USDA 9002	II	25*
BR836	F14.5, USDA 9003	II	25*
BR837	F44.3	II	25*
BR839	F22.3, USDA 9004	II	25*
BR840	F110.3	II	25*
BR842	F107.2, USDA 9005	II	25*
BR843	F31.5, USDA 9006	II	25*
BR844	F7.3	II	25*
BR846	F37.2, USDA 9007	II	25*
BR10014	CAR22	II	24
BR10015	CNPAF119, LMG 9502	II	6
BR10016	CFN299, CENA183	II	25
BR10031	W12.16SA	II	25*
BR10032	W45.5	II	25*
BR10033	W39.4	II	25*
BR10034	W1.18	II	25*
BR10035	W4.73	II	25*
BR10036	W52.20	II	25*
BR10037	W84.13, USDA 9021	II	25*
BR10038	W13.11SB	II	25*
BR10039	W49.14, USDA 9022	II	25*
BR10040	W21.2SA	II	25*
BR10041	W7.5SA	II	25*
BR10042	W67.10	II	25*
BR10043	W38.18, USDA 9023	II	25*
BR10044	W71.6, USDA 9024	II	25*
BR10045	W87.16, USDA 9025	II	25*
R. tropici IIB			
BR322^T	CIAT899^T	III	25
BR847	F93.2, USDA 9008	III	25*
BR848	F93.1, USDA 9009	III	25*
BR849	F51.4	III	25*
BR851	FIIT.3, USDA 9011	III	25*
BR852	F51.1, USDA 9012	III	25*
BR854	FIIT.2, USDA 9013	III	25*
BR855	F98.5	III	25*
BR856	F93.3	III	25*
BR859	F98.1, USDA 9016	III	25*
BR863	F58.2, USDA 9019	III	25*
Sinorhizobium meliloti			
BR7412	RM1021	IV	6
Sinorhizobium sp.			
BR2406	NGR234	V	11

* Strain isolated in the course of this study.

Procedure used to group strains was based upon their ability to grow on Luria-Bertani agar medium and DNA colony hybridization with specific gene probes. Group I: do not grow in LB; hybridize with *nodABC* and ORF3 but not with *nodSU* and *hupSL*. Group II: do not grow in LB; hybridize with *nodABC*, *nodSU*, *hupSL* but not with ORF3. Group III: grow in LB, hybridize with *nodABC*, *nodSU*, *hupSL* but not with ORF3. Group IV: grow in LB; hybridize with *nodABC* but not with *nodSU*, *hupSL* and ORF3. Group V: do not grow in LB; hybridize with *nodABC*, *nodSU* but not with *hupSL* and ORF3.

efficient than strain CIAT899 in BNF with bean plants, independent of their ability to fix nitrogen in *Leucaena* nodules. RE values were determined for 42 of these isolates and in most cases they were superior to 0.75 which may reflect the presence of a functional hydrogen uptake system in these strains (35). This result is in agreement with the detection of DNA homologous to hydrogenase structural genes in 93% of these isolates. The detection of *hupSL* genes in all *R. tropici* reference strains is not in agreement with results reported by van Berkum et al. (39), but this may be due to differences in *hupSL* probes and hybridization conditions used in both studies. Waelkens et al. (43) have shown that *nodS* gene is essential for bean and *Leucaena* nodulation by *R. tropici* strain CIAT899. This is in accordance with the presence of DNA homologous to *nodSU* in all *R. tropici* reference strains and in 99% of *Leucaena* isolates.

The same typing procedure was applied to 61 strains isolated from nodules of bean plants inoculated with the same rhizosphere soil samples. Bean isolates were more heterogeneous than *Leucaena* isolates: 36% of them could be typed as *R. tropici* (of which 59% could grow in LB medium) or as type I strains, while 49% could not be typed as any of the previous species. In contrast, we could not determine the species status of only 10% of *Leucaena* isolates. As *nodABC* genes are highly conserved between *S. meliloti* and *Rhizobium* spp. (23), but not necessarily among all bean nodulating rhizobia, the great number of bean isolates with no DNA homology to *nodABC* may reflect the host promiscuity of this species and/or loss of symbiotic information by the isolates.

R. tropici IIA and IIB strains were present in all sites sampled and it is not clear whether the isolation of 6 fold more strains from the first group is a consequence of differences in competitiveness or of the number of viable cells present in soil. Data on competitiveness obtained with *R. tropici* strains are very contradictory (24, R. Straliotto, unpublished data) and, according to results obtained by Vlassak et al. (42), it appears that *R. tropici* IIB strains do not persist well in fields continuously planted with beans. Recently, Amarger et al. (1) reported the occurrence of *R. tropici* IIA and the absence of IIB strains in France. They also showed that more than half of the isolates tested were moderately effective or ineffective in BNF. We have also observed frequent loss of effectiveness and/or efficiency by *R. tropici* IIA stock cultures, while *R. tropici* IIB stock cultures are very stable. In addition, further selection of *Leucaena* isolates identified at least one *R. tropici* IIB strain, F98.5, that is as competitive as CIAT 899 (42) but more efficient and heat-tolerant than this strain (F. M. Mercante, unpublished data).

The *Phaseolus* bean nodulating rhizobia isolated from *Leucaena leucocephala* constituted the genetic library for the description of *R. tropici*. The isolates are well adapted to acid soils and high temperature and may constitute an important source of strains for bean inoculation in the tropics.

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RESUMO

Uso de *Leucaena leucocephala* na obtenção de estirpes de *Rhizobium tropici* nativas do Cerrado brasileiro

Leucaena leucocephala foi usada para recuperar 422 estirpes de rizóbio de solos da rizosfera de feijoeiro (*Phaseolus vulgaris* L.), cultivado em regiões do Cerrado brasileiro. Os locais amostrados foram de áreas tradicionalmente produtoras de feijão sem histórico de inoculação de rizóbio. Hibridizações de colônia com sondas de gene específico foram usadas para identificar estirpes que poderiam ser agrupadas como *R. tropici*, estirpes tipo I (*R. leguminosarum* bv. *phaseoli* ou *R. etli*), ou como outra espécie de *Rhizobium* não determinada. O mesmo procedimento foi aplicado a 61 isolados de uma ampla coleção de estirpes isoladas de feijoeiro, inoculado com as mesmas amostras de solo. *Rhizobium tropici* estava presente em todos os locais amostrados. A população de estirpes de rizóbio isoladas de feijoeiro foi mais heterogênea do que a população de rizóbio isolada de *Leucaena*. Mais de 90% dos isolados de *Leucaena* poderiam ser agrupados como *R. tropici*. As estirpes agrupadas como *R. tropici* hibridizaram com genes estruturais *hup* de *R. leguminosarum*, mas não com ORF3 de *R.*

leguminosarum bv. *phaseoli*, e a maioria delas apresentaram uma eficiência relativa (ER) superior a 0,75. Todas as estirpes isoladas de *Leucaena* nodularam eficientemente o feijoeiro, e a maioria delas proporcionou uma produção de matéria seca da parte aérea superior às plantas inoculadas com a estirpe CIAT899 de *R. tropici*.

Palavras-chave: *Rhizobium*, diversidade genética, *Phaseolus vulgaris*, hibridização de colônia.

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BOVINE TALLOW IN RATIONS FOR LACTATING COWS: ALTERATIONS IN THE RUMEN MICROBIAL ECOSYSTEM

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ABSTRACT

The effect of consuming concentrated rations supplemented or not with bovine tallow on pH, ammonium nitrogen concentration, protozoa populations and efficiency of nitrogen compound synthesis by microorganisms of the bovine rumen were determined in two experiments. In both studies, offering the animals corn silage combined with concentrates containing 4 to 10% bovine tallow caused a reduction in ammonium nitrogen and in ruminal protozoan populations. The daily production of microbial mass per gram total digested carbohydrate in the rumen was 0.416 g in the absence of tallow, 0.607 g in the treatment containing 4% tallow, and 0.790 g in the treatment containing 10% tallow. The estimate of pH in the ruminal fluid and of the efficiency of synthesis of microbial nitrogen compounds obtained with the ruminal submodel CNCPS (Cornell Net Carbohydrate and Protein System) was found to be compatible with *in vivo* observations.

Key words: Lipids, pH, ammonium nitrogen, rumen protozoa, rumen.

INTRODUCTION

Milk production, specially in cows of higher genetic merit, is limited both by the amount of energy consumed at the beginning of the lactation cycle and by the mobilization of the body tissues stored before parturition. An adequate energy level in the diet is an essential measure to support high milk yields (7). Lipids are used in the diet when higher energy ingestion is desired during the early phase of lactation. The metabolic utilization of these nutrients at the tissue level is more efficient than that of volatile fatty acids, the major source of energy for ruminants (9, 13).

The utilization of fatty acids originating from lipids by the ruminal microbiota is small, a fact that causes a lower energy loss due to methanogenesis. The ruminal metabolism of lipids does not contribute to heat generation in the process of ruminal fermentation (5). These factors contribute to increased milk yields due to the better utilization of dietary energy (5, 7, 8).

Bovine tallow could be used as a source of lipids for the diet of lactating cows because it is a relatively inert food in the ruminal environment (8). The validation of its use in diets is made by investigating the microbiological aspects of the ruminal ecosystem. Fibrolytic and methanogenic bacteria and most protozoan populations are reduced after lipid ingestion and an increase in bacterial biomass occurs. These events result in a greater supply of nitrogen of microbial origin in the small intestine and in a reduction of organic matter degradation in the rumen (16). The changes in ruminal pH and in ammonium

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nitrogen concentrations caused by tallow ingestion are considered to be inconsistent (8, 18).

The objective of the present study was to investigate the possible changes in the ruminal ecosystem caused by dietary supplementation with bovine tallow.

MATERIALS AND METHODS

Experiment 1 consisted of a balanced 4 x 4 latin square using four non-fistulated cows with a mean live weight of 480 kg and mean daily milk yield of 25 kg. Each experimental period consisted of 21 days, 14 of them for adaptation and 7 for the collection of the samples needed for bromatologic analysis.

In experiment 2, owing to the loss of one animal, a 3 x 3 latin square was set up using three heifers fistulated in the rumen and in the abomasus. The experimental periods lasted 21 days, 14 of them for adaptation and 7 for the collection of fecal samples and of digested matter samples from the abomasus.

During the experimental period, corn silage (*Zea mays L.*) was offered as the only bulk fodder. Were used four concentrates with an isoprotein formulation containing 22.5% crude protein (CP) as natural matter (Table 1). In experiment 1 the rations contained no tallow (R0) or 4% (R4), 7% (R7) and 10% bovine tallow (R10). In experiment 2, tallow was provided at percentages corresponding to R0, R4 and R10 (Table 1). These rations, combined with silage, represented treatments T0, T4, T7 and T10 (Table 1).

Feed was offered twice a day at 9:00 a.m. and at 3:00 p.m. using the complete mixture system with a concentrate: silage ratio fixed at 45: 55 %. The animals had free access to water throughout the experiment.

Silage, concentrate and food leftovers of each animal were sampled daily during the phase of adaptation and collection and stored in a refrigerator at -5°C. At the end of each week, individual samples were homogenized and used to prepare a compound sample for each of the three materials.

Dry matter (DM), organic matter (OM), ether extract (EE), crude protein (CP), calcium (Ca) and phosphorus (P) were determined (1). Neutral detergent fiber (NDF) was obtained by the method of Van Soest *et al.* (17). For all samples of each feedstuff, during neutral detergent extraction, an adequate amount of thermoresistant amylase was used.

In experiment 1, ruminal fluid was removed by suction via an esophageal tube using a manual

Table 1. Percentage of ingredients in concentrete rations expressed on natural basis. Bromatological components: Dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), ether extract (EE), total carbohydrate (TC), Calcium (Ca), and Phosphorus (P) of concentrate ratios and corn silage (SILAGE).

Ingredients (%)	Concentrate Rations				
	R 0	R 4	R 7	R 10	
Ground corn	57.18	52.25	48.55	44.85	
Soybean meal	39.19	40.12	40.82	41.52	
Bovine tallow	—	4.00	7.00	10.00	
Mineral mix[2]	3.63	3.63	3.63	3.63	
Bromatologic Components					SILAGE
DM[3]	88.99	89.55	87.47	88.57	36.89
OM[4]	92.90	93.46	93.82	94.39	96.66
CP[4]	24.71	24.42	24.78	24.94	6.94
NDF[4]	10.90	9.58	9.99	9.80	64.26
EE[4]	2.81	6.58	8.98	11.93	2.73
TC[4]	65.38	62.46	60.60	57.52	86.99
Ca[4]	1.36	1.29	1.32	1.32	0.39
P[4]	0.43	0.46	0.42	0.45	0.23

1 - R0: concentrate ration containing absence of tallow; R4: concentrate ration containing 4% of tallow; R7: concentrate ration containing 7% of tallow; R10: concentrate ration containing 10% of tallow.
2 - Percentual composition: sodiun chlorine 1.03; calcium carbonate 0.30; bicalcium phosphate 2.08; magnesium oxide 0.19; zinc sulfate 0.025; copper sulfate 0.0040; cobalt sulfate 0.0025; potassium iodate 0.0015; sodium selenite 0.0015.
3 - In %
4 - In % of the component on DM

vacuum pump, and in experiment 2 it was directly collected through a fistula. Collections were made before the first offer of feed and 2, 4 and 6 hours later. The fluid was immediately filtered through a double gauze layer and 40 ml of the filtrate were destined to ammonium nitrogen analysis (3). One ml 50 % sulfuric acid (v/v) was added to this fraction for storage at -5°C (8). Thirty ml of recently filtered ruminal fluid were used for pH measurement with a digital Digimed/DMPH1 ph-meter, and for protozoan counts.

In experiment 2, approximately 3 liters of liquid and solid ruminal material were removed at the end of each experimental period in order to obtain samples of bacterial biomass and to determine the efficiency of nitrogen compound synthesis in this biomass. The ruminal material was processed and stored at -5°C (4). At the end of the experiment, the efficiency of synthesis of bacterial nitrogen compounds was determined by analyzing purine nitrogen in bacterial biomass samples and in samples from the abomasus (15).

Protozoa from the ruminal fluid were isolated and fixed by the method of Dehority (6). Counts were performed in a Neubauer chamber using a Rossbach S.A./Kyowa binocular microscope with a 30x objective and a 10x eyepiece.

The ruminal submodel denoted Cornell Net Carbohydrate and Protein System (CNCPS) was used to predict pH, efficiency of synthesis of microbial nitrogen compounds and daily production of microbial biomass (2). The estimated data were compared to those observed *in vivo* to determine the ability of the ruminal submodel for the prediction of these ruminal parameters.

Data were analyzed statistically using the Statistical and Genetic Analysis System, version 5.0. The data of experiment 1 were interpreted in terms of regression analysis and the Newman-Keuls test was used to discriminate the differences between averages, assuming $P < 0.01$ (**) or $P < 0,05$ (*). In the second experiment, due to the loss of one animal, the data were described as observed values.

RESULTS AND DISCUSSION

In experiment 1, the concentration of ammonium nitrogen (N-NH3) in the ruminal fluid was higher in the animals that received lower amounts of tallow (Table 2 and Fig. 1). This behavior has been described previously (16). Normally, as observed in this study, 4 hours after feeding a reduction in N-NH3 concentration occurs in the ruminal environment of the animal (9). The values detected (Fig. 1) were within the 5-9 mg/100 ml range, which has been reported to be sufficient to support microbial growth in the rumen (14).

The lower N-NH3 concentration in the rumen of animals that received treatments with tallow was

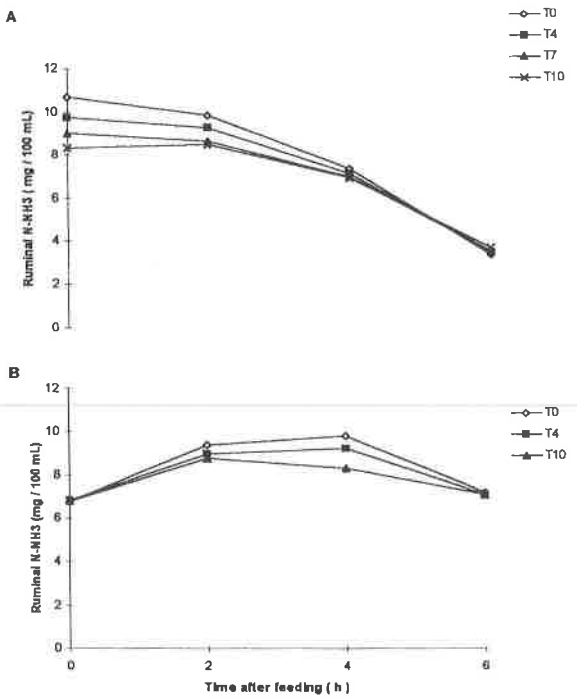

Figure 1. *A* - Ruminal ammonia nitrogen estimative: $\hat{Y}$ ammonia (mg / dl) = 10,660 - 0,236 * x Treatment - 0,599^{n} x Time - 0,208** x Time^2 + 0,048** x Treatment x Time (R^2 = 67,4), experiment 1. ***B***. Observed values of ruminal ammonia nitrogen concentration, experiment 2.

Table 2. Ruminal ammonia nitrogen, pH and protozoa counts observed in the experiment 1.

Treatments	N-NH3 (mg/100 ml)					pH					protozoa (x1000 / ml)				
	0	2	4	6	Mean	0	2	4	6	Mean	0	2	4	6	Mean
T0	11.8	12.8	9.9	8.7	10.8^{a}	6.8	6.7	6.8	6.8	6.7^{a}	563	518	514	552	536.7^{a}
T4	9.7	10.1	9.7	8.3	9.4^{b}	6.9	6.6	6.6	6.6	6.6^{a}	373	329	325	362	347.2^{b}
T7	8.4	10.1	7.1	7.6	8.3^{c}	7.1	6.7	6.6	6.6	6.7^{n}	231	187	182	220	205.0^{c}
T10	7.1	10.0	7.2	7.0	7.8^{d}	7.4	6.9	6.7	6.7	6.9^{a}	89.4	44.5	40.8	78.5	63.3^{d}

Means with unlike superscripts within column are differents by the Newman-Keuls test ($P < 0.05$).

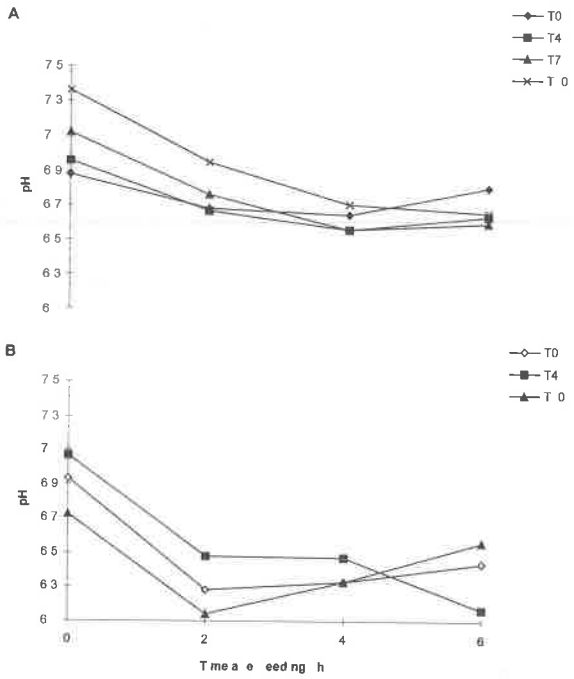

Figure 2. *A* - Ruminal pH estimative: $\hat{Y}$ pH (pH) = 6,888 - 0,010^{n} x Treatment - 0,149** x Time + 0,005** x Treatment^2 + 0,003* x Time^2 - 0,011** x Treatment x Time R^2 = 71,7), experiment 1. *B* - Observed values of ruminal pH., experiment 2.

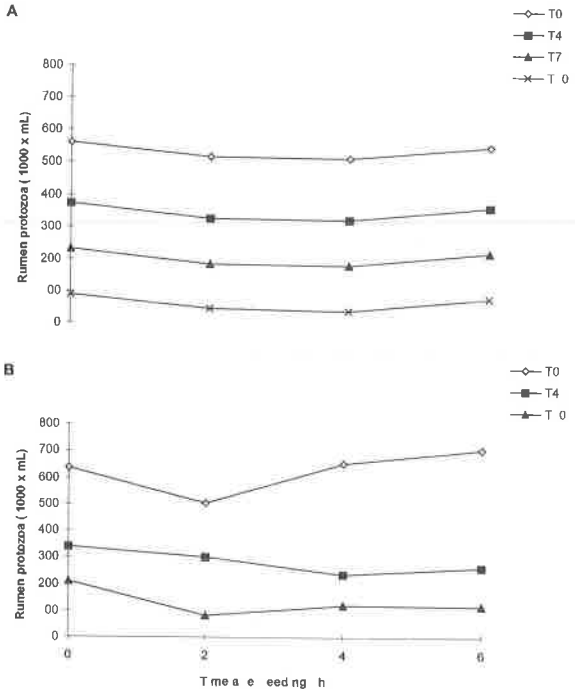

Figure 3. *A* - Rumen protozoa population estimative: $\hat{Y}$ protozoa (Cel/ml) = 563157,00 - 47370,40** x Treatment - 31806,20* x Time + 5163,92* x Time^2 (R^2 = 97,1), experiment 1. *B* - Observed values of rumen protozoa, experiment 2.

probably due to the lower ruminal digestion of crude protein in these treatments (11). This fact may be attributed to the reduction in the population of ruminal protozoa (Table 2 and Fig. 3). The proteolytic activity of protozoa is considered to be important for the generation of ammonium nitrogen in the rumen (9).

In both experiments, the pH values decreased during the 4-hour period after animal feeding (Table 2 and Fig. 2), indicating fermenting activity of the microorganisms. In experiment 1, although not significant, the higher pH values observed during the first 2 hours (Fig. 2a) in treatments T10 and T7 could be explained by the addition of tallow causing a reduction in microorganism access to the substratum. The fermenting activity continued during the 2 subsequent hours in all treatments, and was more evident in the treatments with tallow (Fig. 2a), in which there was a reduction of pH in relation to the control treatment. Four hours after feeding, the pH value observed in treatment T0 increased, whereas it remained practically unchanged in the diets with tallow.

The pH values in the ruminal fluid were close to neutrality (Table 2 and Fig. 2). In all treatments, pH was favorable for cell wall depolymerization, especially between 2 and 4 hours after feeding. The pH values were close to those needed for the activity of ruminal fibrolytic microorganisms (13).

The use of the CNCPS ruminal submodel (2) to estimate the pH of the ruminal fluid obtained with the diets under study resulted in pH values from 6.2 to 6.6. The estimated values were close to observed values (Fig. 2). The utilization of this submodel was considered to be useful to determine the pH values of the ruminal fluid of non-fistulated animals.

The addition of tallow to the concentrates, resulted in a drastic reduction ($P < 0.05$) of protozoan populations (Table 2 and Fig. 3). There was an approximately 10-fold decrease in cell number after treatments T0 and T10, a fact that may be attributed to the release of fatty acids caused by lipid hydrolysis, similar to the behavior described by Van Nevel and Demeyer (16). The immediate consequence of the reduction in number of protozoa in the ruminal ecosystem is the filling of this domain with ruminal fungi and bacteria, especially the latter microbiota (12). It is known that an increase in bacterial populations can produce an increase in the

Table 3. Bacterial abomasal dry matter (MSbAb), microbial nitrogen (NM), ruminally digested total carbohydrate (CHTR), ruminally digested organic matter (MODR), and rumen microbial efficiency of synthesis of the nitrogenous compounds expressed in gNM / kgCHTR and in gMSbAb / gCHTR. Experiment 2.

Treatments	MSbAb (g/day)	NM (g/day)	CHTR (kg/day)	MODR (kg/day)	NM / CHTR (g/kg)	MSbAb / CHTR (g/g)
T0	2907.1	152.0	7.10	7.76	21.41	0.410
T4	3718.0	196.3	6.12	6.89	32.08	0.608
T10	3404.6	186.2	4.32	5.14	43.10	0.788

flow of nitrogen compounds of microbial origin toward the intestine (16).

Although any conclusion in the second experiment must be drawn with caution, the efficiency of microbial nitrogen compound synthesis, numerically increased with increasing tallow levels (Table 3). The reductions observed in the protozoan populations (Fig. 3) are related to the lower ruminal digestion of OM (Table 3) and to the increased flow of microbial nitrogen compounds towards the duodenum (Table 3). A similar behavior was observed by others (16).

Microbial efficiency, expressed as gram bacterial DM (gDM) per gram total carbohydrate digested in the rumen (gTCHR), was numerically higher in all treatments (Table 3) when compared to the mean 0.40 gDM/g value for fermented hexose (13). The values obtained in treatments T4 (0.608) and T10 (0.788) were higher than the mean values reported by CNCPS (0.40), probably due to the unit adopted, gDM/gTCHR, rather than gDM/g fermented hexose.

The values for the bacterial biomass produced in the rumen, expressed as grams DM/day (Table 3) were 2907.1 for T0, 3718.0 for T4 and 3404.6 for T10. The ruminal submodel of CNCPS predicts values of 3257.0 for T0, 3326.4 for T4 and 3179.8 for T10 (2). The difference between predicted and observed values was approximately 12% higher for T0, 10.5% lower for T4 and 6.6% lower for T10. The variations between predicted and observed values were attributed to the fact that the feedstuffs used in the CNCPS feed library are chemically different from that used in the present study in terms of the amount and degradation rates of the fractions that make up the nutrients.

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RESUMO

Sebo bovino em rações para vacas lactantes: alterações no ecossistema microbiano ruminal

Os efeitos do consumo de rações concentradas, adicionadas ou não de sebo bovino, sobre o pH, a concentração de nitrogênio amoniacal, as populações de protozoários e a eficiência de síntese de compostos nitrogenados por microrganismos do rúmen bovino foram determinados em dois experimentos. O fornecimento da silagem de milho associada a rações concentradas que continham de 4 até 10% de sebo bovino, em ambos estudos, causou redução nos níveis de nitrogênio amoniacal e nas populações de protozoários ruminais. A produção diária de biomassa microbiana por grama de carboidrato total digerido no rúmen foi de 0,416 gramas na ausência de sebo, 0,607 g no tratamento contendo 4% de sebo e 0,790 g no que continha 10%. A estimativa do pH no líquido ruminal e da eficiência de síntese de compostos nitrogenados microbianos obtida com o submodelo ruminal CNCPS (Cornell Net Carbohydrate and Protein System) mostrou-se compatível com as observações *In Vivo*.

Palavras-chave: Lipídios, pH, nitrogênio amoniacal, protozoários ruminais, rúmen.

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A LATEX AGGLUTINATION TEST FOR THE DIAGNOSIS OF *BRUCELLA OVIS* INFECTION IN RAMS

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ABSTRACT

A simple latex agglutination (LA) using a heat-extracted antigen for the detection of antibodies against *Brucella ovis* in ram serum was developed. The LA showed high sensitivity and specificity on the basis of positive and negative control sera tested. The antigen-sensitized latex particles were stable at 4°C for almost one year. The use of LA, ELISA and gel diffusion (GD) in conjunction with clinical palpation (CP) of rams for epididymal lesions as a means to detect diseased animals from infected flocks showed that LA/CP performed similarly to ELISA/CP and better than GD/CP. These results indicated that LA can be used as an alternative serological test for diagnosis of *B. ovis* infection in rams.

Key words: *Brucella ovis*, latex agglutination, ELISA, gel diffusion, ram epididymitis

INTRODUCTION

Brucella ovis infection of the genital tract of rams is an important economic problem in most sheep raising countries because it impairs semen quality and reduces fertility. Although *B. ovis* has also been reported to cause abortion in ewes, the major disease produced by this organism is epididymitis of rams (16). The diagnosis of epididymitis based solely on palpation or bacteriological examination of semen is difficult because of transient and asymptomatic infections, and of intermitent shedding of *B. ovis* that induces to false negatives results. Due to these drawbacks the disease is usually diagnosed using serological tests in conjunction with palpation (4). The serological techniques that have been used for detection of antibodies against *B. ovis* in infected rams include complement fixation (8), gel diffusion (GD) (10), indirect hemagglutination (12), immunofluorescence (1), and enzyme-linked immunosorbent assay (ELISA) (11, 6, 15). These techniques, however, have several limitations due to the need of expensive equipment, use of unstable reagents and difficulty to perform with minimal facilities. Little attention has been given to tests simple, rapid and feasible to perform in field conditions such as those based on the agglutination of latex particles (13). In this paper we report the results obtained by a latex agglutination (LA) test using *B. ovis* heat-extracted antigen for the detection of infected rams.

MATERIALS AND METHODS

Serum samples. The following groups of sera were used: (i) negative controls obtained from 60 young rams with no clinical signs of epididymitis and serologically negative by GD; (ii) positive controls obtained from 20 mature rams with palpable lesions of epididymitis and serologically positive by GD; (iii) sera from 230 mature rams belonging to 20 flocks where cases of epididymitis had previously been detected.

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Heat-extracted antigen. *B. ovis* strain CPZ11 was grown on Trypticase Soy Agar with 5% fetal bovine serum for 120 h at 37°C in a 10% CO_2 atmosphere. Cells were harvested in saline, centrifuged (10000 g/20 min), washed three times, standardized to 14% transmission at 600 nm, and autoclaved at 120°C for 20 min. The bacterial suspension was centrifuged again and the supernatant was used as antigen in the serological tests at appropriate dilutions.

Latex sensitization. A suspension of red latex particles (diameter 0.8 µm, Bangs Laboratories Inc., USA) was diluted 1:30 in 0.1 M Tris buffer, pH 7.4, and washed three times by centrifugation at 6000 g for 20 min. Ten milliliters of this suspension were mixed with an equal volume of the heat-extracted antigen diluted 1:40 in Tris buffer and left 4 h at 25°C with agitation. The centrifugation was repeated, the pellet resuspended in 10 ml of 0.05 M Tris buffer, pH 7.2, containing 0.1% BSA, 0.02% PVP-40 and 0.02% sodium azide (Tris). The suspension of sensitized particles thus obtained was stored at 4°C until used.

LA test. Twenty microliters of the stock suspension of latex particles were placed in different wells of a Kline plate and mixed with an equal volume of test serum undiluted and diluted 1:2 and 1:4 with Tris. Positive and negative controls were set up on different wells with known positive and negative sera. After rotating for 2-3 min the agglutination pattern was examined macroscopically and test sera showing a definite clumping of particles such as in positive control sera were regarded as positive.

GD test. The GD test was carried out according to Alton et al. (2). Briefly, 100 mm diameter Petri dishes containing a layer of 3-4 mm of agarose in borate buffer (0.03 M, pH 8.3, NaCl 5%) were used. Seven-well patterns consisting of a central well surrounded by six equidistant wells were cut in the agar. Fifty microliters of heat-extracted antigen diluted 1:4 in saline were placed in the central well. Wells in positions equivalent to 6 and 12 hours received 50 µl of sera under test. The plates were kept in a moist chamber at room temperature and the reactions read after 24, 48 and 72 hours.

ELISA. The ELISA was performed according to established procedures (14). Polyvinylchloride microtiter plates (Hemobag, Brazil) were coated overnight at 4°C with heat-extracted antigen diluted 1:100 in 0.05 M carbonate-bicarbonate buffer (pH 9.6), washed three times with PBS containing 0.05 per cent Tween 20 (PBS-T) and blocked with albumin at 1%. The plates were used or kept at 4°C for not more than one month. Serum samples diluted 1:100 in PBS-T were added to four wells in different quadrants of the plate and incubated for 90 minutes at room temperature. The washing was repeated, peroxidase-conjugated polyclonal rabbit anti-sheep immunoglobulins (Dako, Denmark) was added, and the plates were incubated as above. After washing five times, o-phenylenediamine-substrate was added, left to react for 10 minutes in the dark, and the optical densities (OD) read at 492 nm. Volumes of 50 µl were used with all reactants but blocking solution. Each plate included four wells with positive and four wells with negative control sera. Serum samples yielding mean OD at least two times higher than the mean OD of negative controls were considered positive.

LA sensitivity and specificity. The sensitivity and specificity of the LA test were calculated as follows:

$$\text{Sensitivity (\%)} = \frac{\text{N° of positive sera that tested positive}}{\text{N° of positive sera tested}} \times 100$$

$$\text{Specificity (\%)} = \frac{\text{N° of negative sera that tested negative}}{\text{N° of negative sera tested}} \times 100$$

RESULTS

Sensitivity and specificity. The LA test carried out with 20 positive and 60 negative sera showed 100% of sensitivity and 100% of specificity, respectively. Since the reactions were not different in the three dilutions of sera tested, the highest dilution (1:4) was chosen for use in the comparative study.

Comparison of LA, ELISA and GD with CP. Two hundred and thirty ram sera from 20 flocks with histories of cases of ram epidydimitis were used to examine the application of the LA test, GD test, and ELISA, in conjunction with clinical palpation (CP) of the epidydimis, in the identification of *B. ovis* infected animals (Table 1). The prevalences of rams with positive serological reactions were 20% by GD and 25% by ELISA and LA. Of the 45 rams with clinically diagnosed epidydimitis, 30 were positive by GD, 40 by ELISA and 41 by LA, which results in 15, 5 and 4 false-negatives, respectively. On the other hand, the number of false-positives was similar among the three serological tests. Thus, compared to clinical results, the efficiencies of identification of infected and healthy rams by the GD, ELISA and LA tests were 86%, 90% and 90%, respectively.

Stability of the LA particles. Aliquots of antigen sensitized particles were kept up to 11 months at 4°C and their reactivity with positive and negative control sera examined monthly. No reduction in sensitivity or tendency to agglutinate with negative sera were observed during that period.

DISCUSSION

The components of the *B. ovis* heat-extracted antigen, mainly cell-surface structures such as LPS and outer membrane proteins (7), were easily adsorbed on the surface of the latex particles yielding a stable reagent that reacted with anti-*B. ovis* antibodies in positive control sera for almost one year. The use of heat-extracted antigen to sensitize latex particles was supported by its utilization for coating polystyrene microplates for ELISA, and from previous reports on the successful use of *B. abortus* LPS-coated particles for diagnosis of brucellosis in mice and humans (9, 5).

The results of the LA test with positive and negative-control sera showed that the test had both high sensitivity (100%) and high specificity (100%). Even considering the small number of control sera tested, it was possible to establish the optimal conditions of the LA test, such as proper concentrations of latex particles and antibodies and proper reaction time, in order to obtain maximum clumping of particles. The final test procedure achieved was one in that positive serum developed a clear cut agglutination pattern which could be easily recognized against a white background under normal ilumination conditions after 2 to 3 minutes of reaction.

Clinical palpation in combination with serological techniques such as complement fixation test (CFT) or ELISA has been shown to be effective in detecting *B. ovis* infected flocks (3, 15). We have been routinely using palpation in conjunction with a GD test or ELISA instead of CFT as a criterium to diagnose *B. ovis* infected rams. However, neither of these serological techniques are practical for routine work in unspecialized laboratories. Thus, the LA test developed showed to be a practical technique that can be performed in minimal complexity laboratories giving results comparable to those of the more complex and time consuming serological tests in use. The sensitivities and specificities obtained in the experiment with sera from infected flocks showed that the CP/LA criterium for identification of diseased rams performed similarly to CP/ELISA and better than the CP/GD.

In conclusion, *B. ovis* antibodies are easily detected by the LA test described. The test is simpler and faster than GD and ELISA and may be an alternative to use together with clinical palpation to detect *B. ovis* infected animals in programs seeking the control of ovine brucellosis in infected flocks.

Table 1. Comparison of results of different diagnostic tests used for detection of *Brucella ovis* infection in 230 rams from infected flocks

	GD		ELISA		LA	
	Pos	Neg	Pos	Neg	Pos	Neg
Clinical palpation (CP)						
Positive	30	15	40	05	41	04
Negative	17	168	17	168	18	167
Sensitivity (%)	67		89		91	
Specificity (%)	91		91		90	
Efficiency (%)	86		90		90	

GD: Gel immunodiffusion ELISA: Enzyme-linked immunosorbent assay LA: Latex agglutination
Sensitivity: (N° positive in CP which were positive by GD, ELISA or LA/Total positive by CP) x 100
Specificity: (N° negative in CP which were negative by GD, ELISA or LA/Total negative by CP) x 100
Efficiency: percentage of agreement between results of serological tests and clinical palpation

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RESUMO

Um teste de aglutinação de látex para o diagnóstico da infecção por *Brucella ovis* em carneiros

Foi desenvolvido um teste (LA) que se baseia na aglutinação de particulas de látex sensibilizadas com um antígeno termoextraído para detectar anticorpos anti-*Brucella ovis* em soro de carneiros. O testes LA mostrou alta sensibilidade e especificidade quando foram examinados soros controles positivos e negativos. As partículas de látex sensibilizadas com antígenos se mostraram estáveis por cerca de uma ano a 4°C. A detecção de animais doentes em rebanhos infectados através do uso de LA, ELISA e difusão em gel (GD), em conjunto com palpação clínica (CP) para verificar lesões do epidídimo,

mostrou que LA/CP teve um desempenho similar a ELISA/CP e melhor do que GD/CP. Estes resultados indicam que o teste LA pode ser uma técnica alternativa para o diagnóstico sorológico da infecção por *B. ovis* em carneiros.

Palavras chave: *Brucella ovis*; aglutinação de látex; ELISA; gel difusão; epididimite.

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CULTURAL CONDITIONS FOR PIGMENT PRODUCTION BY *MONASCUS PURPUREUS* IN RICE

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ABSTRACT

The use of natural compounds is a trend observed in the food industry world-wide. The pigments produced by *Monascus purpureus* are a promising alternative to artificial colorants. The aim of this work was to determine the effect of pH (5.0, 6.0 and 7.0) and initial moisture content (60% and 70%) of broken rice "canjica" on the production of pigments by *Monascus purpureus* NRRL 1992. After 7, 10, 13, 16 and 19 days of fermentation, the pigments were ethanol-extracted and the optical density was measured by spectrophotometry at 400 and 500 nm for orange and red pigments, respectively. The absorbance values of orange were always higher than those of red pigments. Pigments yield was maximised until 16 days incubation when substrate was adjusted to an initial pH 6.0 and initial moisture content of 70%. Ater 19 days of incubation highest yield was obtained with initial pH 6.0 and 60% moisture. The initial pH 7.0 gave the highest proportion of red to orange pigments (A_{500nm}/A_{400nm}) of all initial pH tested.

Key words: Natural colorant, *Monascus purpureus*, fermented rice

INTRODUCTION

The use of natural compounds is a trend observed in food industry world-wide and this is an attribute that has become very important to the consumer. Among the natural sources, the microbial one is emerging as a promising alternative to artificial colorants. The advantages are shorter time for pigment production due to the fast growing of the micro-organism and continual production not subject to the vagaries of nature (17).

The best known microbial colorants are those produced by moulds of the genus *Monascus*, specially *Monascus purpureus* species, including corresponding synonyms such as *M. anka, M. rubiginosus* and *M. kaoliang* (10). The mould grown on rice produces a red mass which can be dried, powdered and incorporated into foods. This coloured product is called "Ang-Khak" and has been used for centuries in the Far East (8).

Depending on the cultural conditions, the mould can produce different proportions of yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubrin) and red (rubropuntamine and monascorubramina) pigments (1, 16, 19). These pigments are secondary metabolites synthesised in a polyketide pathway (14), production which is highly sensitive to fermentation conditions (12).

The use of low-quality rice may result in decreased production costs and turn into an interesting value-adding alternative for the rice processing industry.

The aim of the work was to determine the effect of the initial pH (5.0, 6.0 and 7.0) and initial moisture

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content (60 and 70%) on pigment production by *Monascus purpureus* NRRL 1992 using broken rice "canjica" in a solid state fermentation.

MATERIALS AND METHODS

Substrate for fermentation

Broken rice "canjica" sized 1/2 to 1/4 the length of the whole grain was used as substrate for fermentation.

Microorganism

Monascus purpureus NRRL 1992 was obtained from Northern Regional Research Laboratory (Peoria, IL, USA) and was maintained on potato dextrose agar (PDA) (Oxoid, Unipath LTD, Basingstoke, Hampshire, England) slants at 4°C. An inoculum was obtained by growing the mould on slants of PDA for 7 days at 30°C. The surface of the agar was washed out with a solution 0.05% of Tween 80 (v/v) and the suspension obtained was used as the inoculum for fermentation. The number of propagules in the suspension was determined by surface plate count on PDA.

Experimental design

Each 250 ml Erlenmeyer flask was filled with 50 g of rice adjusted to the desired moisture content (60% and 70%), and to the desired initial pH (5.0, 6.0 and 7.0) with 1N NaOH or 1N HCl.

The flasks were sterilised at 121°C for 30 minutes, inoculated to a final concentration of *ca* 2 x 10^4 propagules.g^{-1} and incubated at 30°C.

After 7, 10, 13, 16 and 19 days of incubation, production of pigments was evaluated by blending 10 g of the fermented medium with 100 ml of ethanol A.R. (Reagen, Quimibrás Indústrias Químicas S.A., Rio de Janeiro, RJ, Brazil) in a Waring blender (Eletro Indústria Walita - model LBS, São Paulo, SP, Brazil) for 1 minute. The extract was then filtered through qualitative paper filter (Reagen, Quimibrás Indústrias Químicas S.A., Rio de Janeiro, RJ, Brazil) and diluted as required. The same procedure was followed with non inoculated rice which served as a blank. The pigments concentration was measured by spectrophotometry (ultra-violet/visible CG-UV 8000, CG Analítica Ltda, São Paulo, SP, Brazil) at 400 and 500 nm for orange and red pigments respectively, previously determined as the maximum absorption peaks. The results were expressed as units of absorption (U) (4, 13).

Besides pigment production, pH and moisture content were also determined. For pH determination, 50 ml distilled water was added to the remaining fermented subtract in the Erlenmeyer flask. A blank of non inoculated rice was also prepared. After 30 min. shaking (orbital shaker Kleine-model 255) at 150 rpm, the pH value was measured using a pHmeter (Digigraf LCD). The moisture content of rice samples was determined by gravimetric method (11). Each measurement was done in triplicate.

Statistical analysis

The experimental data were submitted to analysis of variance using a randomised-block model of factorial experiments. The comparison of the mean values was done by Tukey test (9), with confidence intervals to the level of 5%. The program SANEST 2 (20) was used for this purpose

RESULTS AND DISCUSSION

A significant interaction ($P < 0.05$) was observed between moisture content, pH and time of incubation for production of pigments (Tables 1 and 2).

Effect of initial pH on pigment production

Similar profiles for production of orange (Figs. 1 and 2) and red (Figs. 3 and 4) pigments were obtained with the substrate adjusted to initial pH values of 5.0, 6.0 and 7.0, but absorbance values for orange pigments were higher than for red.

The production of orange and red pigments was favoured by setting rice initial pH at 6.0, high yields being obtained with both moisture levels (Figs. 1, 2, 3 and 4). Similar results were obtained by Johns and Stuart (8) when working with rice at initial pH 3.4, 6.0 and 7.0. These authors found that the best performance of the mould was at initial pH 6.0, which is the natural pH of rice.

Media containing 70% moisture and adjusted to initial pH 5.0 resulted in slow increase in absorbance values at the beginning of the incubation period followed by a sharp increase after 13 days incubation, reaching levels of the media adjusted to initial pH 6.0 at the 19 day of fermentation (Figs. 2 and 4). On the other hand, when low initial pH (pH 5.0) was combined with low initial moisture content (60%) the increase in the absorbance values was small and gradual until the 13th day of incubation, when begun to decrease (Figs. 1 and 3). Apparently, the mould could not overcome the adverse conditions, grew very little and then autolysis of fungal cells may have

Table 1. Analysis of variance of absorbance values of orange pigments (400 nm), obtained by factorial combination between different initial pH and initial moisture levels.

Sources of variation	Degrees of freedom	Sums-of-squares	Mean-squares	F value	Prob.>F
Moisture	1	37.0742192	37.074219	373.5773	0.00001
pH	2	86.2889090	43.144454	434.7438	0.00001
Time	4	185.025254	43.256313	466.1004	0.00001
Moisture*pH	2	5.6055195	2.8027597	28.2419	0.00001
Moisture*Time	4	3.6830628	0.9207657	9.2781	0.00004
pH*Time	8	43.1565636	5.3945705	54.3582	0.00001
Moisture*pH*Time	8	58.0732340	7.2591542	73.1467	0.00001
Residue	60	5.9544657	0.0992411		
Total	89	427.8612281			

Final mean = 3.049511 Variation coeficient = 10.33

Table 2. Analysis of variance of absorbance values of red pigments (500 nm), obtained by factorial combination between different initial pH and initial moisture levels.

Sources of variation	Degrees of freedom	Sums-of-squares	Mean-squares	F value	Prob.>F
Moisture	1	8.0532670	8.0532670	227.5588	0.00001
pH	2	25.9433390	12.971669	366.5366	0.00001
Time	4	71.5556797	17.888919	505.4819	0.00001
Moisture*pH	2	2.3753379	1.1876690	33.5596	0.00001
Moisture*Time	4	1.9779414	0.4944854	13.9725	0.00001
pH*Time	8	11.3356347	1.4169543	40.0385	0.00001
Moisture*pH*Time	8	23.2848542	2.9106068	82.2442	0.00001
Residue	60	2.1233898	0.0353898		
Total	89	146.6494437			

Final mean = 1.864467 Variation coeficient = 10.090

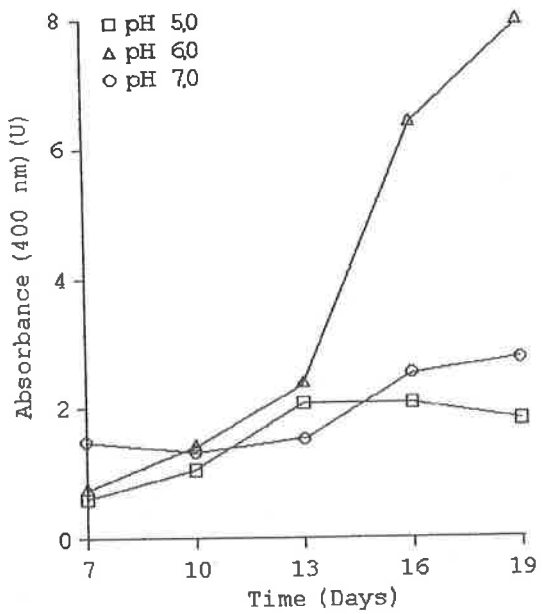

Fig. 1. Production of orange pigmentos by *Monascus purpureus* during fermentation on broken rice "canjica" at 60% moisture content and initial pH values adjusted to 5.0, 6.0 and 7.0. Absorbance readings represent means of three replicates.

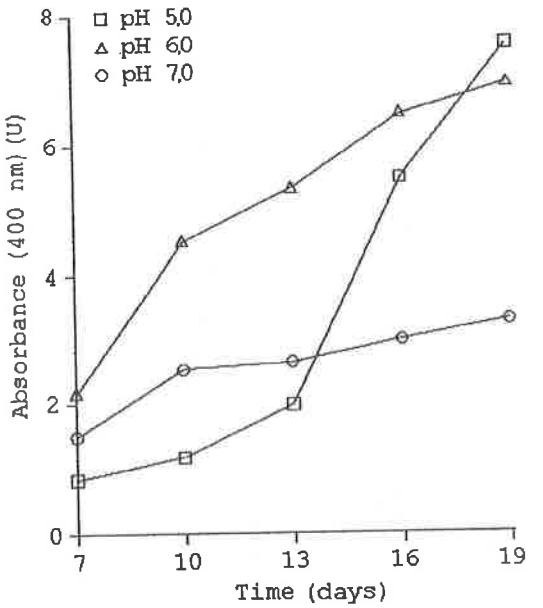

Fig. 2. Production of orange pigmentos by *Monascus purpureus* during fermentation on broken rice "canjica" at 70% moisture content and initial pH values adjusted to 5.0, 6.0 and 7.0. Absorbance readings represent means of three replicates.

started, as suggested by the increase of the final pH of the medium.

As mycotoxins, *Monascus* pigments are also secondary metabolites. Toxigenic strains of *Aspergillus flavus* and *A. parasiticus* normally have a period of active synthesis and accumulation of aflatoxins in the medium, which is followed by reabsorption and metabolism by the producer mycelium (7). In the present work, the combination of initial pH 5.0 with 60% moisture did not result in active synthesis of pigments. Therefore, it is more likely that the small amount of pigments that was produced started to be degraded expontaneously due to chemical changes in the medium.

The media with initial pH 7.0 and 60% moisture content showed absorbance values intermediaries to the ones with pH 6.0 and 5.0 (Figs. 1 and 3).

Production of pigments at 60 and 70% initial moisture

Mould growth and pigment production were highly affected by the initial moisture level. Rice adjusted to 70% moisture content favoured overall pigment production at all initial pH values (Figs. 2 and 4). With 60% moisture an adaptation period was necessary before the mould start actively synthesising the pigments (Figs. 1 and 3).

The combination of high moisture (70%) and pH (7.0) resulted in a small overall production of pigments. Different profile was observed with initial pH 6.0 when higher yields were obtained until 16 days incubation and with pH 5.0 where pigment production was initially inhibited, but increase sharply after 13 days, even overcoming the values obtained with rice at initial pH 6.0. (Figs. 1, 2, 3 and 4).

Different results were reported by Church (5). According to this author, increasing the water level of sterilised rice from 25 to 30% restricted *Monascus* growth to localised areas. Church (5) than concluded that for a satisfactory production of pigments, rice should be added to less than 25% of water, since higher contents were inhibitory to mould growth.

However, findings of Johns and Stuart (12) corroborate with our results. They also noticed that the production of pigments is favoured by moisture content enhancement, when working in the range of 38 to 56%. Micelial growth was not observed in rice with 38% moisture content after 2 weeks incubation. At initial moisture contents of 38-39,5%, mould growth was restricted to the outer layers of the rice grains and when this reached 56%, growth and pigment production was stimulated due to a better penetration of mycelia throughout the grains.

These such findings can be related to the physiological state of the fungi. When under favourable conditions the fungi is capable to produce secondary metabolites, but under stressing conditions

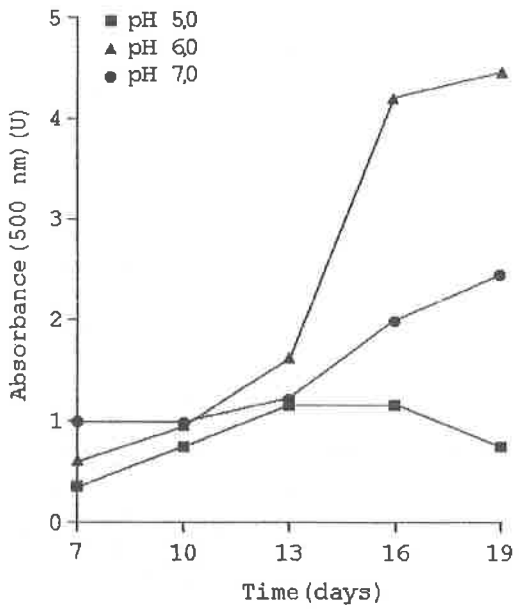

Fig. 3. Production of red pigmentos by *Monascus purpureus* during fermentation on broken rice "canjica" at 60% moisture content and initial pH values adjusted to 5.0, 6.0 and 7.0. Absorbance readings represent means of three replicates.

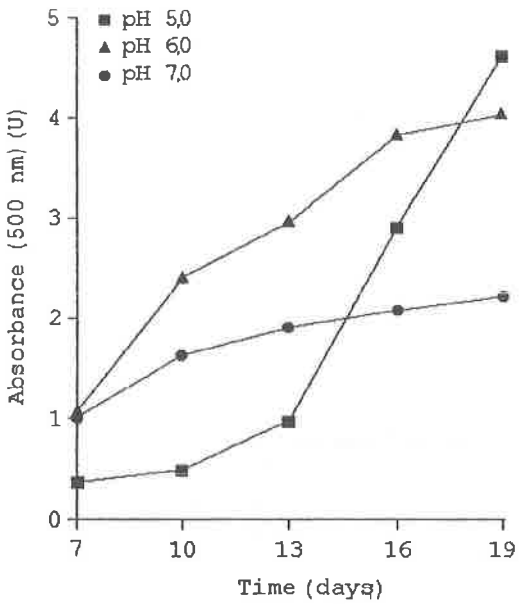

Fig. 4. Production of red pigmentos by *Monascus purpureus* during fermentation on broken rice "canjica" at 70% moisture content and initial pH values adjusted to 5.0, 6.0 and 7.0. Absorbance readings represent means of three replicates.

Table 3. Effect of the initial pH on the relative content of red to orange pigments (A_{500nm}/A_{400nm} Comparison between mean values by the Tukey test.

Days	Substrate Conditions	pH 5,0	pH 6,0	pH 7,0
7°	60% of moisture	0.593 Ba	0.794 Aa	0.657 Aa
	70% of moisture	0.436 Ba	0.494 Bb	0.677 Aa
10°	60% of moisture	0.720 Aa	0.673 Aa	0.725 Aa
	70% of moisture	0.416 Bb	0.531 ABb	0.639 Aa
13°	60% of moisture	0.558 Ba	0.680 ABa	0.763 Aa
	70% of moisture	0.486 Ba	0.556 Bb	0.722 Aa
16°	60% of moisture	0.556 Ba	0.655 ABa	0.784 Aa
	70% of moisture	0.529 Ba	0.591 ABa	0.693 Aa
19°	60% of moisture	0.408 Cb	0.556 Ba	0.895 Aa
	70% of moisture	0.610 Aa	0.581 Aa	0.679 Ab

The means followed by different letters differ at a level of significance of 5%. Small letters = vertical comparison, Capital letters = horizontal comparison.
D.M.S. 5% for different moisture levels = 0.11758
D.M.S. 5% for different initial pH values = 0.14127

the effort was turned to adaptation, reducing pigments yield.

When studying the effect of organic acids on aflatoxin production, some authors observed that sub-inhibitory levels of propionic and acetic acids caused an extended lag phase which after being overcome resulted in large amounts of aflatoxin (15; 18). In this study, the low initial pH induced a lag in mould growth, followed by a sudden increase in pigment production after 13 days incubation when rice initial moisture was 70%. This was not observed at 60% moisture content when the mould did not overcome the unfavourable condition impaired by low initial pH.

The production of orange and red pigments was affected in the same way within the same time interval by different initial pH and moisture levels, showing that the production of both pigments is inter-related.

Changes in pH during fermentation

Rice adjusted to initial pH 6.0 and 7.0 at 60% initial moisture content showed a steady reduction on pH throughout fermentation. Differently, media with initial pH 5.0, alternate increase and decrease in pH values during fermentation (Fig. 5).

Rice containing 70% moisture and adjusted to initial pH 5.0, 6,0 and 7.0 showed a pH reduction until the 10th day of the fermentation period and an increasing pattern at the following days of fermentation (Fig. 6)

The absorbance readings for orange and red pigments and pH value at the end of fermentation period were inversely related. The higher pH value at 19 day incubation (Fig. 5), the lower value of absorbance at the final day of fermentation (Figs. 1 and 3). This increase observed in medium pH might indicate the mould had started autolysis and consequently was metabolically inactive. According to Ciegler et al. (6) the increase observed in the final pH is due to ammonium liberation during autolysis of fungal cells.

Relationship between initial pH, moisture content and the ratio A_{500nm}/A_{400nm}

The red pigments are of particular interest, because red is the most popular food colour and true red natural pigments suitable for food use are difficult to obtain (3).

The comparison between mean values of A_{500nm}/A_{400nm} ratio showed that they were significantly ($P < 0.05$) lower in rice at pH 5.0 as compared with rice adjusted to initial pH 7.0 (Table 3). Rice with 60% initial moisture content and pH 5.0, exhibited decreasing values for the A_{500nm}/A_{400nm} ratio. The same trend was observed when media was adjusted to initial pH 6.0 (Table 3). Differently, with the media adjusted to initial pH 7.0, the ratio A_{500nm}/A_{400nm} increase steadily during fermentation (Table 3).

The increase in moisture content caused an inversion in the profile of A_{500nm}/A_{400nm} values for rice with initial pH 5.0 and 6.0. With rice at 70% initial moisture content, an increasing A_{500nm}/A_{400nm} ratio was observed throughout fermentation.

The combination of initial pH 7.0 and 60% moisture content gave the highest proportion of red to orange pigments (A_{500nm}/A_{400nm}).

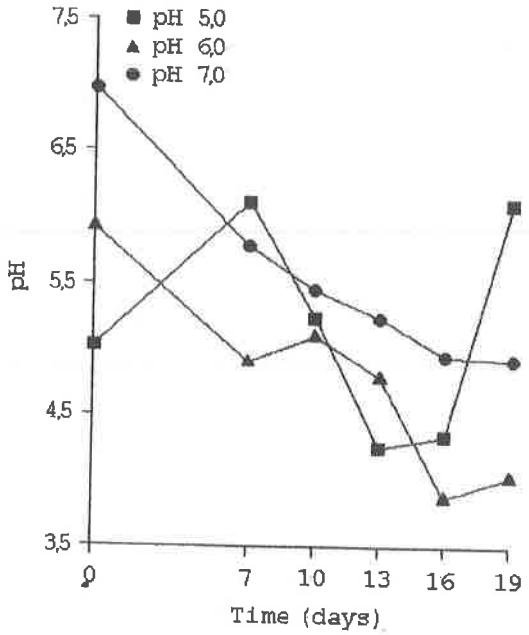

Fig. 5. pH profiles during fermentation of the broken rice "canjica" with 60% moisture content and differents initial pH values by *Monascus purpureus*. Values quoted are means of three replicates.

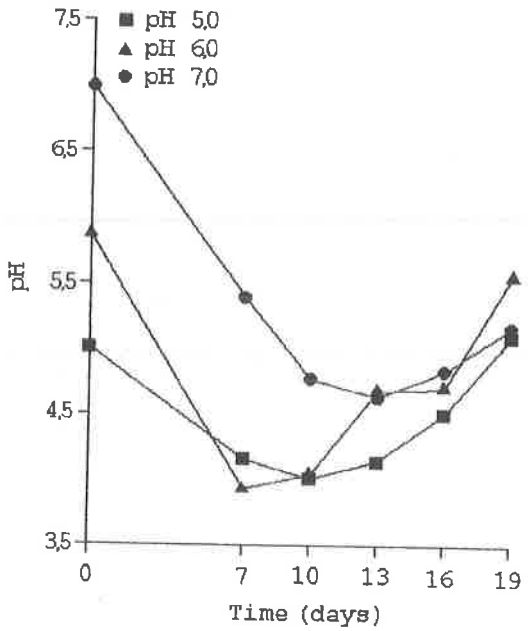

Fig. 6. pH profiles during fermentation of the broken rice "canjica" with 70% moisture content and differents initial pH values by *Monascus purpureus*. Values quoted are means of three replicates.

The findings of Johns and Stuart (12) are somehow in agreement with ours. They worked with rice in the range of 38 to 56% moisture content and reported a maximum A_{500nm}/A_{400nm} ratio when the rice moisture content was 39.5%, which is a low moisture content.

CONCLUSIONS

The initial fermentation conditions strongly affected pigment yield, showing that the right settings must be applied to the fermentation medium for maximised production of pigments. It is concluded that under the conditions of this experiment the rice adjusted to 70% moisture content and initial pH 6.0 is the most favourable substrate for rapid pigment production (16 days or less) whereas the combination of 60% moisture and pH 6.0 is the most indicated when higher total pigment content is sought at the end of incubation period (19th day).

The absorbance readings of red pigments were always lower than that of orange pigments for all samples.

At 7.0 as initial pH the production of both pigments was reduced, but the relative content of red to orange pigments (A_{500nm}/A_{400nm}) was higher. This was because the production of orange pigments increase less at initial pH 7.0 than when at pH 5.0 or 6.0.

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RESUMO

Condições de fermentação para produção de pigmento por *Monascus purpureus* em arroz

É crescente a tendência mundial quanto a utilização de compostos naturais na indústria alimentícia. Os pigmentos produzidos por *Monascus purpureus* estão surgindo como uma alternativa promissora aos corantes artificiais. Este trabalho objetivou determinar o efeito do pH (5.0, 6.0 e 7.0) e da umidade inicial (60% e 70%) sobre a produção de pigmentos por *Monascus purpureus* NRRL 1992 utilizando arroz quebrado "canjica" em fermentação

semi-sólida. Após 7, 10, 13, 16 e 19 dias de fermentação os pigmentos foram extraídos com etanol e a densidade ótica determinada espectrofotometricamente a 400 e 500 nm para pigmentos alaranjados e vermelhos, respectivamente. A absorbância para os pigmentos alaranjados foi superior a dos vermelhos em todas as amostras analisadas. O ajuste do substrato a 70% de umidade inicial e pH 6,0 proporcionou uma mais rápida produção de pigmentos, atingindo os valores mais elevados até o 16º dia de incubação. No final do período de incubação (19 dias) a produção de pigmentos foi maior em arroz ajustado a pH inicial 6.0 e 60% de umidade. O pH inicial 7.0 resultou em maior produção de pigmentos vermelhos em relação aos alaranjados (A_{500nm}/A_{400nm}).

Palavras-chave: Corante natural, *Monascus purpureus*, arroz fermentado.

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CHARACTERISTICS OF A *YERSINIA ENTEROCOLITICA 1/ O:5/ Xz* STRAIN ISOLATED FROM HUMAN DIARRHEIC FECES IN ARGENTINA.

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ABSTRACT

The isolation and characterization of a *Yersinia enterocolitica 1/O:5/Xz* strain isolated from a sample of human diarrheic feces, in Buenos Aires, Argentina, is reported. This strain was the only microorganism in the sample and probably responsible for the diarrhea in the patient although the virulence associated tests were negative.

Key words: *Yersinia enterocolitica B1, O:5, Xz,* human diarrheic feces, virulence tests

Yersinia enterocolitica is a microorganism that appears to be widely distributed in nature (3). It is recognized as an important pathogen organism which causes a variety of human infections. The infections due to *Y. enterocolitica* may manifest themselves in a number of forms depending upon the strain of *Y. enterocolitica*, the dose, genetics factors and the age and immunologycal conditions of the host. It causes an enterocolitis usually acompained by abdominal pain in the lower right quadrant, fever and diarrhea (11). Diarrhea could be absent, however and because of pain localization and fever, Yersiniosis may be misdiagnosed as acute appendicitis (3). Outbreak follow up studies have reported many needless appendectomies.

The serotypes most commonly isolated from humans and considered to be human pathogens are: O:3; O:8; O:5; O:5,27; and O:9 (2,3).Other strains of *Y.enterocolitica* and the other *Yersinia* species are considered environmental microorganisms (18).

The major mechanism of virulence of pathogenic *Yersinia* species is invasiviness, which is mediated by genes "ail", "inv" and "yad A", the first two chromosomal and the last related with the presence of a functional plasmid of 40-48 MDa (3,7).This plasmid also codifies the production of proteins associated to the outer membrane (Yops) (19).

The secretion of these proteins (mainly Yad A) leads to the increase of hidrofobicity (9); the autoagglutination (10) is also promoted and they confer the bacteria all the ability of resisting phagocitosis and the bactericidal effects of the normal human serum (14). Some of these properties are phenotypic characteristics of virulence. The involvement of chromosomal genes in the pathogenicity of *Yersinia* was shown to be related to certain phenotypic characteristics such as activity of the enzime pyrazinamidase and the abilities to hidrolize esculine and to ferment salicine (1,3).

In this paper, we report the isolation and characterization of a *Y. enterocolitica* strain isolated from human diarrheic feces. We also report some tests of virulence associated characteristics.

A total of 90 fecal samples collected from patients with diarrhea in the Hospital de Clínicas, Buenos Aires-Argentina were examined during the period January/1996 to March/1997. *Y. enterocolitica* and other pathogen microorganisms were searched. Stool

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samples were cultured for cold enrichment of *Yersinia* on PBS-Sorbitol-Bile Salts-Casein hydrolyzed-Medium (4,13) up to 21 days at 4°C. After the incubation a loopful of enrichment was streaked directly onto Cefsulodin-Irgasan-Novobiocin (CIN - Merck) (17)-Agar plate and Mac Conkey Agar plate for isolation. Agar plates were incubated for 48 hours at 28°C.

The 90 stool samples were also processed by technics of conventional coprocultive. All samples were negative for *Salmonella* spp., *Shigella* spp., enteropathogenic and enterohaemorragic *E.coli*, *Vibrio cholerae* and *Aeromonas* spp. as well as for parasitological analysis.

One sample, belonging to a 19 years old patient, presented colonies with *Yersinia* characteristics onto CIN Agar. The colonies were preliminary identified by the API 20-E system. The biochemical pattern, serotyping and phagetyping determinations were done at the Laboratório de Referência de *Yersinia* - Araraquara - São Paulo - Brasil.The strain was identified as *Y. enterocolitica* biotype1, serotype O:5 and phagotype *Xz (Ye 1/O:5/Xz) (BC 39).*

Therefore the *Y. enterocolitica* strain was the only isolated microorganism in the sample *BC 39* probably responsible for the diarrhea in the patient. Antibiotic susceptibility pattern of the *BC 39* strain determined by semiquantitative method (12) is shown in Table 1.

Virulence factors of *Y. enterocolitica* strain were studied: pyrazinamidase activity, esculin hydrolisis, and salicin fermentation (8); red Congo binding and calcium dependency (16); autoagglutination (10), and the presence of 40-48 MDa plasmid (6).

Reference strains used were *Y. enterocolitica 4/ O:3/ VIII (FCF 396); Y. enterocolitica 4/ O:3/ VIII (FCF 525),* gently provided by Dr. Deise Pasetto Falcão. Both were also used as positive controls for the presence of the virulence plasmid.

The results of the general characteristics associated to virulence of the strains of reference and the strain under study are shown in Table 2.

Virulence tests for *Y. enterocolitica 1/O:5/Xz* strain were negative. This strain may be considered not adapted to man yet, although it comes from a case of human diarrhea.

Similar results were obtained by Bauab and Falcão (2), who found *Y. enterocolitica* O:5 strains, isolated from human diarrheic feces, beared out no virulence-associated factors.

Ratman et al. (15) described a nosocomial infection due to an "avirulent" *Y. enterocolitica* biotype 1, serotype O:5, similar to the one found by us. Besides, in agreement with our results the strain described by Ratman was also isolated from the stools only after enrichment.

Nowadays is well know that one of the main mechanisms of virulence of pathogenic *Yersinia* species is related with invasiviness / adherence genes located either in the chromosome (inv, ail) or in the plasmid (yad A). The virulence factors encoded by these genes mediate functions such as resistance against phagocytosis, complement lysis and iron

Table 1. Susceptibility of *Y.enterocolitica BC39* to antimicrobials.

Antibiotics	ug/ml	
Amikacin	8	S
Ampicillin	16	R
Ampicillin-Sulbactam	16/8	R
Cefoperazone	4	S
Ceftazidine	4	S
Ceftriaxone	4	S
Cephalotin	16	R
Ciprofloxacin	1	S
Chloramphenicol	8	R
Colistin	1	S
Gentamicin	4	S
Imipenem	4	S
Meropenin	4	S
Nitrofurantoin	64	R
Norfloxacin	4	S
Piperacillin	16	S
Trimethoprim-Sulfamethoxazole	0,5/9,5	S

S: sensitive
R: resistant

Table 2. Virulence propierties of *Yersinia enterocolitica* 1/O:5/Xz (BC39) and *Yersinia enterocolitica* reference strains

Tests	*Y. enterocolitica* BC 39 *	FCF 396 **	FCF 525 ***
Pyrazinamidase activity	+	-	-
Esculin hydrolisis	+	-	-
Salicin fermentation	+	-	-
Calcium dependency at 37°C	-	+	+
Congo Red binding	-	+	+
Autoagglutination at 37°C	-	+	+
Plasmid (40-48 MDa.)	-	+	+

*: *Y. enterocolitica 1/O:5/Xz.*
**: *Y. enterocolitica 4/O:3/VIII*
***:*Y. enterocolitica 4/O:3/VIII*

uptake and thus promote extracellular survival of *Y. enterocolitica* in infected host tissue (3,5,7).

The chromosomal genes are considered important because of their stability. On the other hand plasmidial genes even though they participate in the pathogenic mechanism they may be lost easily during laboratory cultivation (1).

We test some phenotipic characteristics encoded in chromosomal and plasmidial genes. Our results show that the strain *BC 39* would be classified as "avirulent" or non adapted to human yet.

As was suggested by Bottone (3), further studies would be necessary to show that the so-called "avirulent *Y. enterocolitica*" or non-enterocolitica species may induce gastrointestinal disturbances analogous to those due to enteropathogenic microorganisms through enteroadherence or some other mechanism.

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RESUMO

Características de uma cepa de *Yersinia enterocolitica 1/O:5/Xz* isolada de fezes diarreicas humanas na Argentina

Neste trabalho foi descrito o isolamento e a caracterização de uma cepa de *Yersinia enterocolitica 1/O:5/Xz* isolada de fezes diarreicas humanas en Buenos Aires, Argentina. A cepa isolada foi o único microrganismo na amostra e provavelmente responsável da diarréia, embora os testes de virulência fossem negativos.

Palavras-chave: *Yersinia enterocolitica B1, O:5, Xz,* fezes diarreicas humanas, testes de virulência

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NUMERICAL ANALYSIS VARIATIONS OF SDS-PAGE PROTEIN PATTERNS USING DIFFERENT CULTURE MEDIA FOR THE CULTIVATION OF *CANDIDA* FROM THE ORAL CAVITY

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ABSTRACT

The relationships among 12 oral yeast strains belonging to 5 yeast species were determined by one-dimensional electrophoresis of SDS-solubilized whole-cell proteins using different culture medium in the cultivation of the cells. The phenograms were made using Simple Matching coefficient (SM) and UPGMA clustering method. The strains investigated represented species from the genus *Candida*, species *C. albicans, C. tropicalis, C. guilliermondii, C. parapsilosis*, and *C. krusei*, all isolated from the oral cavity of normal and healthy people. The data obtained from the phenograms showed a high difference in protein patterns when all the species were compared using different culture media, suggesting that different nutritional compositions led to the expression of different proteins derived from alternatives metabolic pathways expressed by the electrophoretic profiles. When looking to the different phenograms most species are not delineated as separate entities. A considerable protein electrophoretic heterogeneity is observed among strains of single species. From the data presented it seems that SDS-PAGE is characterizing at the strain level (unique profiles are obtained for each individual strain). Consequently, no taxonomic conclusions can be draw at the species level. Therefore the construction of a database of protein fingerprints is an important tool for the identification and characterization of these organisms.

Key words: Yeasts, *Candida*, culture medium, whole-cell protein electrophoregrams, identification, classification

INTRODUCTION

The Fungi, specially yeasts belonging to the genus *Candida* are potencially pathogenic agents capable of inducing to many pathologies on the living organisms behaving as an opportunists. With the AIDS arisement the researches using such microorganisms have increased in the physiological, biochemical, serological, genetic and epidemiological points of view (Davemport, 1970; Axelsen, 1973; Faux et al., 1975; Greenfield and Jones,1981; Dreizen 1984; Gatermann et al., 1986; Brondz and Olsen, 1991; Allen, 1992 and Challacombe, 1994). In the oral cavity, they are responsible for those pathologies affecting buccal structures justifying the increase in interest in the possible involvement of yeasts in oral disease.

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The form-genus *Candida* includes a heterogeneous group of 81 asporogeneous yeast species (Van Uden and Buckley, 1970). *Candida* species, in addition to forming yeast-like budding cells, have the ability to produce pseudo mycelium and true mycelium (Ajello et al., 1963). Conventional methods for classifying species of *Candida* are based particularly on morphological and physiological characteristics. During the last decades several specific techniques have been developed and applied to taxonomic studies of such organisms as the analysis of the electrophoretic profiles of soluble proteins (Fregerslev, 1969) and isoenzymes (Berchev and Izimirov, 1967); serological features (Tsuchuya et al., 1965; Biguet et al., 1962; Rimbaud and Nakan, 1966); proton magnetic resonance (PMR) spectra of mannans and mannose-containing polysaccharides (Spencer and Gorin, 1969, 1971); immunoelectrophoretic studies of antigens (Gabriel-Bruneau and Guinet, 1984; Biguet and Andrieu, 1962; Manning and Mitchell, 1980); chromosomal DNA banding patterns (Viljoen et al., 1989a; Magee and Magee, 1987); assesment of base-sequence homology by means of DNA-RNA hybrid experiments (Landau et al., 1968) and long-chain fatty acid analyses (Viljoen and Kock, 1989; Viljoen et al., 1989a).

High resolution polyacrylamide gel electrophoresis (SDS-PAGE) of proteins, combined with computerized analysis of patterns, has been used increasingly in microbial systematics (Barns et al. 1991; Hall 1969; Hall et al. 1969; Johnston et al., 1988; Jordan 1994; Magee et al., 1988; Meyer et al., 1993; Okiri and Kawamoto 1995). Protein patterns offers considerable potential for typing strains of clinical interest and for taxonomic purposes, especially for the studies of the quantification of biodiversity among microorganisms (O' Donnel et al. 1994; Barns et al. 1991; Blignaut and Koch, 1992; Booth, 1979). In this sense, the delineation of the electrophoretic clusters of the yeasts studied have demonstrated that a database of SDS-PAGE-protein patterns provides a valuable tool for the identification of such microorganisms. The grouping of the protein electrophoregrams, has been used to built phenograms showing the protein electrophoretic relationship among all the strains studied of a major taxonomic and systematic purpose.

The literature data have been based on the criteria of using a specific culture medium in the cultivation of the cells. Nevertheless, studies in this area of research, show that differents types of such culture medium have been used for such purpose. The aim of the present study was to compare the phenograms of some oral *Candida* species through the analysis of the electrophoregrams of such yeast cells grown on different culture medium and to evaluate its implications on the taxonomy purposes.

MATERIALS AND METHODS

Strains used. A total of 12 strains of 5 *Candida* different yeast species were investigated, *C. albicans* (E-37, 97-a, 17-b and F-72), *C. tropicalis* (1-b and FCF-430), *C. guilliermondii* (FCF-152 and FCF-405), *C. parapsilosis* (21-c and 7-a), and *C. krusei* (1M-90 and 4-c). All strains were isolated from normal and healthy patients of the Odontological Clinic, School of Dentistry, UNESP, São José dos Campos, Brazil.

Cultivation of the cells. Two days old cultures, grown on different cultures media as 1) Yeast Complete Medium (YCM) agar slants (per 500ml of distilled water: 5g neopeptone, 5g yeast extract, 0.25g KH_2PO_4, 10g glucose, 10g agar); 2) Bacto Sabourand Maltose Agar (BSMA), 5g neopeptone, 20g maltose, 7.5g agar, pH 5.6-5.8; 3) Sabourand Dextrose Agar (SDA), 5g peptone, 20g dextrose, 10g agar; 4) Pagano Levin (PL), 5g peptone-bacto, 0.5g yeast extract bacto, 20g dextrose-bacto, 7.5g agar-bacto, added of 50 µg of chloride 2,3,5 tripheniltetrazolium plus 500 µg of neomicin/ml; 5) Fungi Selective Agar (FSA), (per 1000ml of distilled water: 10g soya peptone, 10g dextrose, 15.5g agar, 0.4g cicloreximida; 0.05g chloramphenicol, pH 6.9; 6) Bacto Candida (BCG) Agar Base, 10g peptone, 1g yeast extract, 40g dextrose, 0.02g bromocresol green, pH 6.1-6.2 added of 500 µg of neomicin/ml, incubated in a shaking machine at 37°C, were centrifuged and the cells were harvested in the logaritmic growth phase and washed three times in 0.85% saline steril solution (per liter distilled water: 8.5g NaCl).

Preparation of the cell-free extracts. Yeasts cells (200µL/500 µl of distilled water) maintained in liquid nitrogen (-196°C) were powdered for 3 minutes approximately (Gomes, 1995) and centrifuged at 3.500 rpm (3000g) for five minutes and the supernatant was stored at –80°C. The preparation of the cells for electrophoresis was made according Bruneau and Guinet, (1989) and the supernatant was kept at –80°C before use.

Protein electrophoresis. One-dimensional SDS-PAGE was performed according to the technique of Laemmli (1970), in a discontinuous gel system. The

final polyacrylamide gels concentrations were 12.5% (w/v) for the running gel and 4.5% (w/v) for the stacking gel. Further details of the methods used in gel preparation and electrophoresis were described previously (Moore et al., 1980).

Quantitative comparison of the electrophoretic patterns. Proteins electrophoregrams were scanned and processed using the simple matching coefficient (SM) and UPGMA clustering method led to analysis of the similarity among all strains.

RESULTS AND DISCUSSION

Many researches have been done on the last years with the genus *Candida* using SDS-PAGE with emphasis on systematics (Maiden and Tanner, 1991.; Epstein et al., 1980.; Lehmann, et al., 1989.; Shen et al., 1988). Such technique has been successfully used for classification and identification of these organisms.

A total of 12 isolates belonging to 5 different yeast species were compared. The results show the electrophoretic differentiation of SDS-solubilized whole-cell proteins using different culture medium for the cultivation of the cells. The results obtained with Simple Matching coefficient presented in the Fig. 1 show that the significant features of these phenograms are the higher and clear difference order of clustering and the division of the various isolates into different groups are not the same when compared.

The principal criteria used in fungal taxonomy is the form of reproductive structure and the manner in which these structures develop (Glynn and Reid, 1969). These authors have made a statement on the seventies that identification of fungi, both in culture and in natural substrates, is frequently impossible because of the absence of such reproductive structures or doubt whether the nature of the reproductive units formed are normal, i.e., have the conditions under which the fungus have grown allowed development similar to that found under "natural conditions"? They showed that since the soluble protein must reflect the physiological state of the cell rather than morphological structure, it seems reasonable to expect a variation in the type of protein patterns obtained, depending on the physiological state of the cell at the time of harvesting. Also considerations made by Fink (1970) in terms of the growth parameters of yeast cells, demonstrated that the wild-type yeast strain S-288C has a doubling time of 90 minutes in YEPD medium and 120 minutes in minimal culture medium. A saturated YEPD culture contains approximately $4x10^8$ cells/ml, whereas a saturated minimal medium culture contains approximately $8x\ 10^7$ mL. One liter of a satured YEPD culture yields approximately 17 g of cells, wet weight; a liter of minimal grown cells yields 3.4 g of cells.

The groupings of *Candida* isolates derivated from numerical analysis of the electrophoretic data, showed a highly different protein patterns among all the species by using different culture medium for the cultivation of the cells, suggesting that different nutritional compositions of the culture media led to the expression of different proteins derived from alternative metabolic pathways expressed by the electrophoretic profiles. When looking to the different phenograms most species are not delineated as separate entities. A considerable protein electrophoretic heterogeneity is observed among strains of single species. From the data presented it seems that SDS-PAGE is characterizing at the strain level (unique profiles are obtained for each individual strain). Consequently, no taxonomic conclusions can be draw at the species level. Early investigations made by Shechter (1973), in an attempt to identify *Candida* species by comparative protein disc electrophoresis, found that results are highly dependent on culture conditions, the age of the cultures and the morphological stage of the yeast strain studied. Although, Vancanneyt (1991) has found that independently grown cells of the same strain have approximately the same range of reproducibility as the electrophoretic technique itself.

According to Shechter (1972), the value of numerical taxonomic classification in *Candida* will be determined by its capacity to incorporate new taxonomic characters and additional taxa rather than its concordance with existing classifications. The continuing accumulation of new biochemical and immunological characters in *Candida* will present more opportunities for application of numerical taxonomy.

Researches have been done during the last years with the genus *Candida* by using SDS-PAGE near of others approaches, with emphasis on systematics (Böhler et al., 1994; Agudo et al., 1993; Gambale et al., 1977; Greenfield and Jones, 1981; Monod et al., 1990; Brondz and Olsen, 1995). Such technique has been successfully used for classification and identification of many species from this group of microorganisms.

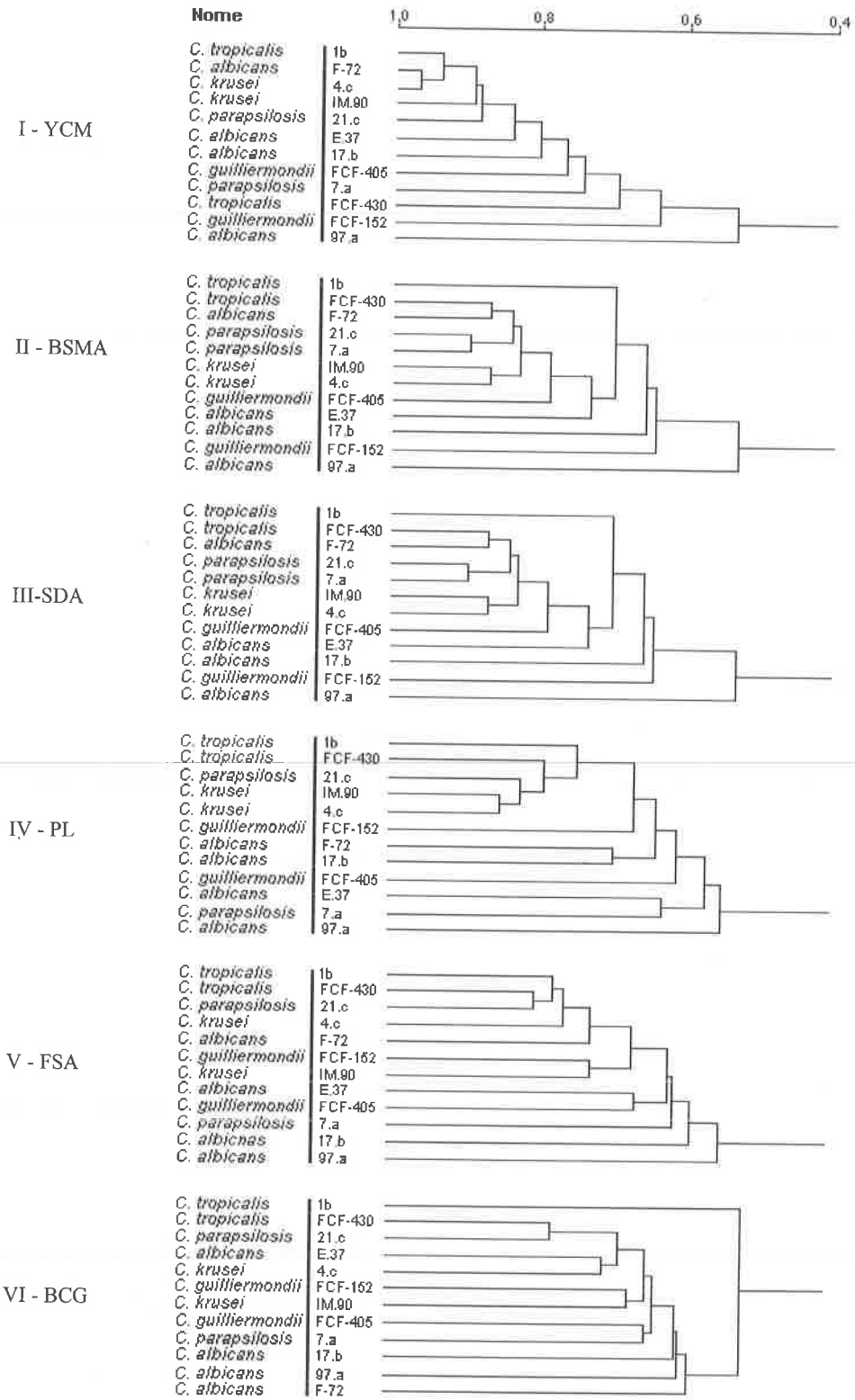

Figure 1. The protein electrophoretic relationships between 12 yeast strains using different culture media for the cultivation of the cells. The similarity coefficient was used and clustering was achieved by the UPGMA method. Roman numerals indicate culture media and respective individual phenograms.

The SDS-PAGE technique combined with numerical analysis of electrophoregrams usually allows valid deductions for taxonomic purposes or microbial classification. It is an useful and practical tool for screening multiple isolates for identification or to verify the authenticity of strains once a database of reference protein electrophoregrams has been constructed or is available. Nevertheless, the non-aquaintance or the non observation that culture conditions as well as others growing agents can be a limitable factor in this kind of research will certainly induce to erroneous interpretations.

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RESUMO

Variações na análise numérica de SDS-PAGE de proteínas intracelulares de leveduras orais

O relacionamento fenético entre 12 amostras de leveduras isoladas da cavidade bucal de humanos, pertencentes a 5 espécies, foi determinado empregando-se a técnica de eletroforese em gel de poliacrilamida (SDS-PAGE), de proteínas intracelulares, usando-se diferentes meios de cultura para o cultivo das células leveduriformes. Os fenogramas foram construídos usando-se o coeficiente similaridade - *Simple Matching* (SM) e o sistema de agrupamento UPGMA. As amostras investigadas representaram o gênero *Candida*, espécies *C. albicans, C. tropicalis, C. guilliermondii, C. parapsilosis,* e *C. krusei,* todas isoladas da cavidade oral de pacientes saudáveis. Os dados obtidos dos fenogramas mostraram diferenças significativas no grau de relacionamento fenético entre essas espécies, quando comparadas usando-se diferentes meios de cultura para o cultivo das células leveduriformes, sugerindo que diferentes nutrientes presentes na composição desses meios levam à expressão de diferentes proteínas derivadas de mecanismos metabólicos alternativos, expressos nos perfis eletroforéticos. Observando-se os diferentes fenogramas, muitas espécies não estão delineadas como entidades separadas. Uma considerável heterogeneidade nos padrões protéicos entre as espécies foi observada e os dados demonstraram que os perfis eletroforéticos obtidos estão delimitados à cada amostra em particular. Consequentemente, conclusões com ênfase taxonômica - ao nível de espécie - não puderam ser obtidas. Entretanto, a construção de um banco de dados obtidos a partir de perfis protéicos de células leveduriformes é, ainda, um importante recurso para a identificação e caracterização desses microrganismos.

Palavras-chave: Leveduras, *Candida*, meio de cultura, proteínas de células totais, eletroforegramas, identificação, classificação

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