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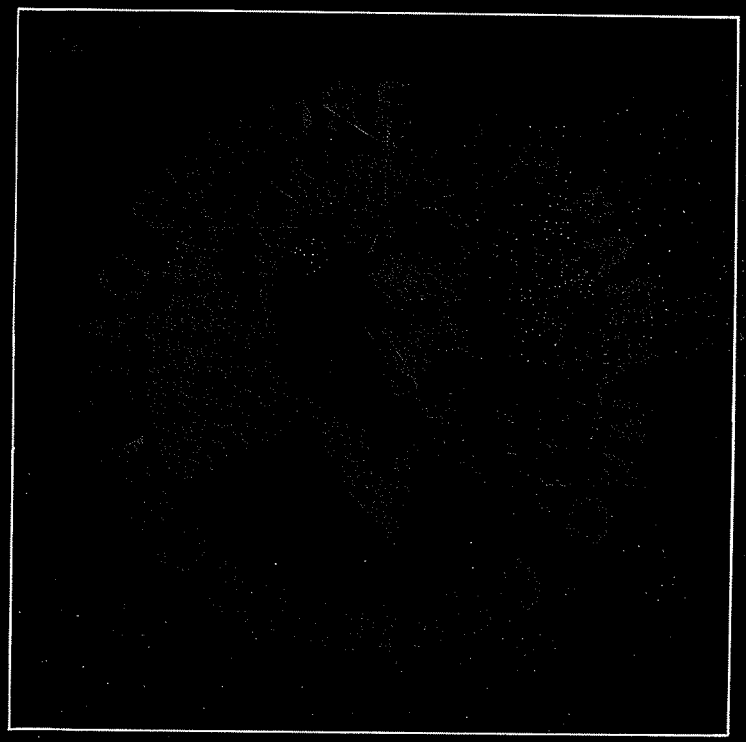

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PRESERVATION OF THE HUMAN AMNIOTIC MEMBRANE INTERFERON

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SUMMARY

Investigations into the preservation of the human amniotic membrane interferon (IFN-AM) were undertaken to permit further research into its purification, chemical and biological properties, and clinical trials. Rapid heating inactivating tests were performed to provide immediate results. IFN-AM was prepared by infecting amnions with Newcastle disease virus. Assay for antiviral activity of IFN-AM was done in Vero cells infected with encephalomyocarditis or Sindbis virus. For the inactivation tests, IFN-AM preparations containing 0; 1 and 2% of sheep serum were heated at temperatures ranging from 35°C to 100°C for various time intervals and the residual antiviral activities were determined. Based on these data, Arrhenius' constant of heating decay was calculated, plotted against the absolute temperature and the data obtained from high temperatures (45°C and above) extrapolated to low temperatures (37°C and below). Results showed that IFN-AM was better preserved at pH 2 in the presence of serum. The data obtained at 45°, 55° and 65°C permitted the calculation of the Arrhenius' constant for lower temperatures by extrapolation. At -4.2°C, IFN-AM should not loose any activity.

Key words: Preservation, interferon, human, amniotic membrane.

INTRODUCTION

The use of proteins in prophilaxys and as therapeutic agents demands appropiate preservation from production to application. Interferons (IFN) are used clinically for the treatment of certain viral infections (19) and tumors (17), and they can be derived from human cells, for instance peripheral blood leukocytes (2), fibroblasts (1), tumor cells (23) and also from genetically engineered bacteria (6).

The human aniotic membrane makes IFN in similar amounts to leukocytes or fibroblasts. In addition, the process is simple and inexpensive (11), permitting its industrial production. Therefore, methods for its production (11) and purification (18) were devised. Suitable preparations were developed for skin or eye viral infections and some physico-chemical properties of this

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IFN were described recently (4).

The preservation of the biological activities of the human amniotic membrane interferon (IFN-AM) is essential to carry out further research. Thermal degradation experiments using high temperatures provide an accurate and rapid test for the preservation of viruses and other biological products (9) and they have been used for vaccines (7), immunological products (12) and interferons (10, 21). In this paper, we show that in IFN-AM preparations containing serum in low pH the antiviral activity should be preserved at temperatures lower than -4.2°C for very long periods.

MATERIAL AND METHODS

Interferon – The production of IFN-AM was done essentially as described (11). Briefly, the amnions were separated from the placentas, washed, fragmented and primed for one hour at 37°C with Newcastle disease virus, Texas 2 strain, previously diluted 10 times. Medium was added and removed after one hour. The tissue was then induced to produce IFN by a second infection with undiluted virus. Media with or without sheep serum were collected after 24 and 48 hours of incubation and the pH was adjusted to 2. Stocks were kept at het -70°C. IFN titration was performed by a microtechnique assay, using a Vero cell-encephalomyocarditis or Sindbis virus system (5), including an internal standard for every titration. In this paper, IFN titers were adjusted to the average titer of this standard. The variation coefficient of the assay was 45%. One laboratory unit was equivalent to approximately 2.8 units of the international standard for human IFN 'Research Standard B, 69/19'.

Protein – The method of Lowry et al. (13) and later its modification by Hartree (8) were used.

Inactivation tests – IFN stocks were thawed overnight, centrifuged at 4°C, 20 min, 3,000 x g and dispensed in 1.0 ml aliquots. Samples were heated in water-baths (±0.5 C) in times and temperatures indicated and imediately cooled (0°C), diluted in medium containing 5% serum and kept at -20°C until titrated for antiviral activity.

Arrhenius' constant of heating decay was calculated according to the formula (7):

$$K = \frac{(XY) - \frac{(X)(Y)}{n}}{X^2 - \frac{X^2}{n}}$$

where: "X" is the time of treatment in hours; "Y" is the logarithm of the percentage of the residual activity after the time "X"; "n" is the number of values obtained at the same temperature in different experiments.

The values obtained in at least three different temperatures were plotted: the Arrhenius' heating decay constant values in the abscissa axis and the absolute temperature in the ordinate axis. The curve obtained was prolonged and by extrapolation, values in lower temperatures for the constant and temperature were found. When the decay constant is zero, in the ordinate axis, there should be no loss of activity.

Materials – Tissue culture media were from DIFCO, Detroit, Michigan, USA. Chemicals from E: Merck, Darmstadt, Germany.

RESULTS

The first experiments were performed in order to set experimental conditions. Aliquots of one preparation of IFN-AM containing 1% serum in pH 2 were adjusted to various pHs and heated at 65°C. Samples were taken at appropriate time intervals and the residual antiviral activities were determined. Fig. 1, panel A shows the results: acid pH, specially pH 2, preserved the antiviral activity. Fig. 1, panel B, displays the behaviour at 65°C of one preparation without serum as compared to an IFN containing 1% serum; the effect of protein on the preservation is striking. Tests using temperatures of 37°, 75° and 100°C showed similar results (data not shown).

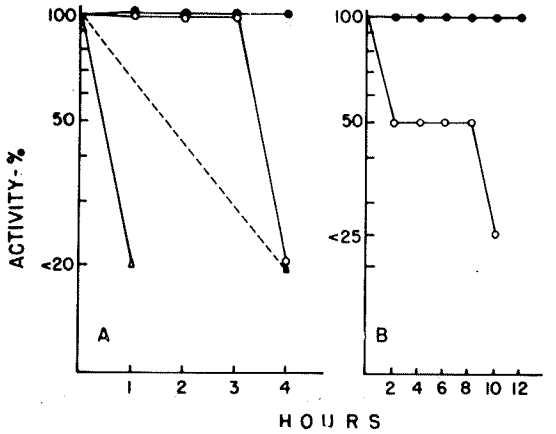

FIGURE 1. Inactivation of the human amniotic membrane interferon at 65°C. Panel A – Aliquots containing serum and treated when their pH was 2 (- -●-●- -); 4 (—o-o—); 5 (- -▲-▲- -); 6, 7, 8, 9, 10 (—Δ-Δ—). Panel B – Aliquots in pH 2 with (— ●-●—) or without serum (—o-o—).

In other experiments, heating six preparations of IFN-AM at 45°C and submitting a second preparation of IFN-AM without serum at 35°, 37°, 45° and 55°C revealed comparable results (data not shown) demonstrating that, under these conditions, the behaviour of diverse IFN-AM preparations was equivalent.

A preparation of IFN-AM, containing 1% serum in pH 2, was heated to 45° and 55°C for various time intervals and the results of the remaining IFN titers of three experiments are shown in Fig. 2. In Fig. 3, eight different preparations of IFN-AM in pH 2 and with 1 or 2% serum were submitted to 65°C. The inactivation of IFN shows a biphasic curve at the three temperatures used in the experiments represented in Fig. 2 and 3. A first phase shows an exponential decay of antiviral activity, and, if the heating period was prolonged a second phase, during which the loss of activity was negligible. Deviations in the curves can be explained by the variation of the antiviral assay.

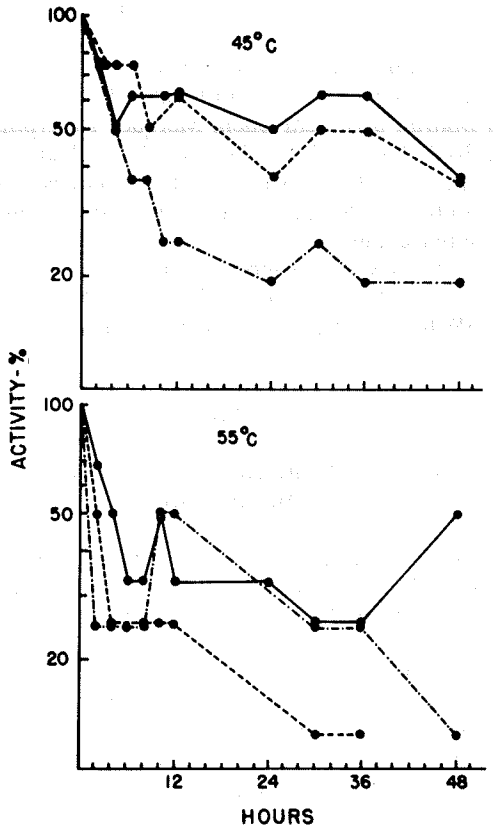

FIGURE 2. Inactivation of the human amniotic membrane interferon at 45°C and 55°C. The preparation contained serum and its pH was 2. The lines represent different experiments.

Since the interest for preservation is to maintain the highest level of activity, and the rapid inactivation tests rely on an exponential decay, the data of the first phase were taken after 8 hours at 45°C; 6 hours at 55°C and 4 hours of 65°C. These data are organized in Table 1, and the Arrhenius' decay constants were calculated: -0.030 for 45°C; -0.060 for 55°C and -0.091 for 65°C.

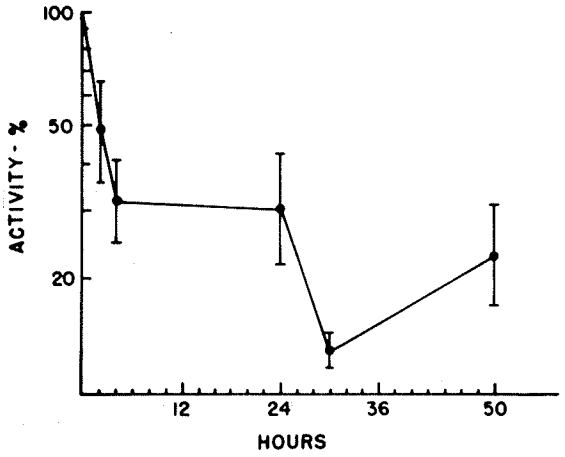

FIGURE 3. Inactivation of the human amniotic membrane interferon at 65°C. The preparation contained serum and its pH was 2. Each point represents the average titer of at least seven titrations and the vertical bar shows the standard deviations.

TABLE 1 – Values for the calculation of the decay constant (a)

45°C		55°C		65°C	
X(b)	Y(c)	X	Y	X	Y
2	1.87	2	1.82	2	1.82
2	1.79	2	1.57	2	1.57
2	1.87	2	1.69	2	1.09
4	1.69	4	1.69	2	1.69
4	1.69	4	1.57	2	1.61
				2	2.00
4	1.87	4	1.39	2	1.39
6	1.79	6	1.34	2	1.69
6	1.57	6	1.57	4	1.52
6	1.87			4	0.96
8	1.57			4	1.39
8	1.69			4	1.27
				4	1.87
				4	1.39
				4	1.57

(a) Data taken from Fig. 2 and 3
(b) Time in hours
(c) Log of the percentage of the residual activity after time x

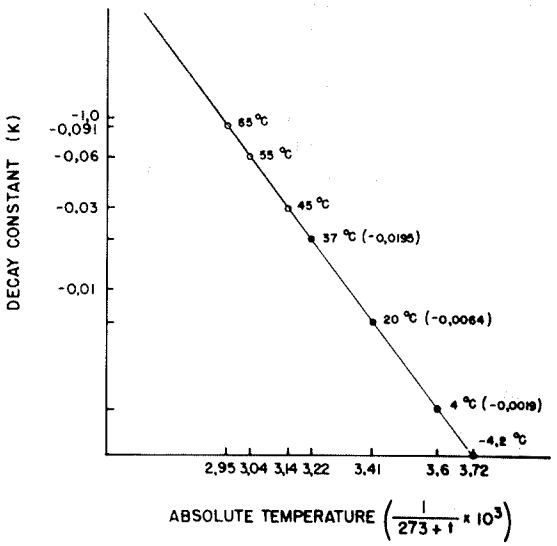

Figure 4. Relationship of the decay constant of the human amniotic interferon submitted to 45°C, 55°C and 65°C (data taken from Fig. 2 and 3) and the absolute temperature, according to the Arrhenius formula (see Material and Methods). Decay constants were calculated from the data (45°C, 55°C and 65°C). For the lower temperatures, the data were extrapolated.

These constants and the absolute temperature were plotted (Fig. 4). The extrapolation of the curve permits the calculation of the decay constants for lower temperatures, for example – 0.0195 for 37°C; -0.0064 for 20°C and -0.0019 for 4°C. At -4.2°C the IFN activity would, at least theoretically, be maintained without loss.

DISCUSSION

The increased stability of IFN-AM seen in pH 2 (Fig. 1, panel A), was also reported for human beta IFN (21) and murine IFN (10).

According to the literature, the stability of IFN is also increased in the presence of protein. Our IFN-AM preparations without serum had 20 to 50 micrograms of protein per ml; in the stocks made with 1% serum, the amount of protein was 200 to 500 micrograms per ml. Sedmak et al. (21) reported that increasing amounts of protein from 5 to 500 micrograms per ml resulted in an enhanced stability, as it happens with the IFN-AM when serum is added (Fig. 1, panel B). Other authors (14, 22) have shown that the protein content does not influence IFN titers when submitted to heat, or that IFN preparations with less protein are more stable (15). It is conceivable that the protein reaches its highest protective effect in certain concentrations. Moreover, for some preparations of IFNs, the presence of proteases or inhibitors of IFN action will require the removal of contaminants for its better preservation.

The biphasic nature of the curves of IFN-AM inactivation (Fig. 2 and 3) was seen with other IFNs (10, 21) and this might be due to the generation of an unfolding intermediate in the process of the unfolding of IFN molecules during the thermal denaturation (16). Because IFN-AM with 1% in pH 2 could theoretically be preserved without loss -4.2°C, and since these requirements are easily achievable, no experiments were performed with other methods of preservation, such as the addition of chemicals like tween 80 (10), tioctic acid (3) or rare earth salts (20).

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RESUMO

Preservação de interferon de membrana amniótica humana

Foram realizadas investigações sobre a preservação do interferon de membrana amniótica humana (IFN-AM) a fim de permitir pesquisas posteriores a respeito de sua purificação, propriedades químicas e biológicas e ensaios clínicos. Testes de inativação rápida foram realizados para ensejar resultados imediatos. IFN-AM foi preparado pela infecção de âmnios pelo vírus da doença de Newcastle. A atividade anti-vírica do IFN-AM foi determinada em um sistema de células Vero-vírus da encefalomiocardite de camundongo ou vírus Sindbis. Para os testes de inativação, preparações de IFN-AM contendo 0; 1 e

2% de soro de carneiro foram aquecidas em temperaturas de 35°C a 100°C em tempos variáveis e foi medida a atividade antivírica residual. Destes dados, foi calculada a constante de Arrhenius de queda de atividade pelo calor, que foi representada graficamente contra a temperatura absoluta. Os dados de temperaturas altas (45°C e acima) foram extrapolados para temperaturas baixas (37°C e abaixo). Os resultados mostraram que o IFN-AM preservou-se melhor em pH 2 em presença de soro. Os dados obtidos nas temperaturas de 45°, 55° e 65°C permitiram o cálculo de constante de Arrhenius de queda para baixas temperaturas por extrapolação. A -4,2°C não deverá ocorrer perda de atividade do IFN-AM.

Palavras-chave: preservação, interferon, membrana amniótica humana.

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STANDARDIZATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE SERODIAGNOSIS OF *MYCOPLASMA BOVIS*

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Ernest Boughton[2]

SUMMARY

An indirect ELISA was developed for the serodiagnosis of *Mycoplasma bovis*. The use of bovine serum albumin (BSA) as a blocking agent was evaluated. Two types of microtitre plates were evaluated in combination with whole cell antigen and sonicated antigen as coating preparations. Results indicated that the use of BSA did not improve the sensitivity or specificity of the test; that sonicated *M. bovis* antigen was the most satisfactory antigen for coating plates and that microtitre plates made of polyvinyl chloride were superior to those made from polystyrene. The optimal concentration of reagents is discussed.

Key words: Mycoplasmosis, cattle, immunology.

INTRODUCTION

The Enzyme-Linked Immunosorbent Assay (ELISA) has been used for the serodiagnosis of mycoplasma infections in man (3), poultry (2), pigs (12), rats and mice (9), sheep and goats (7), horses (1), and cattle (6).

The ELISA is objective and quantitative and results obtained using a single serum dilution can give a relative measure of antibody (4). The specificity and sensitivity of the test is influenced by the quality of the antigen which is applied to microtitre plates, the most popular form of solid phase used for ELISA which have 96 wells moulded from polystyrene or polyvinyl chloride.

The pathogenicity of *Mycoplasma bovis* has been demonstrated at a variety of anatomical sites and next to *M. mycoides* subsp. *mycoides* (also perhaps serogroup 7), it is probably the most pathogenic of the species of mycoplasma isolated from cattle (5). It therefore is important to develop an ELISA for the sero-diagnosis of infections caused by this organism, and introduce this as a routine test.

MATERIAL AND METHODS

For stamdadization of the test reagents chequerboard titrations were made and ELISA results were based on numerous replicate tests. Results were expressed as absorbance values obtained at 490nm, using an ELISA plate reader (MR 600 Dynatech) with the aid of a computer (Hewlett Packard HP 85 B). The indirect ELISA was performed by the method of Engvall and Perlmann (4) using microtitre plates, as follow:

One hundred µl of antigen diluted in 0.1M carbonate-bicarbonate buffer at pH 9.6 (CBB) was added to each well of the microtitre plates, which were then incubated ovenight at 4°C. The proce-

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dure for washing the plates consisted in tapping out excess antigen onto a paper towel. The wells were then refilled with buffer and emptied 3 times. One hundred µl of the positive or negative reference serum diluted in 0.01M phosphate buffered saline at pH 7.4 containing 0.2 per cent polyethylene sorbitan monolaurate (Tween 20 – PBST), was placed in the appropriate wells. The plates were incubated for 30,45 and 60 min at 37°C, and then washed 3 times with PBST. One hundred µl of freshly prepared horse-radish peroxidase-conjugated rabbit anti-bovine IgG diluted in PBST was added to each well and the plates incubated at 37°C for 90 min. The plates were again washed 3 times in PBST and 100 µl of freshly prepared orthophenylene diamine (OPD substrate), was added to each test well. The plates were kept at room temperature in the dark for10 min, and 30 µl of 2.5M sulphuric acid was added to each well to stop the enzymatic reaction.

The indirect ELISA was standardized for:

Antigen – *M. bovis* strain 5B 146 (Central Veterinary Laboratory Culture Collection) was cultured in E broth (11) to produce the seed stock. The seed stock was checked for sterility by means of subculture in ordinary bacterium culture medium (blood agar). The whole cell antigen was prepared following the recommendation of Nicolet et al. (10). The sonificated antigen was prepared according to the technique described by Liberal (8). Both antigens were used to coat microtitre plates overnight at 4°C, diluted in 0.1M CBB from 1/50 to 1/600.

Control sera – a) Positive Control Serum – Seven positive bovine field sera tested at Complement Fixation Test (CFT titres of 1/80 and 1/160) and Disc Film Inhibition Test (DFIT), were mixed together and used as a pooled bovine positive serum.

b) Negative Control Serum – Seven negative bovine field sera tested also at CFT and DFIT were mixed together and used as a pooled bovine negative serum.

Positive and negative control sera were diluted in 0.01M PBST from 1/10 to 1/1280. They were distributed in 100 µl amounts and incubated for 30 min, 45 min and 60 min at 37°C, to find the best incubation time for differentiation between positive and negative sera.

Conjugate – HRP rabbit anti-bovine IgG diluted form 1/200 to 1/6400 in PBST was distributed along the micro-plates.

Bovine Serum Albumin (BSA) – A 3% solution of BSA was used as blocking step in one set of experiments by adding 100 µl of solution to each well of the microtitre plate, immediately after washing the antigen from the plate and before adding the serum. Plates were then incubated for 1 hour at 37°C and washed with PBST and the subsequent steps were performed as described elsewhere. In another set of experiments the BSA incubation step was eliminated and washing was done with PBST.

Microtitre plates – Three different makes of 96 well flat – bottomed microtitre plates were tested: 1) PST1; 2) PST2 and 3) PVC. Plates type 1 and 2 are made of polystyrene and plate 3 was a flexible plate made of polyvinyl chloride. Identical tests were made using positive and negative control sera for *M. bovis* antibodies testing the whole cell and sonicated antigens in all three types of plates.

RESULTS AND DISCUSSION

Determination of optimal antigen concentration – The working dilution of each antigen was determined by testing double dilutions of positive and negative control sera. The working dilution of whole cell antigen was found to be 1/350 and for sonicated antigen 1/550 when "PVC" plates were used. From the results it was clear that the sonicated antigen was the most effective for coating microtitre plates, because it could be used at higher dilution than whole cell antigen. With sonicated antigen the conjugate could be diluted further (Table 1) which also means a more economical test. Sonicated antigen used in a concentration of 5.7-µg/ml resulted in a satisfaetory binding of IgG antibodies to carry out the ELISA test for *M. bovis* serodiagnosis.

Determination of optimum serum concentration – After several repetitions using the optimum concentration of sonicated antigen for the *M. bovis* ELISA, the working dilution of serum was determinated. Based on the results of these experiments, serum dilution of 1/320 and 1/640 were the dilutions of choice for diagnostic purposes. In an attempt to reduce the cost of the assay and to examine more serum samples in each microtitre plate, a single serum dilution of 1/320 was chosen. In this way the absorbance value determined for the positive and negative control serum could be interpreted quatitatively for comparison with field serum samples.

Optimal incubation time for serum – The best incubation for serum was evaluated using three different times (30 min, 45 min and 60 min) at 37°C. The values obtained were not significantly different and an incubation time of 45 min was selected for the test.

Determination of optimal conjugate dilution – Conjugate dilutions ranging from 1/200 to 1/6400 were added to double dilutions of positive and negative sera that had been reacted with different concentrations of antigen. Chequerboard titrations were made using the microtitre plates under study. It was observed that with high concentrations of conjugate, high titres of antibodies were detected but the absorbance values of dilution of 1/4000 provided maximum resolution between *M. bovis* positive and *M. bovis* negative sera where sonicated antigen was used to coat "PVC" plates (Table 1).

Evaluation of BSA to improve sensitivity and specificity – The results obtained shown an inespecific increase in OD values, either in positive or in negative samples, when BSA was used as a blocking agent. Therefore, the use of BSA did not improve the sensitivity of the ELISA test when whole cell antigen was used, nor when sonicated antigen was used to coat the plates (Figure 1), when BSA was

TABLE 1 – Comparison between polyvinil chloride and polystyrene plates, using whole cell and sonicated M. bovis antigens.

Plate	Protein concentration		Antigen dilution		Conjugate dilution	
	Whole cell Ag	Sonicated Ag	Whole cell Ag	Sonicated Ag	Whole cell Ag	Sonicated Ag
"PST$_1$"	9.5 µg/ml	6.5 µg/ml	1/100	1/200	1/2000	1/2500
"PST$_2$"	8.2 µg/ml	6.1 µg/ml	1/200	1/350	1/2500	1/3000
"PVC"	6.2 µg/ml	5.7 µg/ml	1/400	1/550	1/3500	1/4000

Serum at 1/320 dilution.

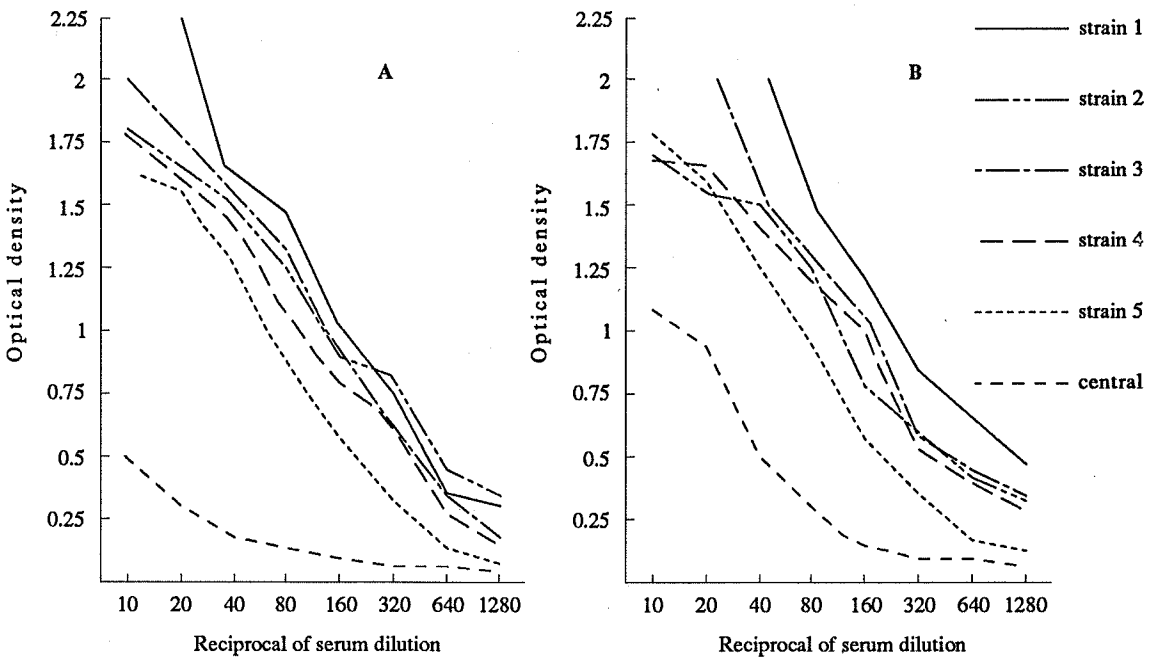

FIGURE 1 - Elisa results withou BSA (A) as blocking agent, and results with the use of BSA (B), testing five strains of *M. bovis*.

used with different *M. bovis* strains and *A. laidlawii* as a control, BSA did not improve the specificity or sensitivity of the test. After the results obtained in this experiment, the use of this blocking agent was eliminated from this ELISA, which saved a considerable amount of time.

Evaluation of polystyrene and polyvinyl chloride micrititre plastes – Control sera positive and negative for *M. bovis* (in different dilutions) were titrated against several concentrations of whole cell and sonicated antigens, using chequerboard design procedure. Chequerboard titrations were also carried out to find the working dilution for the conjugate. Whole cell and sonicated antigens were tested at different dilutions using the three different microtitre plates, with serum at 1/320. The standard dilution of conjugate and antigen (whole cel and sonicated) was determined for each type of microtitre plate. The polyvinyl chloride (PVC) gave better results than polystyrene plates for antigen and cojugate concentrations and for visual reading. The flexibility of the "PVC" plates gave some handling difficulties at the beginning of the experiment especially during the washing steps and the fitting of the plates in the ELISA reader tray. However, with practice the use of this type of plate was satisfactory. As a result of this study "PVC" plates were chosen to perform the *M. bovis* serodiagnostic ELISA test.

After the standardization of the indirect ELISA for *M. bovis*, 11 positive field sera and 11 negative field sera, tested by CFT and DFIT, were used in a standard plate lay-out to allow a uniform reading of each plate and the stablishment of a computer program using duplicates of the positive and the negative control sera. The program calculated the average net OD for each serum pair and adjusted the values to the previously stablished mean absorbance value for the positive control serum (OD = 1.400).

To accurately monitoring the reference sera a correction factor was used and it was multiplied by the net OD of each serum, to derive the corrected OD value. The same positive and negative control sera were retested to check the within-day and day-to-day reproducibility of the assay, testing the control sera 6 times in the same day and at 6 different days. The coefficient of variation and standard deviation presented for day to day and between days results were very small, which means that the OD values obtained were very similar and linear, showing excellent reproducibility.

This indirect ELISA was used to test 1.300 bovine field sera and it was compared with CFT and DFIT. It proved to be a specific and sensitive technique and these results will be described in another publication.

In conclusions, the indirect ELISA protocol was obtained by chequerboard titration and standardization procedures. The final *M. bovis* ELISA protocol is a follow: the antigen used to coat the plates was *M. bovis* 5B 146 sonicated cells diluted 1/550 in CBB. "PVC" plates were used to carry out the tests using sera diluted to 1/320 in PBST, and incubated for 45min at 37°C. Conjugate was diluted 1/4000 in PBST and it was incubated for 90 min at 37°C. The use of BSA as a blocking agent did not improve the sensitivity and specificity of this Elisa. This ELISA proved to be a very promissing test for the serodiagnosis of *M. bovis* infections, as it is a timple, quick and specific technique.

RESUMO

Padronização do teste Elisa indireto para sorodiagnóstico de Mycoplasma bovis.

Foi desenvolvido um teste Elisa indireto para o sorodiagnóstico de *Mycoplasma bovis*. Foi avaliado o uso de albumina de sorobovino (BSA) como agente bloqueador. Dois tipos de microplacas foram avaliadas em combinação com as células como antígeno bem como, fragmentadas após sonicação. Os resultados mostraram que o uso de BSA não melhorou a sensibilidade e a especificidade do teste; o antígeno de *M. bovis* sonicado foi o mais satisfatório e que as microplacas de cloreto de polivinila são superiores àquelas feitas de polistireno.

A concentração ótima dos reagentes foi discutida.

Palavras-chave: Mycoplasmose, gado, imunologia.

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calves, aging from 4 to 60 days, were collected from 20 dairy herds in Minas Gerais State, Brazil. One swab was inoculated into the modified CHAN & MACKENZIE base medium[6] with the antibiotic supplement described by Skirrow[23] (SKM) and the other in the same modified medium with the antibiotic supplement used in the charcoal-based selective medium CSM[13]. The CHAN & MACKENZIE base enrichment medium[6] that received the antibiotic supplement described by Skirrow[23] was called SKME and the other one, using the CSM mixture, was designated CSM-enrichment (CSME). Inoculated enrichment media were transported at room temperature to the laboratory within 4hr.

Media

The modified media[6] cited above was used as transport and enrichment medium; and contained Brain Heart Infusion broth[a] (3.7 g/l), 0.4% agar, 10% defibrinated sheep blood, the FBP (0.05% each of $FeSO_4.7H_2$, sodium metabisulfite and sodium piruvato) supplement[8], and the antibiotic mixture SKM[23] (vancomycin, 10 mg/l; trimethoprim, 5 mg/l and polymixin B, 2500 IU/l)[b] (SKME), or CSM[13] (celoperazone[c], 32 mg/l; cicloheximide[d], 100 mg/l and vancomycin[e], 20 mg/l) for CSME. The media were dispensed in 4 ml aliquots in 12x75 mm rubber-stoppered tubes.

The plate media used were BHI agar[a], 10% defibrinated sheep blood and the antibiotic supplements SKM or CSM.

Culture conditions

The swabs were direct inoculated onto BHI-blood agar plates with the same antibiotic supplement (agar SKM and CSM) used in the enrichment medium, which were seeded within 4 hours after sampling.

The enrichment tubes were incubated at 42°C for 24hr when swabs were inoculated on another set of plates.

All plates were incubated at 42°C in an microaerophilic atmosphere of 5% 0_2, 10% CO_2 and 85% N_2, obtained by partial evacuation, and checked daily for suspect *Campylobacter* colonies up to 3 days.

a - Difco
b - Merck
c - Pfizer
d - Sigma
e - Ely Lilly

Identification of Campylobacter species

Suspected colonies on selective plates were examined microscopically on Gram stained smears for typical Gram-negative curved bacteria. For genera and species identification the tests of: oxidase, catalase, motility, hippurate hydrolisis, growth at 25° C and 42° C, nitrate reduction, H_2S production on TSI, sensibility to cephalothin (30μg) and nalidixic acid (30μg), tolerance to 1.0% glycine, 1.5% and 2.0% NaCl, anaerobic growth in the presence of 0.1% of Trimethylamine N-oxide (TMAO), and rapid coceal transformation were used[14,19,20].

Statistical analysis

The McNemar test and the Cochran Q test were performed, and $p<0.05$ was regarded as statistically significant[22]. The isolation rate of 100% was taken as the total number of strains able to be isolated.

RESULTS

Thirty-nine strains of *Campylobacter* sp were isolated. CSM was able to isolate 36 strains (92.31%), SKM 12 (30.77%), CSM after enrichment in CSME 16 (41.03%), SKM after enrichment in SKME 7 (17.95%). Three strains (1 *C. jejuni* and 2 *C. lari*) from normal calves were only isolated on CSM after enrichment in CSME.

These strains were classified as *C. lari* (22 strains), *C. jejuni* (8 strains), *C. coli* (6 strains) and *C. hyointestinalis* (3 strains).

Table 1 shows the isolation rates of *Campylobacter* sp. from diarrheic and normal calves on different media.

TABLE 1 - Isolates rates of *Campylobacter* sp. from diarrheic and normal calves using different media and procedures.

media	diarrheic (%)	normal (%)	Total (%)
CSM	20 (51.28%)	16 (41.03%)	36 (92.31%)[A]
SKM	9 (23.08%)	3 (7.69%)	12 (30.77%)[B]
CSME	8 (20.51%)	8 (20.51%)	16 (41.03%)[B]
SKME	2 (5.13%)	5 (12.82%)	7 (17.95%)[B]
CSM + CSME	20 (51.28%)	19 (48.72%)	39 (100.0%)*
SKM + SKME	10 (25.64%)	8 (20.51%)	18 (46.15%)

A $p < 0.001$ $x^2 = 22.04$ df= 1
* $p < 0.001$ $x^2 = 19.05$ df= 1
B not significant

DISCUSSION

The isolation rate from *Campylobacter* sp on CSM (92.31%) in the present trial confirms the results of previous studies[7,13,16] with cefoperazone-containing media and shows its effectiveness for isolating *Campylobacter* sp from diarrheic and normal calves. The other treatments were significantly less suitable ($p<0.001$) for isolating *Campylobacter* sp from calf feces. Nevertheless, three strains (7.69% - 1 *C. jejuni* and 2 *C.lari*) from normal calves were only isolated after enrichment in CSME and the procedure CSM + CSME isolated 39 strains (100,0%), which was significantly better ($p<0.001$) than SKM + SKME (46.15%). Although some authors have pointed out that direct culture is equal or sometimes superior to enrichment procedures[1,12], we observed the value of enrichment procedures for isolating *Campylobacter* sp from normal calves. This is probably due to the low number of *Campylobacter* sp. (10^2-10^4) in the feces of normal calves[9,17].

It is worth noting that the majority of authors[7,13,16] reports the isolation of *Campylobacter* sp. in pure cultures on cefoperazone-containing media in more than 50% of the cases. In this study only one plate of CSM shows *Campylobacter* sp. in pure culture, although, in the other plates, the growth of contaminants was only seen in the first quadrant. This might reflect a higher level of resistance of the contaminant fecal flora to the antibiotics used in the media. The low isolation rate on SKM is also probably due to this higher level of resistance of the fecal flora. The heavy growth of contaminants makes the identification of *Campylobacter* colonies on SKM plates very difficult. The higher number of contaminated CSM plates in this study might also be caused by the use of blood instead of the charcoal from the original formulation.

On the basis of the present data, we conclude that CSM is suitable for routine examination of calf feces for thermotolerant *Campylobacter*. The use of enrichment media could also increase the *Campylobacter* isolation rates when normal calves are taken into account.

RESUMO

Isolamento de *Campylobacter* sp de amostras de fezes de bezerros com e sem diarréia: comparação entre procedimentos.

Diferentes meios para enriquecimento e para o isolamento de *Campylobacter* sp de amostras de fezes de bezerros com e sem diarréia foram avaliados. Dentre os procedimentos utilizados, o CSM mostrou-se superior. Três amostras de *Campylobacter* sp. (2 amostras de *C. jejuni* e 1 amostra de *C. lari*) foram isoladas de animais sem diarréia somente após o enriquecimento em CSME. O procedimento CSM + CSME foi significativamente melhor ($p<0,001$), isolando 39 amostras (100,0%) contra 18 (46,15%) em SKM + SKME.

Palavras-chave: *Campylobacter* sp. termotolerante, enriquecimento, meio seletivo, bezerros normais e com diarréia.

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DETECTION OF A BACTERIOCIN-LIKE SUBSTANCE IN *YERSINIA* Spp.

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SUMMARY

Twenty-two *Yersinia* spp isolates from different sources were tested for the ability to produce bacteriocin-like substances using the spot-test technique with double-layer agar.

The method demonstrated the presence of inhibitory activity only for *Y. intermedia* grown at 25°C but not at 37°C, and against *Y. enterocolitica* serotype 0:8.

Key words: *Yersinia* spp, bacteriocin-like substance.

INTRODUCTION

Bacteriocins are, in general, bactericidal proteins or protein compounds produced by bacteria which show activity not only against their own species but also against other non-related strains (8), being immune to their own product (9); nevertheless, reports have been made of bacteria presenting autoinhibition, mainly Gram-positive microorganisms (11).

Gratia & Frederiko (7) suggested the word *colicin* to name isoinhibitory proteins produced by *Escherichia coli*. Later on, the more generical name *bacteriocin* (8) was introduced, because of its occurrence in other genera of bacteria, e. g. *Neisseria, Enterobacter, Shigella, Salmonella* and *Yersinia*.

Bacteriocin production has been detected in *Yersinia pestis, Yersinia pseudotuberculosis* and *Yersinia intermedia* 2,4,10), but not in *Yersinia enterocolitica* (3).

Genetical information to make cells produce bacteriocin is determined by plasmids both in Gram-negative and Gram-positive bacteria (8,11); reports have been made (5), however, which show the involvement of chromosomal genes acting in the codification of bacteriocin synthesis in *Klebsiella pneumoniae*. The expression of such ability occurs under specific cultivation conditions and growth phases (9). *Y. intermedia* can only present this capacity in rich solid media during the logarithmic growth phase at 25°C; bacteriocin cannot be produced in liquid media (10).

Bacteriocin from *Y. intermedia* can be inativated by chymotrypsin, trypsin and pronase, but it is resistant to lipolytic enzymes, chloroform and other organic solvents, as well as to pH between 3 and 11. The activity of the bacteriocin is located in its protein part (10).

Due to the difficulty found in identifying those substances, some authors (6,10) report bacterial antagonism, but without determining its chemical nature, wherefore the use of the expression "bacteriocin-like substance" is recommended to name bacterial products showing antagonic activity, though incompletely characterized (6,10).

The present paper aims to investigating the possible production of a bacteriocin-like substance in different *Yersinia* spp strains.

MATERIAL AND METHODS

Strains used as producers (P) and/or indicators (I) of bacteriocin-like substances (Table 1).

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TABLE 1 - Strains used as producers (P) and/or indicators (I) of bacteriocin-like substances.

Type	Strains/Serotype	Origin/Identification
p/I	*Yersinia enterocolitica* 0:3	IP - YE 134
p/I	*Yersinia enterocolitica* 0:5	IP - YE 124
p/I	*Yersinia enterocolitica* 0:8	IP - YE 161
p/I	*Yersinia enterocolitica* 0:9	IP - YE 383
p/I	*Yersinia enterocolitica* 0:5, 27	IP - YP 885
p/I	*Yersinia pseudotuberculosis* I	IP - YP I
p/I	*Yersinia pseudotuberculosis* II	IP - YP II
p/I	*Yersinia pseudotuberculosis* III	IP - YP III
p/I	*Yersinia pseudotuberculosis* IV	IP - YP IV
p/I	*Yersinia pseudotuberculosis* V	IP - YP V
p/I	*Yersinia frederiksenii* 0:16-Xo	IM - Diarrheic dog
p/I	*Yersinia frederiksenii* 0:16-Xo	IM - Normal dog
p/I	*Yersinia frederiksenii* 0:16-Xo	IM - Fresh water
p/I	*Yersinia frederiksenii* 0:16-Xo	IM - Fresh water
p/I	*Yersinia frederiksenii* NAG-Yz	IM - Freshwater
p/I	*Yersinia kristensenii* 0:11.24-Xo	IM - Fresh water
p/I	*Yersinia kristensenii* 0:11.24-Xz	IM - Ground meat
p/I	*Yersinia kristensenii* NAG-Xz	IM - Bovine muscle
p/I	*Yersinia intermedia* 0:17-Xo	IM - Diarrheic dog
p/I	*Yersinia intermedia* NAG-Xz	IM - Fresh water
p/I	*Yersinia intermedia* NAG-Xz	IM - Diarrheic human
p/I	*Yersinia intermedia* NAG-Xo	IM - Fresh water
p/I	*Yersinia intermedia* NAG-Xo	IM - White cheese
I	*Enterobacter cloacae*	IM - 770001
I	*Escherichia coli*	ATCC - 25922
I	*Klebsiella pneumoniae*	IM - 778039
I	*Pseudomonas aeruginosa*	ATCC - 15442
I	*Proteus mirabilis*	IM - 77880002
I	*Salmonella cholerae-suis*	ATCC - 10708
I	*Salmonella typhi*	ATCC - 6539
I	*Shigella sonnei*	IM - 11780028
I	*Staphylococcus aureus*	ATCC - 25293
I	*Staphylococcus aureus*	ATCC - 6538

The spot test techinique using double-layer agar (10) was used for production of bacteriocin-like substance in solid medium, as following:

Strains assayed for the production of bacteriocin-like substance were grown on nutrient agar slants (Merck) for 24 h at 25°C and then, inoculated in brain heart infusion (BHI-Merck). Following incubation for 24 hours at 25°C, growth were spread according to the spot technique in equidistant points of plates with BHI added 2% of agar.The plates, in duplicate for each producing sample, were incubated at 25°C and 37°C up to 48 hours. Later on, chloroform (Merk) pre-treatment was carried out, with the disposal of 1 ml. on the lids of the inverted plates. After evaporation of the chloroform, which lasts for approximately two hours, the indicator strain inocula were spread onto the producers.

Strains assayed as indicator were spread onto nutrient agar and incubated, at 37°C; *Yersinia* spp at 25°C, for 24 hours; then inoculated in BHI broth and incubated for 24 hours at the indicated temperatures. After this growth, 0.2 ml were added to 3 ml of semisolid (0.7%) BHI agar mantained at 45°C. This inoculum was poured all over the surface of the medium containing the producers. Plates were then incubated at 25°C and 37°C, according to the initial temperature at which producers had undergone incubation. After 24 to 48 hours incubation the plates were examined for detection of bacteriocin-like substance through the appearance of inhibition zones around the spot inoculum.

Assay for bactericidal or bacteriostatic activity of bacteriocin-like substance producers:

The portion of agar where the inhibition zone occurred (item 2.2) was asseptically removed and put into a tube with BHI, so that maceration with a glass stick could be started. Then, tubes were incubated a 25°C and/or 37°C, according to the temperature used for indicators. The activity of the bacteriocin-like substance was considered bactericidal when there was no growth at all, and bacteriostatic when there was any turbidity indicating growth.

Assay for bacteriophages:

The presence of bacteriophages was assayed in the inhibition zone formed on the agar plates (item 2.2), through the phage propagation method (1). The portion of agar where the inhibition zone occurred was asseptically removed and put into BHI broth to macerate. Chloroform (0.05ml) was added and acted on it for 30 minutes. After this period, the material was centrifuged at 3000g for 5 minutes. A 0.2ml portion of the supernatant was taken and added to the same volume of BHI containing a recent growth (18 hours) of the indicator. This micture was incorporated to semisolid (0.7%) BHI agar kept at 45° and poured onto a plate with BHI containing 2% agar. After 24 hours incubation at 25°C, the presence of lysis plaques was verified. When growth showed alteration resembling lysis, the agar was removed and the phage propagated according to the above mentioned technique.

RESULTS AND DISCUSSION

The 22 strains of *Yersinia* spp (including five *Y. enterocolitica*, five *Y. pseudotuberculosis*, five *Y. intermedia*, three *Y. kristensenii* and four *Y. frederiksenii)* were tested for their ability to produce bacteriocin-like substance. The results achieved could only demonstrate such characteristic in a *Y. intermedia* NAG-Xo sample isolated from white cheese, against the *Y. enterocolitica* serotype 0:8 (strain Y.P.: Y.e. 161). Isoinhibitory activity was demonstrated by an inhibition zone of about 7 mm. This inhibition was observed after 16 hours of incubation at 25°C.

As for heteroinhibitory activity, it could not be detected in the tested strains mentioned, not even after alleged producers were incubated at 25°C and 37°C. These findings are in accordance with the reports of Bottone & Cols. (3), who demonstrated this activity in *Y. intermedia* at 25°C against *Y. enterocolitica* serotype 0:8. e. NAG, *Y. frederiksenii* and *Y.. intermedia* of various serotypes, as well as the investigation of Sandhu & Cols. (10) on *Y. intermedia, who used Y. frederiksenii* Y. e. 867 as indicator - who also failed to detect heteroinhibitory activity.

In the present survey the strain producing bacteriocin-like substance was not active against itself and the possibility of bacterial antagonism or phages acting in the inhibitory process was discarted.

Thus, we may well be before a bacteriocin-like substance; however, the possibility remains that this isoinhibitory activity could mean the synthesis of either a defective prophage or phage components. Minute additional research is needed to better ascertain this inhibition activity and the chemical nature of the substance.

RESUMO

Detecção de uma substância semelhante a bacteriocina em Yersinia spp.

Vinte e duas amostras de *Yersinia* spp foram testadas quanto à capacidade de produzir substância semelhante à bacteriocina pelo método do "spot-test" usando dupla camada de ágar. O resultado mostrou a existência de uma atividade isoinibitória somente em *Y. intermedia* à temperatura de 25°C e não a 37°C, e contra *Y. enterocolitica* sorotipo 0:8.

Palavras-chave: *Yersinia* spp, substância semelhante à bacteriocina.

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CHROMOSOMAL DNA PROFILE AND SUSCEPTIBILITY TO AMPHOTERICIN B OF *CANDIDA ALBICANS* CLONES

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SUMMARY

Chromosomal DNA profile associated to minimal inhibitory concentration and minimal fungicidal concentration of Amphotericin B was studied in 5 clones derived from different *Candida albicans* strains. Based on agar dilution test, similarities among the clones Ca1.2, Ca2.4, Ca3.7 and Ca5.5 were detected for susceptibility to Amphotericin B. From chromosomal DNA digested with the endunuclease Eco RI and hibridized with the DNA probe A-27, similar profiles was detected only for Ca3.7 and Ca4.10. A clear correlation between susceptibility to Amphotericin B and chromosomal DNA profile was not detected in this study.

Key words: Candida albicans; Amphotericin B; Susceptibility to Amphotericin B; Chromosomal DNA profile.

INTRODUCTION

Althrough new antifungal drugs have been comercially available, amphtotericin B is still the most utilized drug for treatment of deep mycoses, including candidiases (7,13,15).

Candida albicans with different "in vitro" susceptibility to amphotericin B has been related (2, 4, 9). The biochemical mechanisms described to explain the antifungal activity of amphotericin B and the resistance of different isolates are related to the ergosterol amount in the cellular membrane and the occurrence of the yeast metabolism oxidative process (5, 8, 14, 19). The presence of these factors may be directed related to the genetic material of the yeast cells (1, 11). However, the study of chromosomal fragments associated to resistance of *C. albicans* to Amphotericin B has not been described.

This study was designed to evaluate the association of resistance to amphotericin B and chromosomal DNA profile of clones from different *C. albicans* isolate.

MATERIAL AND METHODS

1. Microorganisms. Clones called Ca1.2, Ca2.4, Ca3.7, Ca4.10 and Ca5.5, derived respectively

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from 5 clinical isolates of *C.albicans* were selected for studing. Some of these clones were considered, in a previous study, natural variants in the assimilation of some carbon sources (22).

The strain *Saccharomyces cerevisiae* ATCC 9763 was utilized as standard in the susceptibility test.

2. Antibiogram

2.1. Antifungal drugs and stock solution. Amphotericin B (provided by Squibb Indústria Química S/A), as powder, was dissolved in dimetilsufoxide to a final concentration of 6.4 mg/ml (18).

2.2. Minimal inhibitory concentration (MIC). The media utilized was YNB (Difco), with agar, L-asparagine and dextrose to a final concentration of respectively 1%, 0,15% and 1%. Two fold dilutions of the stock solution were prepared in 2 ml distilled water and added to 18ml of media to obtain concentration of 32 µg/ml to 0.06 µg/ml. Five pul of saline containing 5 x 10^3 cells of clone or standard strain were innoculated on the plates. The test was done in dulicate and read after 24-48 h of incubation at 30°C (18).

2.3. Minimal fungicidal concentration (MFC). A subculture of the inocula without growing in the MIC test was prepared in Sabouraud dextrose agar (Difco) and incubated at 30°C for 72 h (3).

3. Chromosomal DNA analysis. A sample from each clone incubated in YEPD broth at 30°C for 24 h was submitted to DNA extraction following a protocol described by Scherer & Stevens (16). The DNA was digested with the endonuclease Eco RI (Boehringer, Mannhein) and submmited to electrophoresis in 0.7% agarose containing ethidium bromide at 40 V for 18 h. The DNA frangments were transfered to nitrocelullose membrane (21), hybridized with the DNA probe A-27, kindly provided by Scherer & Stevens (17), labelled with 32p and submitted to autoradiography.

TABLE 1 – Susceptibility to amphotericin B and chromosonal DNA profile of *C. albicans* clones.

clones	Amphotericin B (ug/ml)		DNA profile
	MIC	MFC	
Ca1.2	0.5	2.0	I
Ca2.4	0.5	4.0	II
Ca3.7	0.5	2.0	III
Ca4.10	2.0	8.0	III
Ca5.5	1.0	4.0	IV

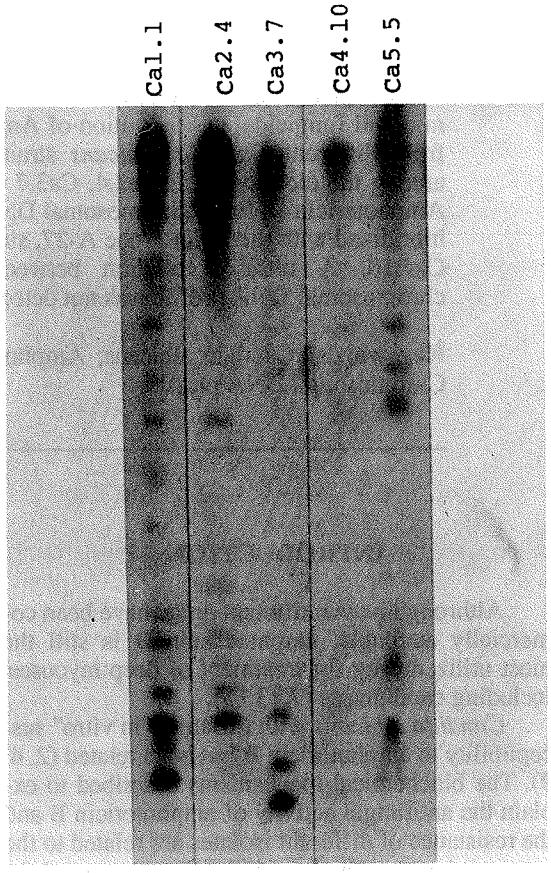

FIGURE 1 – Chromosonal DNA digested with the endonuclease Eco RI and hibridized with the DNA probe A-27 labelled with α ^{32}p. Cal.2 to Ca5.5: profiles of *C. albicans* clones.

RESULTS

The amphotericin MIC was 0.5 µg/ml for the clones Ca1.2 (MFC 2.0 µg/ml), Ca2.4 (MFC 4.0 µg/ml) and Ca3.7 (MFC 2.0 µg/ml). The clones Ca5.5 revealed a MIC of 1.0 µg/ml (MFC 4.0 µg/ml) and the clone Ca4.10 showed the highest MIC, 2.0 ug/ml (MFC 8.0 ug/ml (Table 1).

The DNA analysis revealed 4 different DNA profiles: Ca1.2 with 11 DNA bands; Ca2.4 with 9 bands; Ca3.7 and Ca4.10 with 7 bands; and Ca5.5 with 6 DNA bands (Figure 1).

DISCUSSION

C. albicans strains naturally resistant to amphotericin B have been related (2,4,9). Also, inducible resistance to amphotericin B have been described in naturally susceptible strains (6,11,12,20). In both cases it is believed that the fungistatic activity is depending upon the attachment of amphotericin B to the ergosterol in the cellular membrane detected by the leak of intracellular potassium. The fungicidal activity is more complex and, although depending upon the attachment of the drug to ergosterol, also involves the oxidative metabolism including catalase and peroxidase activities (5,8,14,19).

The yeast genetic material is directelly related with the presence and activity of determinated metabolic pathways (1,10). In this sense, Scherer & Stevens (17), utilizing the probe A-27, demonstrated that among 10 *C. albicans* mutants resistants to 5-fluorocytosine, 9 presented the same chromosomal DNA profile and one mutant had a deletion.

We did not find in the literature studies associating susceptibility of *C. albicans* to amphotericin B and chromosomal DNA profile.

In this research we show that the clones Ca1.2, Ca2.4, Ca3.7 and Ca5.5, which revealed the same or very similar susceptibility to amphotericin B, presented few identical DNA bands being classified in different profiles. However, the clone Ca4.10, which presented the highest MIC (Table 1), revealed a DNA profile similar to the clone Ca3.7 (Figure 1).

Although we did not show association between susceptibility to amphotericin B and chromosomal DNA profile, we should consider that different genes are related with susceptibility to amphotericin B and probably to other antifungal drugs.

Other studies, including more strains, different restriction endonucleases and DNA probes, are warranted for a better understanding of the susceptibility to amphotericin B at a genetic level.

RESUMO

Perfil de DNA cromossômico e sensibilidade à anfotericina B de clones de *Candida albicans*

O perfil de DNA cromossômico e a sensibilidade à Anfotericina B foram estudados em cinco clones derivados de diferentes amostras de *Candida albicans*. Com base no teste de diluição em ágar, os clones Ca 1.2, Ca 2.4, Ca 3.7 e Ca 5.5 mostraram comportamentos similares frente ao antibiótico. A partir do DNA cromossômico digerido com endonuclease Eco RI, hibridizado com "probe" de DNA A-27, apenas os clones Ca 3.7 e o Ca 4.10 mostraram perfis similares. Neste trabalho não se observou uma clara correlação entre sensibilidade à anfotericina B e perfil de DNA cromossômico.

Palavras-chave: *Candida albicans*, anfotericina B, susceptibilidade a anfotericina B, perfil de DNA cromossômico.

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APICULATE YEASTS WITH HIGH GROWTH TEMPERATURES ISOLATED FROM *DROSOPHILA* IN RIO DE JANEIRO, RJ, BRAZIL

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SUMMARY

Some strains of *Kloeckera africana*, *K. apis*, *K. japonica* and *K. javanica* isolated from *Drosophila* in forests and an urban wooded area in Rio de Janeiro, RJ grew at temperatures that exceeded those reported for these apiculate yeasts. These isolates represent new biotypes that may have been selected by high environmental temperatures frequently encountered in the tropical climate of Rio de Janeiro, Brazil.

Key words: Apiculate yeasts, temperature, biotypes, tropical climate.

INTRODUCTION

Bipolar budding yeasts have drawn the attention of researchers since the early studies in taxonomy and ecology of yeasts. This is reflected in the long list of synonyms of the names for the species now accepted (1, 5, 7, 9, 11). The most common apiculate yeasts are included in the imperfect genus *Kloeckera* and their teleomorphs in the genus *Hanseniaspora*. In addition to their distinct morphology, these yeasts are highly fermentative but utilize few carbon sources, most notably glucose and cellobiose, and have unusually high vitamin requirements, including for inositol. Apiculate yeasts are typical of ripe or deteriorating fruits and fermentating fruit juices, and are spread by insect vectors including fruit flies of the genus *Drosophila*. The yeasts probably have nutritional importance for the *Drosophila* making this a good example of mutualistic interaction (12).

Yeast strains have maximum growth temperatures that can be defined to within about 1°C and species have characteristic temperature ranges for growth (3). The maximum temperature for growth is considered to be that located between the lowest temperature at which no growth occurred after 7 days and the next lowest temperature where growth had occurred (5). Standard descriptions of the species of *Hanseniaspora* and *Kloeckera* show that only *Hanseniaspora guilliermondii* and its anamorph *Kloeckera apis* are capable of growth at 37°C. Other species of this group are described as variable or negative for growth at 35°C. Data from the literature of growth at different temperatures for the species isolated in our work are presented in table 1.

Kloeckera javanica and *Kloeckera apiculata* isolated from fruits in northeast Brazil had growth temperatures which were apparently typical of these species (Santos, E. A., unpublished results). However, *Hanseniaspora guilliermondii* and some *Kloeckera apis* strains isolated in Rio de Janeiro, RJ, from ripe and spoiled fruits of cashew and pinneaple have often grown at relatively high temperatures for their species (Oliveira, R. B., unpublished results, 13). Most of the *Hanseniaspora uvarum* strains isolated from wa-

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TABLE 1 – Temperature of growth for *Hanseniaspora* species and their anamorphs in the genus *Kloeckera*.

	Temperature (in °C) (1)					
	30	34	35	37	40	42
Hanseniaspora guilliermondii/ *Kloeckera apis*	+	ND	+	+	–	–
Hanseniaspora occidentalis/ *Kloeckera javanica*	+	ND	–/w	–	–	–
Hanseniaspora osmophila/ *Kloeckera apiculata*	+	–	–	–	–	–
Hanseniaspora uvarum/ *Kloeckera corticis*	+	ND	–	–	–	–
Hanseniaspora valbyensis/ *Kloeckera japonica*	–	ND	–	–	–	–
Hanseniaspora vineae/ *Kloeckera africana*	+	+	–/w	–	–	–

(1) + = positive;
– = negative results;
–/w = some strains present negative, others growth.
ND = No data
Data from references 1, 5.

ters of Rio de Janeiro, RJ, grew well at 37°C (Hagler, A. N., unpublished results). Most of the yeasts isolated from dried fruit beetles in Central California were able to grow at rather high temperatures for yeasts (8). However Heed *et al.* (4) have stated that the high frequency of heat tolerant yeasts is not characteristic of regions with temperate or tropical climate.

Recent studies of yeasts associated with *Drosophila* of an urban wooded area and rain forests of Rio de Janeiro, RJ, confirmed the prevalence of apiculate yeasts in these habitats (10). Isolates of *Kloeckera japonica* from *Drosophila* in this study had typical negative results for growth at 37°C, but *Kloeckera africana* and *Klockera javanica* strains all grew at 37°C, and some cultures of *Kloeckera apis* grew at 40°C. To confirm these unusual results that may have taxonomic and ecological implications we determined growth temperatures of representative cultures.

METHODS

Strains were selected from yeast isolates obtained from fruit flies from Tijuca National Park and Pau da Fome forests and from the Catalão Point area on the Ilha do Fundão in the city of Rio de Janeiro, RJ. Yeasts were characterized and identified according to standard conventional methods (5). Growth temperatures were determined in waterbaths adjusted to 1°C intervals from 34 to 42°C, and allowed to stabilize for three days before the experiments. These were monitored 8 times per day during the experiment with calibrated thermometers. The inoculum was transferred from 24 hr Sabouraud glucose agar slants to Yeast Nitrogen Base suplimented with 0.1% glucose and 0.05% yeast extract and incubated for 5 days at 28°C. Culture tubes (13 mm diameter) containing 3 ml each of 2% glucose, 1% peptone, 0.05% yeast extract broth received 0.1 ml each of innoculum and the presence or absence of growth noted after 3-5 days incubation at the temperatures indicated.

RESULTS

The results of growth tests after incubation at different temperatures are presented in the Table 2. Strains of *Kloeckera corticis* fit the standard description of the species well, including for growth temperatures. Three strains of *Hanseniaspora valbyensis* were also typical of the species in being negative for growth at 30°C but all 86 strains of *Kloeckera japonica* grew at 34°C. Six strains of *Kloeckera africana* and one strain of its teleomorph *Hanseniaspora vineae* did not grow above 34°C, but one isolate grew at 35°C and three grew at 38°C. Among 144 isolates of *Kloeckera apis*, 76 representative strains grew at 37°C but not at 40°C, conforming with the standard description of the species. Of these strains eigth grew at 38°C and 14 at 39°C. Growth at 40°C ocurred for 68*Kloeckera apis* strains and two of these grew at 42°C. Of the 117 strains of *Kloeckera javanica* 111, grew well at 37°C, and two of these grew also at 38°C, above the expected range of this species noted to be negative or weak for growth at 35°C. Strains of *Hanseniaspora occidentalis* grew at 30°C (data not shown), but not at 35°C which is in accord with the standard description. Strains of *Hanseniaspora uvarum* were typical with respect to growth temperature.

DISCUSSION

These strains of apiculate yeasts, independent of their taxonomic significance at the species level, represent distinct thermotolerant biotypes.

TABLE 2 – Growth of *Kloeckera* and *Hanseniaspora* strains isolated from *Drosophila* of Rio de Janeiro, RJ, at different temperatures.

		Temperature (in °C)									
	n	34	35	36	37	38	39	40	41	42	43
Hanseniaspora occidentalis	1	+	–	–	–	–	–	–	–	–	–
	1	–	–	–	–	–	–	–	–	–	–
Hanseniaspora uvarum	1	+	–	–	–	–	–	–	–	–	–
	9	–	–	–	–	–	–	–	–	–	–
Hanseniaspora valbyensis	3	–	–	–	–	–	–	–	–	–	–
Hanseniaspora vineae	1	+	–	–	–	–	–	–	–	–	–
Kloeckera africana	6	+	–	–	–	–	–	–	–	–	–
	1	+	+	–	–	–	–	–	–	–	–
	3	+	+	+	+	+	–	–	–	–	–
Kloeckera apis	40	+	+	+	+	ND	ND	–	–	–	–
	14	+	+	+	+	–	–	–	–	–	–
	8	+	+	+	+	+	–	–	–	–	–
	14	+	+	+	+	+	+	+	–	–	–
	31	+	+	+	+	+	+	+	ND	ND	–
	35	+	+	+	+	+	+	+	–	–	–
	2	+	+	+	+	+	+	+	+	+	–
Kloeckera corticis	2	–	–	–	–	–	–	–	–	–	–
Kloeckera japonica	25	+	–	–	–	–	–	–	–	–	–
	61	+	ND	ND	–	–	–	–	–	–	–
Kloeckera javanica	6	+	–	–	–	–	–	–	–	–	–
	84	+	+	+	+	ND	ND	–	–	–	–
	25	+	+	+	+	–	–	–	–	–	–
	2	+	+	+	+	+	–	–	–	–	–

Symbols: n = number strains tested;
ND = not determined.

These were probably selected by an environment where temperatures of up to 40°C are common, especially during the summer months. Desert climates also seem to favor thermotolerance as noted for cactus yeast isolates (4) and strains isolated from dried fig beetles in central California (8). Some of the *Kloeckera apiculata* strains associated with *Drosophila* from a forest in Brazil (Serra da Cantareira, São Paulo) by Cunha *et al.* (2) could have been positive for growth at 37°C or higher temperatures, but this was not reported. Environments with considerable variation in temperatures, and that can reach about 40°C, appear to put a selective pressure on high temperature growth for yeast species. Confirmation of this requires more extensive data on growth temperatures of yeast isolates from tropical ecosystems.

In yeast species with few positive results for taxonomic tests in their standard descriptions, a striking difference in the temperature of growth can indicate a new species. An example is the species *Hanseniaspora guilliermondii* that to a large degree was first noted as separate from *H. valbyensis* based on ability to grow at 37°C (6). Differences in growth temperature characteristics were found to be more consistant with *in vitro* genetic comparisons to differentiate *Kloeckera africana* from *K. corticis* than were carbohydrate assimilation tests (8). The number and size of ascospores are considered less valuable criteria for differentiating *Hanseniaspora valbyensis* and *Hanseniaspora guilliermondii* than are the assimilation of 2-keto-gluconate and growth at 37°C (7). Non-sporulating strains of apiculate yeasts from

much longer period of time. The observation of colonies of zoosporic fungi on submerged leaves indicated their activity on the leaf decomposition process (14). The difficulty to observe the development of zoosporic fungi on the surface of decaying submerged leaves makes it difficult to assume their characterization as allochthonous substrates decomposers. The use of chitinic, keratinic and cellulosic baits to isolate the colonies of zoosporic fungi has been useful even for fungal succession studies (18, 19). Combining baiting and culture methods, abundant and diversified mycota has been isolated on submerged *Ficus microcarpa* (18) and *Quercus robur* (19) leaves. On these substrates aquatic Hyphomycetes were observed for the first time in Brazil, as well as the active role of the zoosporic fungi on the fungal succession.

The aim of this paper was to compare the aquatic mycota during the decomposition of *Ficus microcarpa, Quercus robur* and *Alchornea triplinervia* leaves submerged in a stream situated in the Atlantic rainforest.

MATERIAL AND METHODS

The stream in the area studied has a width of 4-5m, sandy bed and depth of 30 to 70cm according to rain occurrences.

In March of 1990, leaves of *Ficus microcarpa, Quercus robur* and *Alchornea triplinervia* were collected respectively in the "Parque Estadual das Fontes do Ipiranga" in São Paulo city, in the municipality of Itapecerica da Serra and in the "Reserva Biológica do Alto da Serra de Paranapiacaba" in São Paulo State, brought to the laboratory and dried at room temperature during two weeks to standardize leaf moisture condition. The leaves were disposed in 180 litter bags with 22x10cm with 1mm diam. mesh, 60 for each plant species, enough for an one-year long experiment. Each bag was filled with 15g of previous weighed leaves, to standarize the quantity of available substrate. Five sites along the stream were marked with stakes, on which the bags were fastened with nylon string (1mm diameter), to avoid losses of the samples due to the water flow.

The bags were kept submerged approximately 10cm deep. Monthly, from April to November, 1990, five bags of each plant species were sampled. In the laboratory, the leaves were carefully washed under tap water to remove debris. Fifty disks with 5mm diameter were cut from leaves of each plant species with a cork borer. The disks were incubated at room temperature in petri dishes containing sterilized distilled water during 3-4 days for the observation of aquatic Hyphomycetes (8). Corn leaves, cellophane, snake skin, pollen, shrimp shell, hair and *Sorghum* seeds were added to the dishes as baits for zoosporic fungi (5, 11, 12, 17).

The aquatic Hyphomycetes and the zoosporic fungi were identified by direct observation of the structures on the leaves and baits.

Current literature was used (8, 9, 20, 21) to identify the isolated colonies to species levels, including the baits and pertinent literature (8, 9, 20, 21). The index of similarity of Sorensen (13) was calculated using the total occurrences of the aquatic fungi in each leaf species.

RESULTS AND DISCUSSION

Twenty taxa of zoosporic fungi and eleven of aquatic Hyphomycetes were isolated during the experiment (Table 1). The period of the experiment was expected to be for one year, but after eight months the leaves were almost totaly decomposed. One hundred sixty four occurrences of aquatic fungi were observed, 58% of zoosporic fungi and 42% of aquatic Hyphomycetes. These results do not agree with the ones mentioned in the literature in which the total occurrence of aquatic Hyphomycetes is frequently higher than the one for zoosporic fungi (3, 4, 15, 16, 22), probably due to the use of insufficient types of baits. In the study of the fungal succession in *Ficus microcarpa* (18) and *Quercus robur* (19) leaves similar results were obtained confirming that combined baiting methods must be used to obtain a representative aquatic mycota.

Catenophlyctis variabilis (Karling) Karling, *Polychytrium aggregatum* Ajello, *Rhizophydium sphaerotheca* Zopf were the most common zoosporic fungi isolated from *Alchornea triplinervia* leaves. In *Quercus robur* leaves, *Catenophlyctis variabilis* and *Nowakowskiella elegans* (Nowak.) Schroeter and in *F. microcarpa* leaves *Catenophlyctis variabilis, Polychytrium aggregatum, Dictyuchus* sp. were the most frequent isolated zoosporic fungi. *Catenophlyctis variabilis* followed by *Polychytrium aggregatum* and *Rhizophydium sphaerotheca* were the most frequent taxa isolated in all plant species. *Rhizophydium stipitatum* Sparrow was isolated only from *F. microcarpa* leaves. *Achlya radiosa* Maurizio was observed in the all plant species only in September, while *Achlya bi-*

TABLE 1 – Ocurrence of Aquatic fungi in leaves of *Alchernea triplinerviia, Quercus robur* and *Ficus microcarpa* submerged in a stream in the "Reserva Biológica do Alto da Serr de Paranapiacaba, SP".

Substrate / Fungus	*Alchornea triplinervia*									*Quercus robur*									*Ficus microcarpa*									Total of ocurrences
Month	A	M	J	J	A	S	O	N	Total	A	M	J	J	A	S	O	N	Total	A	M	J	J	A	S	O	N	Total	
Achlya bisexualis	–	–	–	–	–	–	–	–	0	+	–	–	–	–	–	–	–	1	–	–	–	+	–	–	–	–	1	2
Achlya flagellata	+	–	–	–	+	+	–	–	3	+	–	–	+	–	–	–	–	2	+	–	–	–	–	–	+	–	2	7
Achlya radiosa	–	–	–	–	–	+	–	–	1	–	–	–	–	–	+	–	–	1	–	–	–	–	–	+	–	–	1	3
Achlya sp.	–	+	–	–	+	+	–	–	3	–	–	+	–	–	+	–	–	2	–	–	–	+	–	+	–	–	2	7
Aphanomyces sp.	+	+	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	+	–	0	3
Catenophlyctis variabilis	+	+	–	–	–	–	+	+	4	+	+	+	+	–	–	+	+	6	+	–	–	–	+	+	+	–	6	14
Dictyuchus sp.	+	–	+	–	–	–	–	–	2	+	+	–	–	–	–	–	–	2	+	+	–	–	+	–	–	–	2	7
Diplophlyctis sarcoptoides	–	–	–	–	-	–	–	–	0	+	–	–	–	–	–	–	–	1	–	–	–	+	+	–	–	–	1	3
Karlingia rosea	–	–	+	–	+	–	–	+	3	–	–	–	–	–	–	–	–	0	–	–	–	–	–	+	–	–	0	4
Nowakowskiella elegans	–	–	–	–	+	+	–	–	2	–	+	+	–	–	+	–	+	4	–	+	–	–	–	–	–	–	4	7
Polychytrium aggregatum	+	–	+	+	–	+	–	+	5	–	–	–	–	–	–	–	–	0	–	+	+	–	–	+	–	–	0	8
Pythium sp.	–	–	–	–	–	–	–	–	0	–	+	–	–	–	–	+	–	2	+	+	–	–	–	–	+	–	2	4
Rhizophyctis sp.	–	–	–	–	+	–	–	–	1	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	+	–	0	2
Rhizophydium elyensis	–	–	–	–	–	–	–	+	1	–	–	–	–	–	–	–	+	1	–	–	–	–	–	–	–	+	1	4
Rhizophydium sphaerotheca	+	+	+	–	+	–	–	–	4	-	+	–	+	–	–	–	–	2	–	–	–	+	+	–	–	–	2	8
Rhizophydium stipitatum	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	0	–	–	+	–	–	–	–	–	0	1
Rhizophydium sp.	–	–	–	–	+	–	–	–	1	–	–	–	–	–	–	–	–	0	–	–	–	–	+	–	–	+	0	3
Saprolegnia megasperma	–	–	+	–	–	–	–	–	1	–	–	–	–	–	–	+	–	1	–	–	–	–	–	+	–	–	1	3
Saprolegnia parasitica	–	–	–	–	–	–	–	–	0	–	+	–	–	–	–	–	–	1	+	–	–	–	–	+	–	–	1	3
Saprolegnia sp.	–	–	–	–	–	–	–	–	0	+	–	–	–	+	–	–	–	2	–	–	–	–	–	–	–	–	2	2
Total of zoosporic fungi	6	4	3	1	6	6	1	4	31	6	6	3	3	1	3	3	3	28	5	3	3	4	5	8	4	2	28	93
Anquillospora longissima	–	–	–	–	–	–	–	–	0	–	+	+	–	–	–	–	–	2	+	–	–	–	–	–	–	–	2	3
Anquillospora sp.	+	–	–	–	–	–	–	–	1	+	–	–	–	–	–	–	–	1	+	–	–	–	–	–	–	–	1	3
Camposporium pellucidum	+	–	–	–	–	–	–	–	1	+	–	–	+	–	–	–	–	2	–	–	–	–	–	–	–	–	2	3
Dendrospora sp.	+	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	0	+	–	–	–	–	–	–	–	0	2
Lemonniera aquatica	–	–	–	-	–	–	–	–	0	+	–	–	+	–	–	–	–	2	–	–	–	+	–	–	–	+	2	4
Lunulospora curvula	+	–	–	-	–	–	–	–	1	+	+	–	–	–	–	–	–	2	+	+	–	–	–	–	–	–	2	5
Margaritispora sp.	–	+	+	-	–	+	–	–	3	–	+	–	–	+	–	+	+	4	–	–	+	–	–	–	–	–	4	8
Tetrachaetum elegans	+	+	–	-	–	–	–	–	2	–	+	–	–	–	–	–	–	1	–	+	–	–	–	–	–	–	1	4
Triposperum (?)	+	+	+	–	+	+	+	+	7	+	+	+	–	–	–	+	–	4	–	+	+	+	+	+	+	+	4	18
Triscelophorus monosporus	+	+	+	–	+	+	–	–	5	+	+	+	+	+	+	+	–	7	+	+	+	–	+	+	+	–	7	18
Trisulcosporium acerinum	–	–	–	–	+	–	–	–	1	–	–	–	–	–	–	–	–	0	–	–	–	–	–	–	–	–	0	1
Total of aquatic Hyphomycetes	7	4	3	0	2	4	1	1	22	6	6	3	3	2	1	3	1	25	5	4	3	2	2	2	2	2	25	69
Total	13	8	8	1	8	10	2	5	55	12	12	6	6	3	4	6	4	53	10	7	6	6	7	10	6	4	53	164

sexualis Coker & A. Couch, *Achlya flagellata* Coker, *Saprolegnia megasperma* Coker and *Saprolegnia parasitica* Coker occurred sporadically. *Rhizophydium elyensis* Sparrow was isolated at the end of the leaf decomposition. *Diplophlyctis sarcoptoides* (H.E. Petersen) Dogma and *Karlingia rosea* (de Bary & Woronin) Johanson were observed only a few times in the substrates.

Triscelophorus monosporus Ingold and *Tripospermum* sp. were the most frequent isolated aquatic Hyphomycetes in the three plant species (Table 1). *Tripospermum* species were also common on leaves of *Thypha, Pseudotsuga, Acer* and *Alnus* submerged in American streams (1). *Triscelophorus monosporus* has been often mentioned as one of the most common aquatic Hyphomycete in the tropics (15). *Dendrospora* sp. and *Trisulcosporium acerinum* Hudson & Sutton occurred only in leaves of *A. triplinervia*. This is the first record of these taxa in Brazilian continental waters. *Camposporium pellucidum* (Grove) Hughes and *Lemonniera aquatica* Wild were seldom observed in the studied substrates.

The total number of occurrences of zoosporic fungi was higher in *Ficus microcarpa* leaves, whereas *Quercus robur* leaves supported a higher number of occurrences of aquatic Hyphomycetes.

A tendency of higher occurrence of taxa at the beginning of the leaf decay was detected, except by the species of *Ficus microcarpa*, probably due to the capacity of zoosporic fungi and aquatic Hyphomycetes to metabolize simple sugar compounds, characterizing them as pioneers in the decomposition of allochthonous organic matter. On the other hand, the leaf disk washing method probably eliminated propagules adhered to the leaf surface, resulting in the observation of aquatic fungi in more advanced stages of the decomposition process (18, 19), whereas in the present experiment the propagules of the filoplane were immediatly observed.

The index of similarity of Sorensen (13) was 88% between *Quercus robur* and *Ficus microcarpa* leaves, 75% between *Alchornea triplinervia* and *Ficus microcarpa* leaves and 73% between *Alchornea triplinervia* and *Quercus robur* leaves. Although there are not many papers mentioning the Sorensen index for mycota of zoosporic fungi, the similarity indexes found may be considered high, when comparing with the ones given by Christensen (6), indicating the absence of a decisive influence of the plant species on the aquatic fungi population. Similar results, although related to terrestrial fungi were found on *Hortia* and *Licania* leaves in a tropical rainforest in the northeast region of Brazil (10).

In view of the results obtained, the zoosporic fungi play a very important role in the decomposition of leaves. So, combined techniques must be used in order to obtain a representative decomposing mycota of allochtonous organic matter.

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RESUMO

Fungos aquáticos em folhas submersas em um riacho situado na Mata Atlântica.

Objetivando a comparação da micota aquática durante a decomposição de três espécies de plantas, cento e sessenta e dois sacos de tela de náilon, contendo 15g de folhas secas foram distribuídos em 54 unidades para cada espécie vegetal estudada, *Quercus robur* L., *Ficus microcarpa* L. f. e *Alchornea triplinervia* (Spreng.) M. Arg. e submersos a 10cm de profundidade em cinco diferentes pontos ao longo de um riacho situado na Reserva Biológica do Alto da Serra de Paranapiacaba, SP. Mensalmente, de abril a novembro de 1990, quando os substratos estavam em avançado grau de decomposição, 15 sacos foram retirados de cada ponto de coleta. Cinqüenta discos de 5mm de diâmetro de cada tipo de folha foram colocados em placas de petri contendo água destilada esterilizada às quais foram adicionados substratos quitínicos, queratínicos e celulósicos para iscagem de fungos zoospóricos. As placas foram incubadas à temperatura ambiente por 3-4 dias. Os Hyphomycetes aquáticos foram observados diretamente nos discos de folha. Foram isolados 31 táxons, sendo 20 fungos zoospóricos e 11 Hyphomycetes aquáticos. Os fungos zoospóricos *Catenophlyctis variabilis* (Karling) Karling e *Polychytrium aggregatum* Ajello e os Hyphomycetes aquáticos *Triscelophorus monosporus* Ingold e *Tripospermum* sp. foram os táxons de maior ocorrência nos substratos estudados. Encontrou-se maior número de táxons no início de decomposição foliar.

Baseado no índice de similaridade de Soren-

sen, há poucas diferenças entre as micotas aquáticas das diferentes espécies vegetais. Diante dos resultados obtidos ficou evidente a necessidade do uso de técnicas consorciadas para a obtenção de micota efetivamente envolvida na decomposição de folhas submersas, principalmente à vista da representatividade dos fungos zoospóricos demonstrada neste trabalho.

Palavras-chaves: fungos zoospóricos, Hyphomycetes aquáticos, Mata Atlântica.

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INFLUENCE OF BAG MATERIALS ON THE MOISTURE LOSS AND FINAL AFLATOXINS CONTENT OF UNSHELLED PEANUTS STORED WITH HIGH MOISTURE. PRELIMINARY STUDIES

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SUMMARY

In the present work the influence of bag materials on the moisture loss and final aflatoxins content (B1+G1) of stored moist unshelled peanuts (MUP) was studied in the rainy season of 1990, in Marília, SP, and in the rainy season of 1991, in Jaboticabal, SP.

In each season, MUP were ventilated, as they arrived from the field, to get rid of extraneous materials and then put into 200 bags of jute and into 200 bags of polypropilene in 1990 and into 120 bags each material in 1991 stored in stacks. In the rainy season of 1990 (February-April) moisture and aflatoxins were determined at the beginning (average moisture = 14.31%; aflatoxins not detected). Following, moisture was determined twice a week in samples taken from the external part of the stacks during 80 days when the stacks dismantled and moisture and aflatoxins were determined in 6 samples of each stack. In the rainy season of 1991 (February-March), due to operational difficulties, closed mesh jute bags were used (green coffee type) and the experiment lasted only 30 days. Moisture and aflatoxins were determined only at the beginning (average moisture = 15%; aflatoxins not detected). At the end of the storage period three samples were taken from each lot, for moisture and aflatoxins analyses.

The results showed, in both experiments, that in jute bags, even with closed mesh, moisture loss was slightly faster with an average final moisture = 9.68% in jute bags and 10.38% in polypropilene in 1990 and 9.50% in jute and 10.36% in polypropilene in 1991 and that the final aflatoxins content was considerably lower in jute bags with average figures of 51 μg/kg (1990) and 361 μg/kg (1991) for jute against 1,380 μg/kg (1990) and 3,703 μg/kg (1991) for polypropilene.

From the results it was concluded that the use of jute bags is considerably better and can be indicated as the most convenient for preventing aflatoxins build up during storage of unshelled peanuts, specially when threshed and bagged moist.

Key words: Aflatoxins, moisture loss, peanuts, storage, bag material, jute, polypropilene.

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INTRODUCTION

The aflatoxins occurrence and levels in peanuts is dependent of several factors that include pre and post-harvest conditions and practices, of which, storage is one them. It is well known that storage of unshelled peanuts having moisture above 11% and relative humidity of at least 84% (2, 3, 4) has a great chance to permit the development of molds like *Aspergillus flavus* and *A. parasiticus* producers of aflatoxins.

Combine harvester is widely used today in Brazil and in one operation it gathers, thresh and put the unshelled peanuts in bags. It is a commom practice among most producers to make it when peanuts are still high in moisture (14-18% and even more), for they believe, and it is true, that when threshed dry (moisture lower than 11%) the yield of good unshelled peanuts is lower, resulting in lower profits. Efforts have been made lately, to change this practice because it certainly favors mold development with aflatoxins production in high levels. It is also true that, in the rainy season (January/March), the climatic conditions turns almost unavoidable to combine-harvest moist peanuts for it rains very much and do not favors a fast moisture loss for a safe storage. Very often the producers are rushed to remove the product from the field in order to prevent total loss due the germination of the peanut.

In the last ten years, the use of open mesh jute bags was almost completely changed to polypropilene ones for storage of unshelled peanuts for they cost less and last longer. However some middlemen have observed that unshelled moist peanuts seemed to loose moisture faster when in jute bags than when in polypropilene ones. Looking for specific literature, nothing was found about this subject and in order to try to clarify it, we decided to run an experiment on the possible influence of the two types of bag material on the moisture loss in the final aflatoxins content of unshelled peanuts during a storage period.

MATERIAL AND METHODS

Materials

Peanuts: unshelled ventilated with 14.31% (1990) and 15.00% (1991) of average moistures.

Bags: open mesh jute and polypropilene ones, in 1990, and closed mesh jute and open mesh polypropilene, in 1991 (see Fig. 4).

Methods

1990 CROP – Marília

Unshelled peanuts with an average moisture (3 samples) of 14.31%, freshly arrived from the field to the store of a middleman, in the region of Marília, SP, were put into 200 jute bags and 200 polypropilene bags. One sample for sample for aflatoxins analysis was taken from each lot and then stacked close to each other. during 80 days, three samples from each side of the external part of the stacks were taken for moisture determination, twice a week. At the end period the stacks were dismantled and from each one, 5 samples from the inner part and 1 from the outer part were taken for moisture and aflatoxins analyses.

1991 CROP – Jaboticabal

Due to internal problems of the middleman of Marília, in the 1991 crop, the experiment had to be executed in Jaboticabal at a later time. By that time open mesh jute bags were not available and the experiment had to be run with closed mesh jute bags used for storage of green coffee.

Unshelled peanuts with an average moisture (3 samples) of 15.0%, freshly arrived from the field, were put into 120 bags of polypropilene and in 120 bags of jute (closed mesh). As for aflatoxins is concerned, 10 subsamples of 2 kg were drawn, homogenized and then 2 kg were taken for analysis.

No samples were taken for moisture analyses during the 30 days that the peanut remained stored. At the end of this period, the stacks were dismantled and 3 samples of 2 kg were taken from each lot for moisture and aflatoxins analyses.

Analytical methods

Moisture was determined by the oven method (Brasil, 1976), which is the official method.

Aflatoxins were assayed by a modified Pons et alii (1966) thin layer chromatography method. The modifications introduced were: use of the water slurry technique of Velasco & Morris (1976) for sample homogenization, and use of acetone-water (85+15) for aflatoxins extraction and lead acetate clean up solution without heating (Pons et alii, 1972).

RESULTS AND DISCUSSION

The results are in the Tables 1 and 2 illustrat-

TABLE 1 – Final aflatoxinas B1+G1 contents (µg/Kg) in unshelled peanuts stored in two types of bag material, during 80 days, in the region of Marília. Samples taken in May 03, 1990.

Sample Number	Type of material	
	Jute	Polypropilene
1 (i)	153	4,137
2 (i)	ND	592
3 (i)	ND	1,330
4 (i)	153	1,034
5 (i)	ND	837
6 (e)	ND	354
Average	51	1,380

(i) - samples taken from the inner part of the stack
(e) - sample taken from the external part of the stack
ND - Not detected

TABLE 2 – Final aflatoxins B1+G1 contents (µg/kg) in unshelled peanuts stored in two types of bag material, during 30 days in the region of Jaboticabal, SP.

Sample Number	Type of material	
	Jute	Polypropilene
1	108	550
2	433	5,415
3	541	5,144
Average	361	3,703

ed in the Figures 1 to 3. For a better discussion the crops will be treated separately.

1990 CROP

Moisture behavior

Initial moisture content of the three samples varied from 13.40 to 14.94% with a mean value of 14.31%. This means that the lots had sufficient water activity to allow mold growth, as it was desired.

The Fig. 1 shows that within 5 days moisture of the external samples of both lots (not necessarily and probably not in the center of the stack) fell under 11%, considered to be the minimum for mold growth on unshelled peanuts. They show also that in the jute lot moisture loss was just a little bit faster than in the polypropilene one and that the moisture raised in both lots after rainfalls that occurred in the 35th, 65th and in the 75th day of storage, as can be seen also in the Fig. 1. The final moisture content in the inner core of the stacks were 10.38% for the polypropilene bags lot and 9.68% in the jute bags lot.

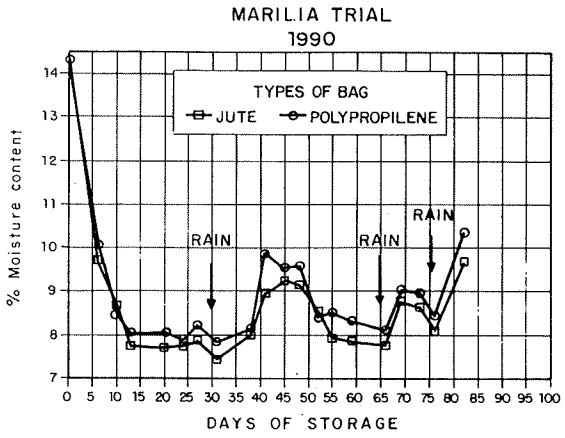

FIG. 1 – Moisture loss in unshelled peanuts stored in jute and polypropilene bags.

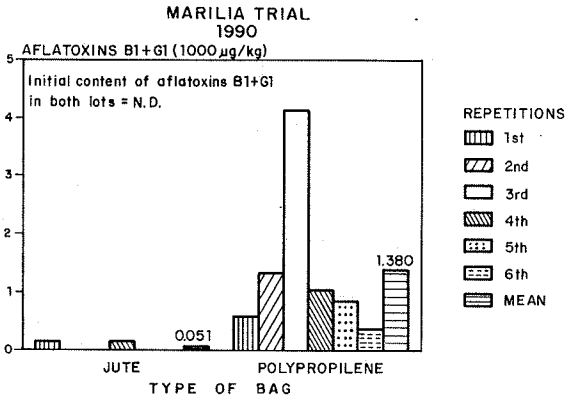

FIG. 2 – Final aflatoxin in unshelled peanuts stored in jute and polypropilene bags.

Aflatoxins behavior

Contrary to the very slight different behavior of both lots regarding to moisture, this was not the case for aflatoxins, that was not detected at the initial stage of the experiment, as it was desired, but its final contents in the two lots were quite differ-

ent: while in the jute bags lot only two, out of six samples (Table 1) yielded 153 µg/kg of aflatoxins B_1+G_1, with an average value of 51µg/kg in the polypropilene bags lot all samples had aflatoxins ranging from 354 to 4137 µg/kg with an average value of 1380 µg/kg, which means 22 times more, what is very significant (see Fig. 2).

1991 CROP

Moisture behavior

The initial average moisture content of three samples taken from the lots was 15.0%. This also means that the lots had sufficient water activity to allow mold growth.

Moisture, as mentioned before, could not be determined during the storage period but only at the end of the experiment which lasted only 30 days. The final moisture fell to an average of 9.50%, ranging from 9.39 to 9.63% in the jute bag lot and to an average of 10.36% ranging from 10.28 to 10.47% in the polypropilene bag lot with the same behavior of the 1990 experiment. No other observation could be made due to the circumstances of the test, but a similar behavior to that of the 1990 test was observed.

Aflatoxins behavior

Also in this test contrary to the very slightly different behavior of both lots regarding to the moisture, it was not the case for aflatoxins that was not also detected at the initial stage of this experiment. The final aflatoxins contents in the two lots were very different. Although all samples (Table 2) from the jute bag lot had aflatoxins they were very much lower than in the polypropilene bag lot. They ranged from 108 to 542µg/kg of aflatoxins B_1+G_1, with a mean value of 361 µg/kg and when compared to the polypropilene bag lot, that showed figures from 550 to 5415 µg/kg of aflatoxins B_1+G_1, with a mean value of 3703 µg/kg, signifies that the total aflatoxins content was around ten times more in the latter, confirming the same behavior observed in the 1990 crop (Fig. 3), although the closed mesh jute bags utilized this year.

No reasonable explanations were found for the dramatic difference of the aflatoxins contents of both types of bag material but the findings are quite encouraging.

Further longer terms and more complete studies will be carried in the 1992 rainy season trying to obtain a better and sharper picture of the situation.

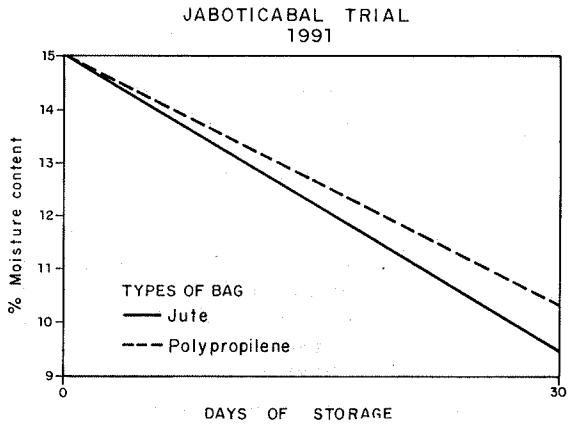

FIG. 3 – Misture loss in unshelled peanuts stored in jute and polypropilene bags.

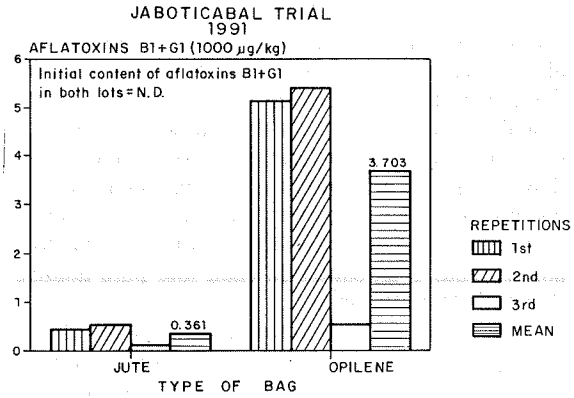

FIG. 4 – Final aflatoxin in unshelled peanuts stored in jute and polypropilene bags.

From the results it was concluded that: the use of jute bags for storage of moist unshelled peanuts allow a slightly better moisture loss when compared to polypropilene bags: in the conditions of this study, aflatoxins contents, at the end of the storage period in both experiments, were 10 to 22 times lower than in the polypropilene bags, and the use of jute bags is considerably better and can be indicated as the the most convenient for preventing aflatoxins contents build up during storage of unshelled peanuts, specially when threshed and bagged moist.

RESUMO

Influência do tipo de sacaria na perda de umidade e no conteúdo final de aflatoxinas de amendoim em casca armazenado úmido.

No presente trabalho foi estudada a influência

do tipo de sacaria na perda de umidade e no conteúdo final de aflatoxinas (B_1+G_1) de amendoim em casca armazenado úmido. O experimento foi realizado em Marília, SP, na safra das águas de 1990 e em Jaboticabal, SP, na safra das águas de 1991.

Em cada safra, o amendoim em casca úmido recém chegado do campo sofreu pré-limpeza para a retirada de material estranho e impurezas em geral e foi colocado em 200 sacos de juta e 200 de polipropileno (safra de 1990) e em 120 sacos de juta e em 120 sacos de polipropileno (safra de 1991) e em seguida armazenados em pilhas. Na safra das águas de 1990 (fevereiro/março) determinou-se, inicialmente, umidade e aflatoxinas em 3 amostras (umidade média = 14,31% e aflatoxinas não detectadas). Duas vezes por semana, durante os 80 dias subseqüentes, determinou-se umidade da parte externa das pilhas, após o que estas foram desmanchadas e determinou-se umidade e aflatoxinas em 6 amostras de cada pilha. Na safra das águas de 1991, devido a dificuldades operacionais, utilizou-se sacaria de juta de trama fechada (para café) e o experimento durou apenas 30 dias. Umidade e aflatoxinas foram determinadas apenas no início do experimento (umidade = 15,0% e aflatoxinas não detectada). No final foram retiradas 3 amostras de cada lote para análise de umidade e aflatoxinas.

Os resultados mostraram, em ambas as safras, que em sacos de juta, mesmo com trama fechada, a perda de umidade é ligeiramente mais rápida, do que em sacos de polipropileno, tendo acusado no final a umidade média de 9,68% na sacaria de juta e de 10,38% na de polipropileno em 1990 e 9,50% na de juta e de 10,36% na de polipropileno em 1991 e que o conteúdo final de aflatoxinas foi consideravelmente menor na sacaria de juta que na de polipropileno com valores médios de 51 µg/kg (1990) e 361 µg/kg (1991) para juta contra 1380 µg/kg (1990) e 3703 µg/kg para polipropileno.

Estes resultados levam à conclusão de que o uso de sacaria de juta é altamente vantajoso e pode ser indicado como o mais conveniente para prevenir o desenvolvimento de aflatoxinas no amendoim em casca, durante o armanezamento quando o mesmo for colhido e ensacado úmido.

Palavras-chave: Aflatoxinas, amendoim, perda de umidade, sacaria, armazenagem, juta, polipropileno.

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COMPARISON OF PRESERVATION METHODS APPLIED TO YEASTS USED FOR ETHANOL PRODUCTION IN BRAZIL

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SUMMARY

The following preservation methods were tested on ten yeast strains used for production of ethanol in Brazil: subculturing; suspension in distilled water; freeze-drying; and cryopreservation in liquid nitrogen.

Five of the strains tested were *Saccharomyces* species obtained from culture collections, and five were commercial brands used in Brazilian distilleries.

Cell viability and ability to produce ethanol from defined culture media were evaluated at different storage periods. Generally speaking, all methods kept ethanol yields at expected levels (70 to 90%) during storage of all strains. Subculturing allowed cell recovery after 10 months without transfer to fresh medium. Suspension in distilled water succeeded in maintaining viable cells for a maximum period of fifty-four (54) months. Although a high cell mortality rate was observed during the freeze-drying process, viability of remaining cells was maintained during storage. Cryopreservation maintained the highest level of cell viability and, from the point of view of maintenance of cell viability, proved to be the most advantageous preservation method.

Key words: preservation, cryopreservation, *Saccharomyces*.

INTRODUCTION

The use of microorganisms in the production of substances useful to man is a secular practice. Some authors consider yeast strains the most important of all the groups of microorganisms (1). Yeast strains have been used to produce proteins, beverages, aromatized compounds, oils (1), fuel (2), fats (1, 16), to the resolution of racemic mixtures (4), and as biotransformation agents. They are also widely used in Genetic Engineering studies (5).

The growing use of microorganisms for industrial processes and the impact of Applied Microbiology and Biotechnology tend to present new issues ranging from the preservation of biotechnological characteristics of improved strains, to legal problems involving patenting, distribution, and availability of active industrial cultures.

The need for optimizing preservation methods has been the object of intensive study in developed

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countries (15, 18). Unfortunately, few studies are to be found in Brazil on the effectiveness of preservation techniques, even though many research projects and industrial processes are based on the action of microorganisms. Therefore, a constant supply of viable and stable cultures of such strains must be given top priority.

Is has been reported that important characteristics have been lost depending on the preservation method employed (3, 7, 8, 10). Such loss justifies the on-going selection and improvement of preservation methods, especially for industrial strains.

The purpose of the present study is to compare the effectiveness of different preservation methods on cell viability and fermentative capacity of yeast strains.

MATERIAL AND METHODS

Microorganisms - Ten yeast strains were studied, the following five of which are commercial strains used in Brazilian distilleries for the production of ethanol from sugar cane juice and molasses: - IZ-1904 AB 008 *(Saccharomyces uvarum* - Instituto Zimotécnico de Piracicaba); - ZANITA AB 013 (Escola Superior de Agricultura Luiz de Queiroz ESALQ - USP), - VALOSEP AB 014 (CRPAA - Cooperativa Regional dos Produtores de Açúcar de Alagoas), - Fermento Fleischmann AB 015 (Produtos Alimentícios Fleischmann e Royal Ltda); and - Fermento Itaiquara AB 016 (Usina Itaiquara de Açúcar e Álcool S. A.).

The other five strains listed bellow are from culture collections or laboratories: - *Saccharomyces cerevisiae* AB 001 (CCY 21.4.36 Czechoslovak Collection of Yeasts); - *Saccharomyces bayanus* AB 09 INRA - Institut National de la Recherche Agronomique, France); - *Saccharomyces sake* AB 010 (INRA - Institut National de la Recherche Agronomique, France); - *Saccharomyces cerevisiae* AB 011 (UG-5 INSA - Institut National des Sciences Apliquées, France); and - *Saccharomyces formosensis* AB 012 (NEDO - New Energy Development Organization, Japan).

Preservation methods

Subculturing - The previously stored culture was periodically transferred (interval at 6 months), a loopful at a time, to tubes containing fresh YM agar (DIFCO - 0712-01-08), incubated at 30°C for 48 hours, and stored in an ordinary household refrigerator (4°C).

Distilled Water - Cells were grown for 48 hours in 100 ml YM liquid medium (30°C at 150rpm), harvested (4°C for 10 minutes at 10.000 rpm), and suspended in sterile distilled water. Five milliliter (5 ml) amounts were dispensed into ten milliliter (10 ml) amber flasks, rubber stoppered, sealed with aluminum caps, and stored at room temperature.

Freeze-drying - Cells grown on YM solid medium at 30°C for 48 hours were carefully removed from the agar surface and suspended in a cryoprotectant solution. The suspending medium used was 10% skimmed milk with 5% sodium glutamate. Four drops (0.1 ml) of the suspension were transferred to sterile glass ampoules, plugged with a cotton plug, and frozen to -50°C (approximately 1°C/min) in a model 75250 Labconco shell-freezer. Lyophilization was carried out in a model 5-75180 Labconco apparatus. Ampoules were vacuum-sealed and stored in a household refrigerator.

Cryopreservation in Liquid Nitrogen - Cultures were grown in YM broth at 30°C under 150 rpm agitation for 72 hours. Suspending medium consisted of a 10% glycerol solution. Equal amounts of suspending medium and culture were aseptically mixed. The mixture was dispensed (in amounts of approximately 0.05 ml) into appropriate drinking straws previously sealed at one end as described in literature (11). The drinking straws were sealed and placed in screw-capped polypropylene vials, frozen at a slow-cooling rate (1°C/min), and stored immersed in liquid nitrogen.

Viability assessments - Each preservation method was submitted to determination of viability by plating samples in YM agar, initially and after several different storage periods (13). Cultures preserved in liquid nitrogen were quick-thawed in a 37°C water bath, diluted in sterile distilled water and plated. Freeze-dried cells were resuspended and diluted in sterile distilled water at room temperature (25°C) and appropriate dilutions were plated. Colony forming units were enumerated (cfu/ml) after 48 hours of incubation at 30°C.

Agar slants were evaluated by visual observation during storage.

Fermentative Capacity - Cells from one straw were inoculated directly into 100ml YM broth and recovered by incubation at 30°C, 48 hours, 150 rpm. A loopful of each subculture or 1ml of each distilled water stock were also recovered in the same way. Cultures were harvested (4°C/10 min/10.000 rpm), and inoculated into 75 ml of fermentation medium containing glucose (100 g/l), ammonium sulfate (1 g/l) in 0.1M, pH 4.5 citrate buffer.

Twenty-five milliliter (25 ml) amounts of cell suspension were then distributed into three separate polypropilene tubes where fermentations were performed at 30°C taking care to prevent evaporation, but allowing elimination of CO_2. The tubes were weighed periodically to constant weight and the fermentation end-point was determined when three identical consecutive weights were obtained (12).

The following parameters were used for initial and final evaluations during the fermentation: number of viable cells determined by staining with a solution of methylene blue (14, 21); and ethanol determined by means of gas chromatography in an adsorption column measuring 1/8" by 1m with 80-100 mesh Porapack Q; respective temperatures of oven, injector, and detector were 190, 270 and 300°C. Initial values of total reducing sugars were approximately 100 g/l and final values 0.4 g/l (19), pH was kept around 4.5.

RESULTS AND DISCUSSION

Table 1 shows initial viability (cfu/ml) and percentage survival of yeast cells after storage during 12 months in liquid nitrogen, freeze-dried and in suspension in distilled water. Table 2 represents yeast viability results obtained after preservation in distilled water (cfu/ml) for 24 months. Each result is the average of nine observations. Results evaluated at the end of fermentation tests, as described in material methods, are shown in Tables 3, 4 and 5. The highest percentage survival was obtained with cells preserved in liquid nitrogen (Table 1). Results are in accordance with those found in literature reporting the superiority of liquid nitrogen preservation (9, 17).

Lyophilization led to decreased viability immediately after drying even though the cells used were agar-grown cells with skimmed milk plus

TABLE 1. Percentage survival of yeast strains after preservation during 12 months in liquid nitrogen, freeze-drying and suspended in distilled water.

	inicial viability (cfu/ml)			percentage survival		
Strain number	liquid nitrogen	freeze drying	distilled water	liquid nitrogen	freeze drying	distilled water
AB-001	6.2×10^7	2.6×10^7	1.0×10^8	100.0	30.4	1.60
AB-008	4.1×10^7	2.1×10^7	6.0×10^8	100.0	22.4	0.23
AB-009	2.0×10^8	2.3×10^7	5.6×10^7	100.0	100.0	4.46
AB-010	1.5×10^8	3.2×10^7	5.5×10^8	100.0	100.0	0.35
AB-011	1.2×10^8	1.7×10^7	8.5×10^8	100.0	100.0	0.14
AB-012	1.5×10^8	2.3×10^7	5.7×10^8	80.0	43.5	–
AB-013	3.7×10^7	1.2×10^6	4.7×10^7	100.0	100.0	0.21
AB-014	1.8×10^7	7.3×10^6	7.1×10^7	100.0	4.9	0.01
AB-015	3.1×10^7	4.6×10^7	2.5×10^8	100.0	100.0	0.10
AB-016	1.1×10^8	5.9×10^7	4.8×10^8	40.9	100.0	0.02

TABLE 2. Viability counts of yeast strains (cfu/ml) after maintenance in distilled water

	Storage time (months)				
Strain number	0	6	12	18	24
AB-001	1.0×10^8	3.7×10^7	1.6×10^6	3.2×10^5	8.5×10^5
AB-008	6.0×10^8	4.3×10^6	1.4×10^6	2.0×10^6	1.3×10^6
AB-009	5.6×10^7	2.8×10^6	2.5×10^6	1.3×10^6	9.5×10^5
AB-010	5.5×10^8	7.5×10^7	1.9×10^6	1.1×10^6	7.6×10^6
AB-011	8.5×10^8	1.6×10^8	1.2×10^6	8.0×10^5	1.0×10^6
AB-012	5.7×10^8	1.2×10^5	—	2.7×10^4	1.9×10^4
AB-013	4.7×10^7	1.0×10^5	1.0×10^5	2.0×10^5	6.0×10^4
AB-014	7.1×10^7	1.1×10^6	7.9×10^5	8.0×10^4	—
AB-015	2.5×10^8	5.7×10^5	2.5×10^5	2.7×10^5	2.8×10^5
AB-016	4.8×10^8	7.0×10^7	1.1×10^5	1.3×10^5	2.2×10^4

TABLE 3. Evaluation of ethanol production using yeast strains preserved by subculturing.

	Strain number and storage time (months)																			
	AB-001		AB-008		AB-009		AB-010		AB-011		AB-012		AB-013		AB-014		AB-015		AB-016	
	6	12	0	12	0	12	0	12	0	12	0	12	0	12	6	12	0	12	0	12
Time to complete fermentation (h)	23.0	26.0	24.0	28.0	23.0	25.0	28.0	21.0	25.0	30.0	25.0	10.0	20.0	26.0	22.0	26.0	24.0	28.0	24.0	20.0
Final ethanol (g/l)	38.1	40.9	39.2	46.3	41.5	44.2	39.2	43.3	44.0	46.5	43.0	45.7	42.4	41.5	37.0	43.6	37.4	40.9	39.0	40.2
Viable Cell number (cellx10^8/ml)	2.2	1.5	3.7	3.1	2.3	2.4	3.5	3.7	2.4	2.0	1.3	1.7	1.4	1.6	1.6	1.1	1.3	1.7	1.2	1.6
Ethanol Yield (%)	73.5	80.9	78.3	92.5	76.8	77.3	79.8	73.1	88.6	81.1	82.2	86.9	81.9	82.4	72.2	88.5	75.1	83.8	84.5	70.2

TABLE 4. Evaluation of ethanol production using yeast strains preserved in distilled water.

	Strain number and storage time (months)																			
	AB-001		AB-008		AB-009		AB-010		AB-011		AB-012		AB-013		AB-014		AB-015		AB-016	
	6	12	6	12	0	12	0	12	0	12	0	14	6	12	0	12	0	14	0	12
Time to complete fermentation (h)	23.0	27.0	18.0	16.0	22.0	27.0	23.0	25.0	21.0	20.0	13.0	17.0	19.0	30.0	20.0	26.3	24.0	43.0	23.0	18.0
Final ethanol (g/l)	40.1	40.5	43.1	43.2	41.9	43.5	41.6	44.8	41.0	45.0	43.0	38.5	41.8	40.8	46.4	40.9	41.9	39.1	48.2	47.3
Viable Cell number (cellx10^8/ml)	2.3	1.5	4.8	3.8	2.0	2.5	3.0	1.7	3.0	3.6	1.7	1.5	2.5	1.4	1.6	3.3	1.8	4.4	2.2	2.2
Ethanol Yield (%)	82.2	85.6	76.7	76.5	87.8	88.3	90.5	86.7	86.5	82.2	80.7	75.6	83.4	85.3	75.6	79.1	84.2	78.3	94.0	85.6

TABLE 5. Evaluation of ethanol production using yeast strains preserved in liquid nitrogen.

	Strain number and storage time (months)																			
	AB-001		AB-008		AB-009		AB-010		AB-011		AB-012		AB-013		AB-014		AB-015		AB-016	
	0	6	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12	0	12
Time to complete fermentation (h)	12.0	23.0	13.0	27.5	14.0	21.0	14.0	22.0	14.0	20.0	12.0	23.5	12.0	21.5	14.0	24.5	13.0	22.0	16.0	18.0
Final ethanol (g/l)	43.2	44.4	44.0	48.0	45.6	43.2	45.0	43.2	42.4	43.8	44.4	45.6	43.5	45.6	45.9	46.2	42.3	44.0	39.0	44.8
Viable Cell number (cellx10^8/ml)	3.5	2.5	5.0	8.2	4.3	6.9	3.9	7.5	2.8	6.1	3.1	5.7	1.6	4.0	4.1	5.9	2.8	4.0	4.4	3.5
Ethanol Yield (%)	90.5	94.9	83.0	90.2	86.3	84.7	90.0	88.4	88.4	87.6	88.5	89.9	82.8	87.9	87.9	87.6	79.9	89.1	79.1	91.4

sodium glutamate as suspending medium, and the preceeding freezing process was carried out at a slow cooling rate - 1°C/min - (conditions that had proved best in previous trials - Ferreira da Silva, unpublished results). After one year of storage, viability counts remained at the same levels for six yeast strains, while four strains showed a decreasing percentage survival from 43.5% to 4.9% (Table 1).

Preservation in distilled water showed the lowest values of cell survival after one year (Table 1). However this method allowed recovery of viable cells even after 24 months of storage (Table 2) and even after 54 months (data not shown). Preservation in water had been reported previously. Some authors considered the utilization of nutrients from dead cells and low metabolic activity as possible explanations for the success of preservation in water (6, 9, 20).

Subculturing and liquid nitrogen preservation produced no changes in fermentative capacity (ethanol production parameters-Tables 3 and 5). However, subcultures must be transferred to fresh media at maximum intervals of ten (10) months when maintained at refrigerator temperatures. Preservation in water is advantageous in this respect because stocks could be renewed at intervals of forty (40) of fifty (50) months after storage at room temperature. Ethanol yields remained approximate to the initial values obtained, although variation was not uniform among the strains (Table 4). Moreover, preservation in water is less expensive and requires no frequent manipulation, sophisticated equipment, or periodic supply of liquid nitrogen, and is therefore more suitable to less well-equipped laboratories.

RESUMO

Comparação de métodos de preservação de leveduras utilizadas industrialmente na produção de etanol.

Dez linhagens de leveduras utilizadas na produção de etanol foram submetidas a diferentes métodos de manutenção. Os métodos empregados foram o subcultivo em meio de cultura sólido, a suspensão em água destilada, liofilização e a criopreservação em nitrogênio líquido.

Cinco das linhagens foram espécies de *Saccharomyces* obtidas em coleções de culturas e cinco a partir de marcas comerciais utilizadas em usinas.

A viabilidade celular e a capacidade de produção de etanol a partir de meio de composição definida foram avaliadas no momento inicial e após diferentes períodos de estocagem.

De modo geral, os métodos mantiveram o rendimento em etanol dentro de níveis satisfatórios, durante o armanezamento, para as diferentes linhagens.

Com relação à viabilidade celular, o subcultivo permitiu recuperar células viáveis por até 10 meses sem necessidade de renovação dos estoques. A preservação em água destilada manteve células viáveis por períodos de até 50 meses, apresentando entretanto relativo decréscimo. Embora a liofilização tenha promovido alta taxa de morte, o nível de viabilidade das células remanescentes permaneceu estável durante o período de estocagem. A criopreservação manteve a viabilidade em níveis elevados, apresentando vantagem neste aspecto sobre os demais métodos.

Palavras-chave: preservação, leveduras, *Saccharomyces.*

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the cellulases, characterization of the enzymes, and possible application of the enzymes in the hydrolysis of cellulose.

MATERIALS AND METHODS

Isolation of thermophilic microorganisms. Approximately 1 g sample from compsot heaps in the São Paulo State, Brazil, was inoculated into 10 ml of the enrichment medium in test tubes, followed by static incubation at 45°C for one week. This medium was described by Mandel and Sternberg (8), substituting cellulose with filter paper (size 0.5 x 6 cm). If during one week of incubation at 45°C, the submerged part of the suspended filter paper in the medium was hydrolyzed, the culture broth was inoculated onto potato dextrose agar (PDA) and incubated at 45°C for one week. The colonies which grew on the plate were transferred to slant culture containing PDA.

Examination of enzyme production. One ml of spore suspension of the isolated strains of microorganism was inoculated into 20 ml of the culture medium described above, containing 1% paper cellulose powder as carbon source, pH 5 in 125 ml Erlenmeyer flasks, and incubated at 45°C with agitation at 110 rpm. After 4 days of incubation, the culture medium was centrifuged to separate the supernatant and the activities of extracellular carboxymethylcellulase or CMCase (endo-1,4-ß-D-glucanase EC. 3.2.1.4), avicelase (exo-1,4-ß-D-cellobiohydrolase EC. 3.2.1.91), and ß-glucosidase EC. 3.2.1.21 (cellobiase) determined in the supernatant.

Assay of enzymatic activities. The activities of CMCase and avicelase were determined by incubating a mixture of 0.1 ml enzyme solution with 0.9 ml of a 1% solution of the respective substrate (carboxymethylcellulose and avicel) in 0.1 M acetate buffer pH 5.0 at 45°C for 10 min. Reducing substances were then quantified using a dinitrosalicylic acid (DNS) solution. The activity of ß-glucosidase was determined by incubating a mixture of 0.1 ml enzyme solution with 0.9 ml of 0.1 mM p-nitrophenyl-ß-D-glucopyranoside (PNPG) in 0.1 M acetate buffer pH 5 at 45°C for 10 min. After incubation, 6 ml of 0.1 N NaOH was added to the mixture and the p-nitrophenol released measured at 420 nm in a spectrophotometer. One unit of ß-glucosidase activity was defined as the amount of enzyme needed to liberate 1 µmole of p-nitrophenol per min under the assay conditions. One unit of activity of CMCase and avicelase was defined as the amount of enzyme which formed 1 µmole of reducing sugar as glucose per min under these conditions.

RESULTS AND DISCUSSION

Isolation and identification of microorganism. Two hundred five (205) thermophilic strains of microorganism were isolated from 35 compost heaps prepared by 16 farms. Seven strains of fungus produced thermostable cellulases. One of these strains, strains No. 10-2, produced cellulases with exceptionally high activity as compared to other 6 strains. The strains was identified as Genus *Aspergillus* in accordance with description by Raper and Fennel (11).

The temperature range for growth of the fungus was 20-50°C, and the optimum growth temperature range was 35-45°C.

Production of cellulases. Table 1 shows the effect of various carbohydrates on the production of CMCase, avicelase, and ß-glucosidase.

It was found that the highest production of three cellulolytic enzymes was obtained when the fungus was grown on culture medium containing paper cellulose powder as carbon source. Either avicel or microcrystalline cellulose also increased the production of CMCase and avicelase but did not induced ß-glucosidase significantly.

Solka Floc. (FC-300), CMC, and xylan slightly induced the production of CMCase, while avicelase was not produced by these carbohydrates.

TABLE 1 – Effect of carbohydrates on the production of cellulolytic enzymes by *Aspergillus* sp.

		CMCase (Unit/ml)	Avicelase (Unit/ml)	β-Glucosidade (Unit/ml)
Paper cellulose powder;				
	1%	1.5	0.4	6.9
	2%	1.0	0.4	3.1
	3%	0.9	0.3	2.2
Microcrytalline cellulose;	1%	1.2	0.4	1.5
	2%	1.0	0.3	0.8
	3%	0.9	0.3	0.8
Avicel;	1%	1.4	0.4	1.5
CMC;	1%	0.6	0	4.4
Xylan;	1%	0.5	0	1.0
Solka Floc; FC-300	1%	0.9	0	1.2

Culture medium described in the text substituting respective carbohydrate was cultivated at 45°C dor 3 days.

Production of ß-glucosidase was highly induced by CMC but slightly by avicel, xylan and Solka Floc (FC-300). The effect of temperature on the production of cellulolytic enzymes is illustrated in Figure 1. Alteration of activities of avicelase, CMCase, and ß-glucosidase was observed when the strain of *Aspergillus* sp., was grown at different temperatures. Maximum production of avicelase was reached after 5th day of incubation at 30, 37 and 45°C and incubation at 37 and 45°C produced more enzyme than at 30°C. Incubation at 45°C produced remarkably high activity of ß-glucosidase as compared to incubation at 37°C, while only a slight production was occurred at 30°C. For the production of CMCase, incubation at 37°C resulted in a higher enzyme production than incubation at 45°C, and the production of the enzyme was significantly decreased at 30°C. The effect of temperature on the production of cellylolytic enzymes by *Aspergillus* sp., No. 10-2 is very important point because temperature of incubation at 45°C induced more ß-glucosidase. Avicelase CMCase and ß-glucosidase act synergistically in the complete degradation of crystalline cellulose (1). However, ß-glucosidase has been considered rate limiting to cellulose hydrolysis (9).

Time course of the production of cellulolytic enzymes by Aspergillus sp., No. 10-2 was compared to Trichoderma reesei QM 9414 using same culture medium described in the method. Temperature of cultivation for *T. reesei* QM 9414 was 28°C, whereas *Aspergillus* sp., No. 10-2 was cultivated at 37 and 45°C respectively. The results were ilustratred in Figure 2, *T. reesei* QM 9414 produced avicelase and CMCase about two times as much as that of Aspergillus sp., No. 10-2. On the contrary, Aspergillus sp., No. 10-2 produced ß-glucosidase exceptionally high activity (13.6 units) as compared to *T. reesei* QM 9414. Maximum production of ß-glucosidase (7 units/ml) by *T. reesei* QM 9414 was reached on third day of cultivation and then gradually decreased to about 0.5 unit/ml.

Properties of cellulolytic enzymes. As shown in Figure 3, optimum pH ranges of avicelase, CMCase and ß-glucosidase were 4.5 - 5.7, 4.7 - 5.3 and 4.7 - 5.3 (Fig. 3-A) and pH stability range of the three enzymes were 4.5 - 8.0 (Fig. 3-B). The effect of temperature on activities of the cellulolytic enzymes were measured at various temperatures under the assay conditions described in the method. Results were shown in Figure 4, optimum temperatures for avicelase, CMCase and ß-glucosidase were 70, 70 and 65°C respectively (Figure 4-A). The effect of temperature on enzyme stability was investigated by incubating culture filtrate pH 5.0 at various temperatures for 30 min and then determined residual activities. As shown in Figure 4-B, avicelase, ß-glucosidase and CMCase were not inactivated at 55°C but 77% of avicelase activity was retained at 65°C whereas 73% of ß-glucosidase ac-

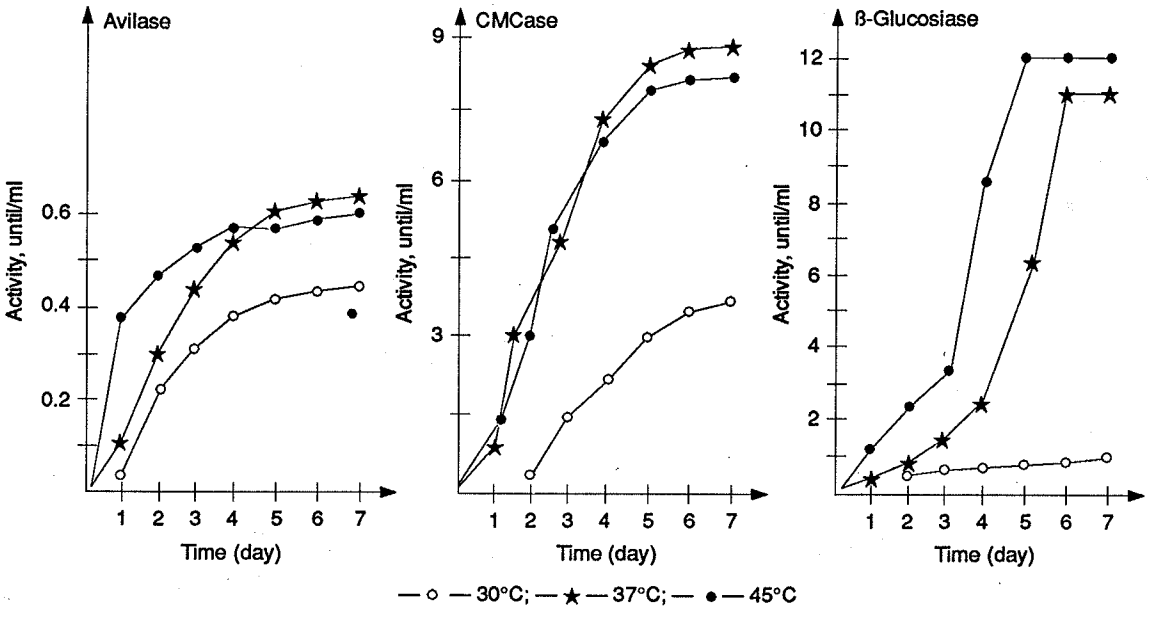


FIGURE 1 – Time course production of cellulolytic enzymes by *Aspergillus* sp., at various temperatures.

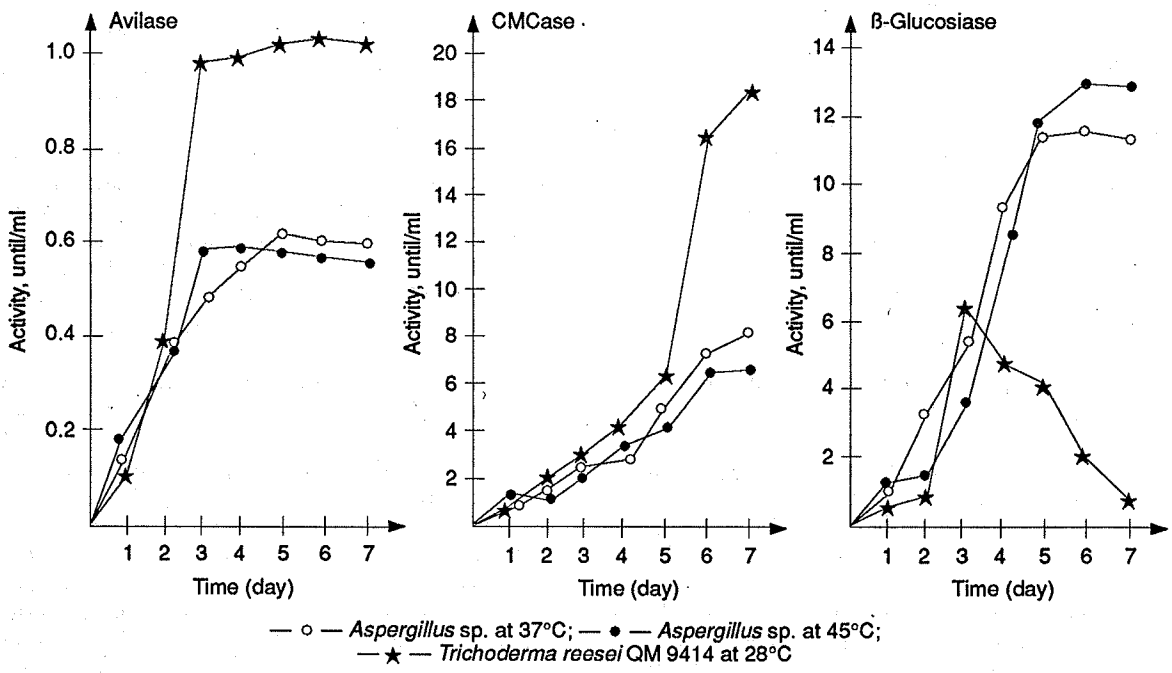
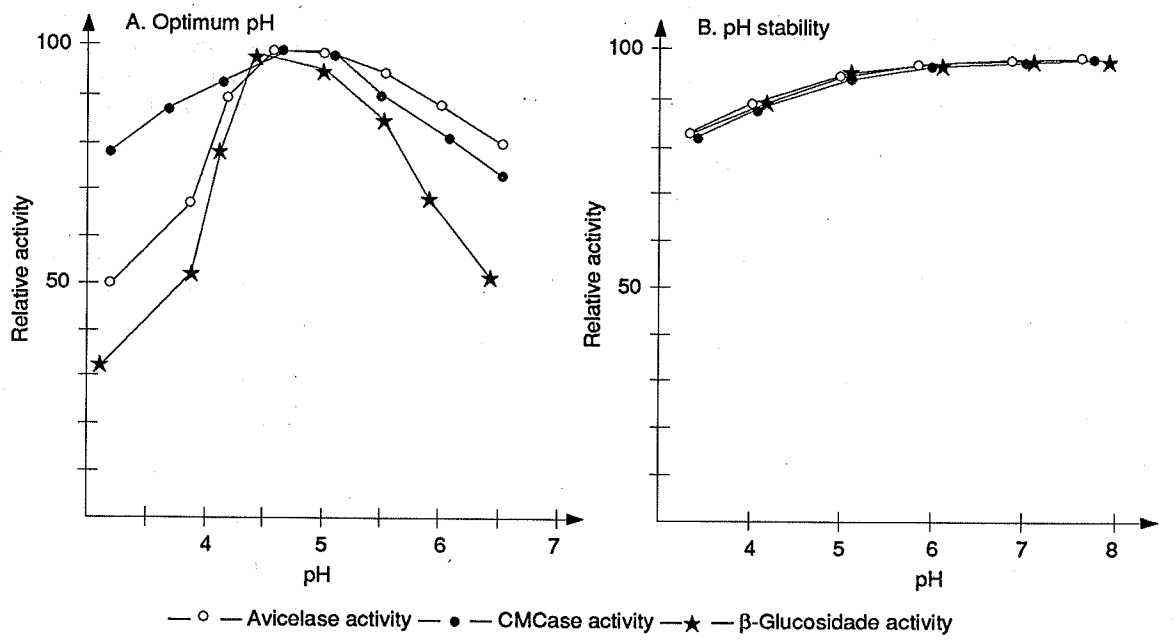


FIGURE 2 – Time course production of cellulolytic enzymes by *Aspergillus* sp., and *Trichoderma reesei* QM 9414.



Enzyme activities were determined as described in text using two different buffers; Acetate buffer 0.1 M, pH 4 - 5.6; Phosphate buffer 0.1 M, pH 5.8 - 8.
pH stability was examined by determining enzyme activities after enzyme solutions (5 mg of crude enzyme powder in 5 ml of each buffers) were held for overnight at room temperature.

FIGURE 3 – Effect of pH on the activity of *Aspergillus* cellulases

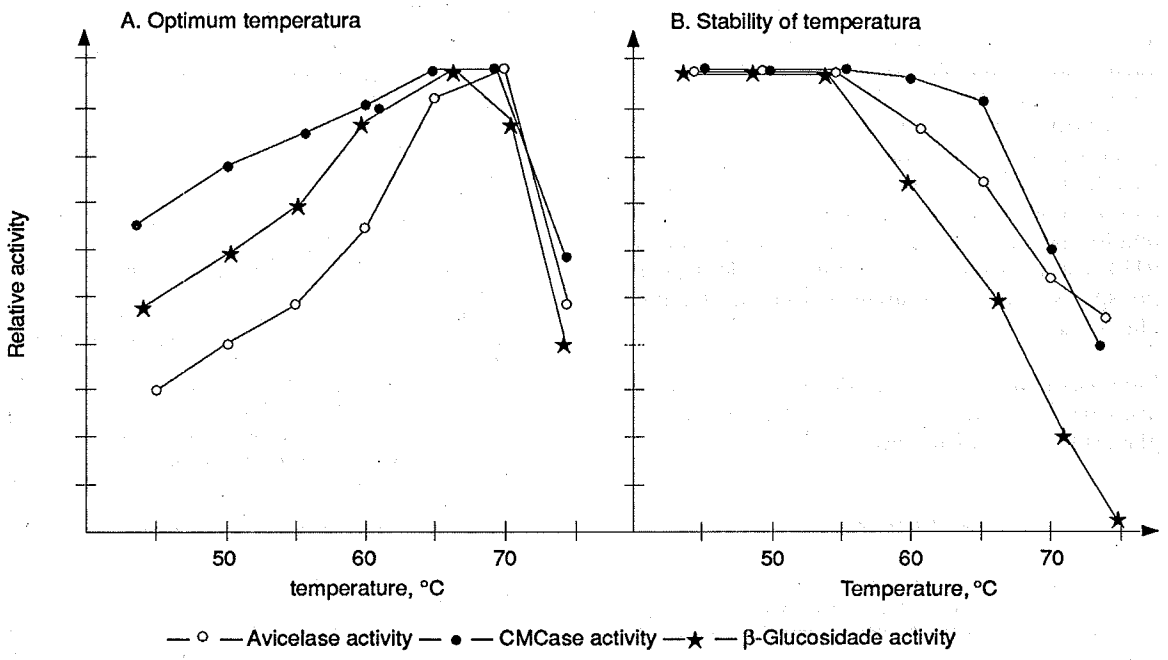


FIGURE 4 – Effect of temperature on activity and stability of *Aspergillus* cellulases

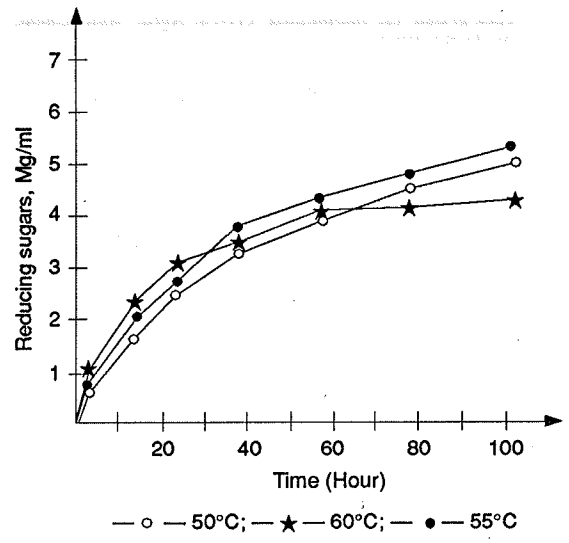


FIGURE 5 – Hydrolysis of Solka-Floc, BW-200 by *Aspergillus* cellulares

tivity retained at 60°C. On the other hand, CMCase was not inactivated at 60°C and 94% of the enzyme activity was retained at 65°C.

Enzymatic hydrolysis of cellulose. Suspension of Solka-Floc, BW-200, 1% in 100 ml of acetate buffer 0.05 M, pH 5 which contained crude preparation of *Aspergillus* sp., No. 10-2 enzyme (avicelase 2 units, CMCase 12 units, and ß-glucosidase 55 units) was incubated at 50, 55, and 60°C respectively and the results were illustrated in Figure 5.

It was found that the optimum temperature for saccharification was 55°C. Paper chromatographic analysis of the resulting hydrolysates have demonstrated predominant glucose spot but trace amounts of cellobiose, whereas hydrolysates by *T. reesei* QM 9414 enzyme consisted of low glucose to cellobiose ratios. This result is due to fact that the *Aspergillus* sp., No. 10-2 produced high activity of ß-glucosidase.

RESUMO

Uma linhagem termofílica do fungo *Aspergillus* sp., No. 10-2, foi isolada de compostos. A faixa de temperatura para crescimento variou de 20 a 50°C sendo que a temperatura ótima ficou entre 35 a 45°C. As produções máximas de endo-1,4-ß-D-glucanase EC. 3.2.1.4 (CMCase), exo-1,4-ß-D-cellobiohydrolase EC. 3.2.1.91 (Avicelase) e ß-glucosidase EC. 3.2.1.21 (Celobiase) foram obtidas quando o fungo foi cultivado em meio

líquido contendo papel de celulose em pó. A maior produção de CMCase foi obtida a 37°C enquanto que uma extraordinária produção de ß-glucosidase ocorreu a 45°C. A temperatura ótima de sacarificação de celulose pela enzima foi 55°C. Ensaios de cromatografia de papel de hidrolisados da celulose demonstraram predominantemente glicose quando comparado a hidrolisados de celulose obtidos por enzima de *Trichoderma reesei* QM 9414. Isto ocorreu porque a linhagem de *Aspergillus* sp., No. 10-2, produziu maiores a enzima ß-glucosidase.

Palavras-chave: Celulases de *Aspergillus*, Celulases de *Trichoderma*, Celulase termoestável, ß-glucosidase, Sacarificação de celulose.

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PRODUCTION OF AMYLASES BY CANDIDA IN LIQUID AND SEMISOLID FERMENTATION

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SUMMARY

Quantitative and qualitative amylases production by *Candida famata* FTPT 1539, *C. fennica* FTPT 1829, and *C. fennica* FTPT 8903 was measured and compared to a standard strain of *Schwanniomyces alluvius* UCD 54-83. The YES and YNB liquid media and semisolid wheat bran medium were used. The semisolid medium was shown to be better for alpha-amylase production. The determination of the residual starch and the appearance of amounts of reducing sugars suggest that *C. fennica* FTPT 8903 expressed both alpha-amylase and glucoamylase.

Key words: alpha-amylase; amylolytic yeasts; *Candida*; glucoamylase; semisolid fermentation; liquid fermentation.

INTRODUCTION

Starch occurs as the major reserve carbohydrate in all higher plants in the form of insoluble granules. Starch is a heterogenous polysacharide which is composed of two high molecular components, amylose, and amylopectin (10).

Four main types of enzymes are responsible for starch degradations: alpha-amylase (alpha-1,4 glucan glucanohydrolase E.C.3.2.1.1); beta-amylase (alpha-1,4 glucan maltohydrolase E.C.3.2.1.2); glucoamylase (alpha-1,4 glucan glucanohydrolase E.C.3.2.1.3); and debranching enzymes, represented by pullulanases and isoamylases, (12). The capacity to degrade starch is not widespread among yeasts, but those which are capable of degrading starch have been investigated as promising microrganisms for single cell protein production as well as ethanol (5). The ability to produce lipids for alimentary or pharmaceutical use from starch degrading oleaginous yeasts also has been considered (4).

Amylolytic yeast strains have been optimized by genetic manipulations, such as protoplast fusion (including intergeneric fusions), which is a valuable technique for the improvement of industrial yeast strains.

In this paper, we report a study for production of alpha-amylase and glucoamylase from three yeast strains which were isolated from different habitats and discuss some characteristics of these enzymes.

MATERIAL AND METHODS

Yeast strains- C. fennica FTPT 1829, *C. fennica* FTPT 8903, and *C. famata* FTPT 1539 were isolated by Leptospirosis Laboratory, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, and identified by Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas,

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Brasil. *S. alluvius* UCD 54-83, used here as a standard strain, was donated by the culture collection of the National Research Council of Canada, Prare Regional Laboratory, Saskatoon, Sasck. The stock cultures were maintained on YEPD slants (1% yeast extract, 2% peptone, 2% dextrose, 2% agar).

Detection of amylolytic enzyme activity - After growth in YES medium (0.5% yeast extract, 1% soluble starch, pH 7.0), for 24 h at 28°C on a reciprocal shaker (160 rpm), the yeast cultures were diluted and then plated on YPS agar (1% yeast extract, 2% peptone, 3% soluble starch) and incubated at 28°C for 96h with subsequeent incubation at 4°C for 48h. The yeast strains producing amylolytic enzymes exhibited clear zones (halos) around the colonies (9).

Production of crude enzyme - Yeasts were grown for 72 h at 28°C on a reciprocal shaker (160 rpm) in 250 ml Erlenmeyer flasks containing 25 ml of liquid media. Two different liquid media were used: YES (pH 7.0) and YNB (Yeast Nitrogen Base (pH 4.5)) according too Wilson and Ingledew, (13). The incubation in semisolid wheat bran medium (37% wheat bran in 1% soluble starch (w/v)) was carried out for 72h at 28°C as described by Linardi and Machado, (6).

The inoculum used in all media consisted of 3.0 ml. of a cell suspension, containing 10^8 cells/ml, obtained after first growing the strains in YES medium for 24 h.

For enzyme determinations wheat bran was mixed with 100 ml of distilled water and agitated for 1 h at room temperature. The mixture was filtered, centrifuged, and the supernatant was examined for enzymatic activity. For liquid media, cell-free supernatants were used.

Enzymatic assays - Alpha-amylase and glucoamylase were assayed as described by Wilson and Ingledew (13).

For alpha-amylasse assay, 2.0 ml of substrate solution (0.2% soluble starch (Merck) in boiling 0.05M KH_2PO_4-NaOH buffer, pH 6.0, and cooled to 40°C) was treated with 1.0 ml. of enzyme for 10 min at 40°C. The reaction was revealed by adding 0.2 ml aliquot of the digest to 5.0 ml. of iodine reagent (1.0 ml of iodine stock solution (0.5% I_2 in 50% KI) diluted with 500 ml. of distilled water containing 5.0 ml of 5M HCl). The absorbance was measured at 620 nm. One unit of alpha-amylase was defined as the amount of enzyme that hydrolyses 0.1 mg of starch in 10 min at 40°C in presence of 40 mg of starch. This assay was also carried out at different temperatures and pH in order to define the optimal conditions. The mode action of amylases in degrading starch were also analysed by this method at different time intervals during a 100 minute period. Samples were removed and the reaction was quenched by boiling for 5 min. Reduction of iodine staining power of starch was measured as described by Stark et al. (11) and the reducing substances determined by the method of Nelson and Somogyi (7).

For glucoamylase assay, the substrate was 0.5% soluble starch (Merck) dissolved in boiling 0.05M KH_2PO_4-NaOH, pH 5.0 and cooled to 40°C. It was added 1.0 ml of enzyme to 5.0 ml of starch substrate and after 10 min of incubation at 40°C the reaction was stopped by boiling for 5 min. After cooling, glucose liberation from starch was measured by the glucose-oxidase assay (ENZ color Kit, BioDiagnostica). One unit of glucoamylase was defined as the amount of enzyme that liberates 1 µmol of glucose in 10 min at 40°C in the presence of 25 mg of starch.

RESULTS AND DISCUSSION

Except for *C. famata* FTPT 1539, all yeast strains showed a well defined clear halo on YPS agar plates with enzyme activity index less than 1.0 (Table 1). Liquid and semisolid media were used for production of amylases (Table 2).

TABLE 1 – Relationship between diameter of colony and halo.

Strains	Mean diameter of colony (mm)	Mean diameter of halo (mm)	Enzyme activity index*
S. alluvius UCD 54-89	2.9	7.5	0.38
C. fennica FTPT 8903	7.8	21.0	0.37
C. fennica FTPT 1829	3.1	7.1	0.44
C. famata FTPT 1539	5.1	np	1.00

* Enzyme activity index = mean diameter of colony (mm)/ mean diameter of halo (mm)
np = not produced

We observed that *C. famata* FTPT 1539 showed no ability to produce alpha-amylase or glucoamylase in both media. *C fennica* FTPT 1829 produced alpha-amylase but not glucoamylase in semisolid medium. It is proved that *C. fennica* FTPT 8903 was better alpha-amylase and glucoamylase producer than other strains in this experiment (Table 2).

TABLE 2 – Production of alpha-amylase and glutcoamylase in liquid and semisolid media.

Strains	YES		YNB		Semisolid	
	AA*	GA**	AA	GA	AA	GA
S. alluvius UCD 54-89	7.4	np	3.3	np	102.0	1.0
C. fennica FTPT 8903	2.8	np	5.2	np	124.7	2.3
C. fennica FTPT 1829	np	np	np	np	58.2	np
C. famata FTPT 1539	np	np	np	np	np	np

* AA (alpha amylase activity) = the amount of the enzyme that hydrolyses 0.1 mg of starch in 10 min at 40° C, in presence of 4.0 mg of starch.

** GA (glucoamylase activity) = the amount of enzyme that liberates 1.0 µmol of glucose in 10 min at 40° C in presence of 25 mg of starch.

np = not produced.

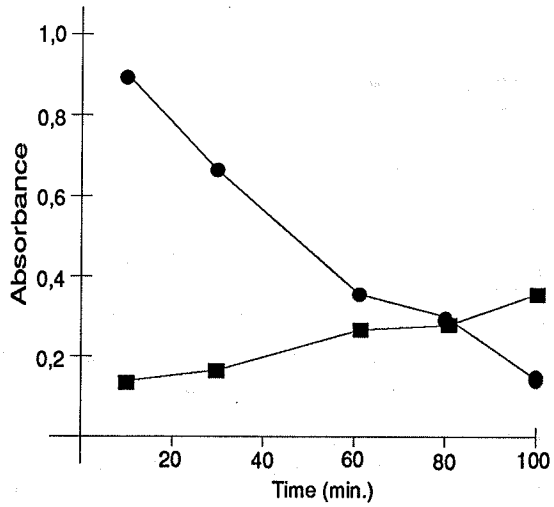

FIGURE 1 – Action of alpha-amylase produced by *S. alluvius* UCD 54-83. (●) Residual starch (600 nm), and (■) appearance of amounts of reducing sugar (540 nm).

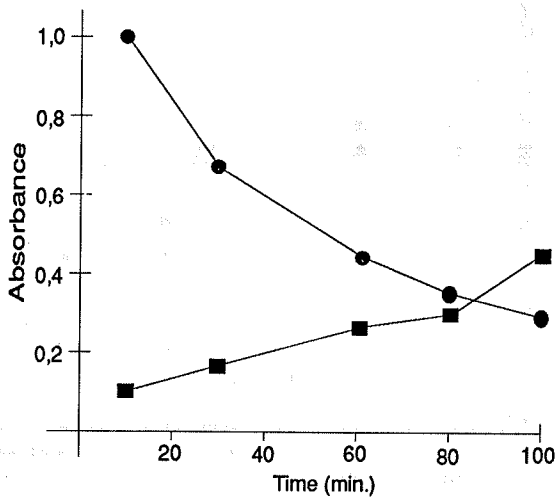

FIGURE 2 – Action of alpha-amylase produced by C. fennica FTPT 8903. (●) Residual starch (600 nm), and (■) appearance of amounts of reducing sugar (540 nm).

Characteristics of production of amylolytic enzymes from this strain is similar too *S. alluvius which* was reported previously as producer of both alpha-amylase and glucoamylase (Wilson and Ingledew (13)). Our data demonstrated that *C. fennica* FTPT 8903 produced these two enzymes (Table 2).

Liquid media did not increase enzyme production. On the other hand, semisolid fermentation has shown to improve enzyme production or to favour enzyme excretion. It had already been reported the influence of the composition of culture medium on biosynthesis and excretion of amylases (8). It could be also considered that the difficulty of diffusion of catabolites as well as released glucose, and ather substances could act as enzymatic inhibitors in semisolid medium (3).

The residual starch content and the appearance of reducing sugars after fermentation with the strains of *S. alluvius* and *C. fennica* FTPT 8903 (Figures 1 and 2), suggest the presence of at least two different enzymes: an alpha- amylase and a glucoamylase. It is known that there is a sinergistic action of alpha-amylase and glucoamylase on hydrolisis of starch (2). This observation could be ascribed by the good performance for *C. fennica* FTPT 8903 in degrading this substrate. The mode of action of amylases were examined by measuring the production of reducing sugars and the decrease of iodine staining power (Figure 3). *C. fennica* FTPT 8903 and *S. alluvius* UCD 54-83 exibit the same profile, but it was not the typical profile obtained by Stark et al. (11) and Fairbain et al. (1) for endo and exoamylases. This could be explained by the use of crude enzyme in our experiments, instead of the purified fraction used by those authors.

The optimal temperature and pH for the action of alpha-amylase from *C. fennica* FTPT 8903 and *S. alluvius* UCD 54-83 in semisolid fermentation were compared (Figures 4 and 5) and the optimal temperature and pH were 40°C and 6.0 for both yeast strains. These data are in agreement with the data obtained by Wilson and Ingledew (13) who worked with purified alpha-amylase of *S. alluvius* UCD 54-83.

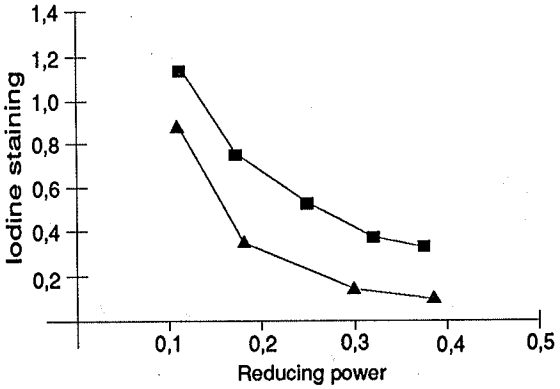

FIGURE 3 – Reducing sugars from starch and reduction of iodine staining capacity during hydrolysis of starch by extracts from semisolid fermentation. (▲) S. alluvius UCD 54-83, and (■) C. fennica FTPT 8903

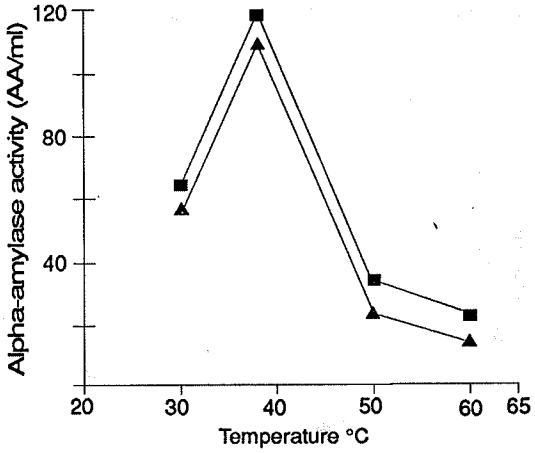

FIGURE 4 – Effect of temperature on the action of alpha-amylase produced by semisolid fermentation. (▲) *S. alluvius* UCD 54-83, and (■) *C. fennica* FTPT 8903

Strains of *C. fennica* and *C. famata* were characterized as prototrophic. These yeasts were found to be resistant to the antifungals nystatin and benomyl. These characteristics are important in subsequent genetic studies and further improvement of the strains can be applied for biotechnological process.

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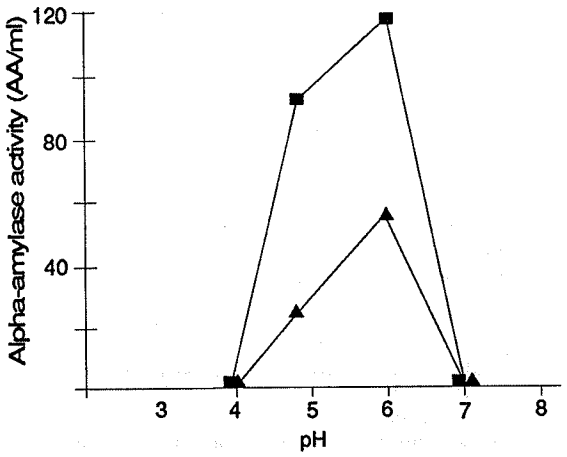

FIGURE 5 – Effect of temperature on the action of alpha-amylase produced by semisolid fermentation. (▲) *S. alluvius* UCD 54-83, and (■) *C. fennica* FTPT 8903

RESUMO

Produção de amilases por Candida em fermentação líquida e semi-sólida

A produção qualitativa e quantitativa de amilases por *Candida famata* FTPT 1539, *Candida fennica* FTPT 1829 e *C. fennica* FTPT 8903 foi avaliada e comparada à linhagem de *Schawanniomyces alluvius* UCD 54-83. Como meios de produção foram usados os meios líquidos YES e YNB, e o semi-sólido de farelo de trigo. O meio semi-sólido mostrou ser o melhor para a produção de alfa-amilase e glicoamilase. A determinação do amido residual e o aumento de açúcares redutores sugere que a *C. fennica* FTPT 8903 prooduz alfa-amilase e glicoamilase.

Palavras-chave: alfa-amilase; leveduras amilolíticas; Candida; glicoamilase; fermentação semi-sólida; fermentação líquida.

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ANTAGONISM BETWEEN TOXIGENIC FUNGI AND A STRAIN OF STREPTOMYCES SP.

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SUMMARY

We aimed at determining whether a *Streptomyces sp.* strain isolated from the soil, which is an antagonistic of the *Aspergillus parasiticus* NRRL 2999 (an aflatoxin producer) and of the *Fusarium tricinctum* NRRL 3299 (a trichothecene producer), had its inhibitory effect through lysis or antibiosis. Turbidimetric and gravimetric studies of inhibition in liquid medium, as well as studies in solid medium, were carried out by confronting the microorganisms on agar plates and slides.

To determine the presence or absence of antifungic substances the *Streptomyces sp* C/33-6 was cultured under different conditions in two production media (GM and BM). The filtrates of these media were confronted in plates with the toxigenic fungi and we aimed at characterizing the antibiotic produced, by means of U.V. spectrophotometry.

The results obtained in the previous tests demonstrated that the fungal growth inhibition was due antibiosis.

Key words: Toxigenic fungi – *Streptomyces*-antagonism.

INTRODUCTION

Mycotoxins, toxic substances produced by certain fungi, are food contaminants, therefore, very dangerous for human and animal health (6).

Aflatoxins constitute a group of secondary metabolites highly toxic and carcinogenic. They are produced by *Aspergillus flavus* and *Aspergillus parasiticus,* which may infect seeds (11).

Fusarium produces a great number of sesquiterpen metabolites called trichothecenes as well as zearalenone, moniliphormin, butenolide and fusarines (9). These toxigenic *Fusarium* have been involved in human diseases such as "toxic food aleukia", esophagus cancer and some animal diseases such as hemorrhagic, estrogenic, hemetic syndrome (5).

These mycotoxins deteriorate food quality and, since their detoxification is almost impossible, contamination by the producer fungic strains is avoided whenever possible (3).

In the near future bio-control agents which will interact with pathogens through antibiosis, competition or exploitation mechanisms will be used in agriculture (8). Exploitation implies the utilization of cells of an organism by another organism which may or may not lyse the exploited cell. Competition may be defined as the rivalry between two organisms for a factor of limited occurrence in the environment, such as space, nutrients,

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etc. (4). Antibiosis is the interaction between organisms where a metabolic agent produced by one of them has a dangerous effect on the other (8).

Among antagonistic microorganisms present in soils where cereals are grown there are many varieties of *Streptomyces* capable of producing inhibitory substances which could affect the viability and dissemination of the toxicogenic fungi as well as their capability to produce mycotoxins (3).

In previous studies, where a *Streptomyces sp.* isolated from the soil, was made to grow together with *Aspergillus parasiticus* NRRL 2999 and with *Fusarium tricinctum* NRRL 3299, the antagonistic power of the *Streptomyces* on the germination and growth of the said fungi was verified "in vitro" (2, 3). Since in those studies it was confirmed that the *Streptomyces* did not exert its control by competition mechanisms, the objective of the present study was to determine whether the inhibitory effect is due to lysis or to antibiosis.

MATERIALS AND METHODS

Microorganisms employed – We used strains of *A. parasiticus* NRRL 2999 (aflatoxin producer) and *F. tricinctum* NRRL 3299 (trichothecene producer), both supplied by the National Regional Research Laboratory (USA), and a *Streptomyces sp.* strain C/33-6, isolated from samples of soil from the Estabelecimiento Experimental Oliveros del Instituto Nacional de Tecnología Agropecuaria (I.N.T.A.), which has a strong antagonistic effect on different fungal strains.

The microorganisms employed were maintained in a Potato Dextrose Agar medium (P.D.A.).

Inhibition in a liquid medium produced by the strain C/33-6

a) Turbidimetric estimation – The antagonistic *Streptomyces sp* was inoculated in a liquid medium (Gregory medium) containing soya flour 20g, dextrose 20g, CaCO3 5g and corn steep liquor 100ml, in 900ml distilled water and incubated in shaker at 28°C for 7 days (10). Then the culture was filtered through a Millipore filter (0.22 um).

Suspensions containing 5,6 x 10^6 *A. parasiticus* conidia/ml and 5,1 x 10^6 *F. tricinctum* conidia/ml were prepared. We filled two hemolysis tubes, each one with 4ml of one of the two suspensions, and 1ml of the culture filtrate of the *Streptomyces* C/33-6 was added to both tubes. Dead cells as well as living cells of the fungi under study were used. The dead cells were killed in a boiling bath for 30 minutes.

The optical density of the suspensions was determined by a photocolorimeter at 450nm with distilled water as reference. Readings were made after incubation at 25°C for 4, 24, 48, 72 hours and a week after the beginning of the experiment.

b) Gravimetric estimation – We prepared a *A. parasiticus* NRRL 2999 conidia suspension in sterile distilled water containing 1,95 x 10^7 conidia/ml and *F. tricinctum* NRRL 3299 conidia suspension containing 9,8 x 10^7 conidia/ml and a suspension of the *Streptomyces sp* C/33-6 containing 3,6 x 10^6 cells/ml.

Aliquots of these suspensions corresponding to 10^7 *Aspergillus* conidia, 10^7 *Fusarium* conidia and 10^6 cells of the *Streptomyces sp* C/33-6 were used to inoculate 250ml erlenmeyers containing 50ml of Sabouraud broth, according to the scheme presented in Scheme 1.

Inhibition in solid medium produced by the strain C/33-6

a) Agar Plates – Two series of experiments were carried out: one with living cells of the said fungi, and the other with dead cells. A suspension containing 2 x 10^4 cells/ml of water was mixed with the Potato Dextrose Agar medium in a ratio 1:5, and placed on Petri plates.

The *Streptomyces sp* C/33-6 was re-suspended in distilled water and streaked on the surface of the culture medium with a sterile swab. The plates were incubated at 28°C for seven days (1).

b) Slide Cultures – *A. parasiticus* NRRL 2999 and *F. tricinctum* NRRL 3299 were sown in Sabouraud medium and incubated at 28°C for two days until the formation of the mycelium was visible. At that moment two suspensions of the micelium of each fungus were prepared in distilled water, reserving one to be used as a live mycelium, and the other to obtain a dead mycelium through heating.

At the same time the *Streptomyces sp* C/33-6 was inoculated in tubes containing Sabouraud and was incubated at 28°C for a week. Later a suspension of the antagonistic was prepared in distilled water. This suspension was added to the Sabouraud medium in a 1:6 ratio, and eight cultures were prepared on slides. After an incubation at 28°C for one day, either live or dead *A. parasiticus* or *F. tricinctum* mycelia were placed on the surface of the slides. Two slides were used in each case.

SCHEME 1 – Gravimetric estimation of inhibiton in liquid medium produced by the *streptomyces* sp. C/33-6 (employed methodology).

Culture Nº											
1	2	3	4	5	6	7	8	9	10	11	12
Sowing I											
Asp	Asp	Fus	Fus	---	---	---	---	---	Str	Str	Str
Culture Nº											
1	2	3	4	5	6	7	8	9	10	11	12
Sowing II											
Str	---	Str	---	Str + Asp	Str + Fus	Str	Asp	Fus	---	Asp	Fus
Incubation II (28°C – 1 week)											
Filtration											
Mycelium dry weight determination (110°C)											

Asp: *Aspergillus parasiticus NRRL 2999*
Fus: *Fusarium tricinctum NRRL 3299*
Str: Streptomyces sp. C/33-6

All the slides were placed in a wet chamber and incubated at 28°C for three days. Then, they were colored with Lactophenol Cotton Blue (LCB) and observed microscopically (7).

Inhibition on plate produced by culture filtrates of the strain C/33-6

a) Preparation of the filtrates – Volumes of 0,5ml of the aqueous suspension of the strain C/33-6 were dispensed in four 125ml erlenmeyers: two of them contained 50ml of Gregory medium (GM)(10) and other two, 50ml of Ball medium (BM) (12). One flask of each medium was mantained in rest state (R) at 28°C and the remaining cultures, in shaker (S) at 30°C for a week.

The cultures were filtered and the filtrate was then divided in fractions (A and B) of 20ml each. Fractions A were extracted with two 10ml n-butanol parts. Fractions B were reserved for the testing of the antifungic power of the filtrates.

b) Test for the inhibitory power of the filtrates – Two Agar Potate Dextrose plates were sown on the surface: one with 0,2ml of the *A. parasiticus* conidia suspension, and the other with 0,2ml of the *F. tricinctum* conidia suspension used in 2.b).

The inibitory power of obtained filtrates was tested placing 0,2ml of each of the four B fractions in the holes made in each of the two plates previously prepared. After an incubation of 72 hours at 28°C the diameters of the inhibition zones formed were measured.

c) *Characterization of the inhibitory substance* – The U.V. absorption spectrum (350-200nm) of the eight fractions obtained from the filtrates was determined in a VARIAN spectrophotometer, series 634. For this purpose, 1:50 dilutions of the fractions previously mentioned were prepared and compared with standards of cicloheximide (5mg/ml of water) and amphotericin B (5mg/ml of butanol).

RESULTS

The results corresponding to the determination of the optical density of the turbidimetric estimation of inhibition are presented in Table 1.

The results obtained through the gravimetric estimation of inhibition are shown in Table 2.

In the inhibition tests on agar plates, zones with no development of the fungi sown around the growth line of the *Streptomyces sp.* C33-6 were observed. However, the conidia present in the inhibition zones showed, when observed microscopically, absence of lysis but lack of germination as well.

The microscopic observation of the cultures in slides demonstrated that there was no growth in the slides where the live mycelia of the *A. parasiti-*

TABLE 1 – Turbidimetric estimation of *A. parasiticus* NRRL 2999 and *F. tricinctum* NRRL 3299inhibition by *streptomyces* sp. C/33-6.

Time (hours)	Optical Density (450 nm) Living Fusarium Cells	Dead Fusarium Cells	Linving Aspergillus Cells	Dead Fusarium Cells
0	0,255	0,250	0,470	0,480
4	0,275	0,260	0,490	0,500
24	0,260	0,270	0,510	0,505
48	0,260	0,255	0,500	0,490
72	0,270	0,265	0,515	0,510
168	0,265	0,255	0,495	0,500

TABLE 2 – Gravimetric estimation of the inhibition produced by the *Streptomyces* sp. C/33-6.

Culture Nº	DRY Weight (g)
1	0,179
2	0,262
3	0,191
4	0,224
5	0,023
6	0,037
7	0,026
8	0,156
9	0,106
10	0,050
11	0,063
12	0,058

TABLE 3 – Inhibition produced the filtrates of cultures of the *Streptomyces* sp. C/33-6 on PDA plates with toxigenic fungi.

Tested Fungus	Filtrate Cº	Diameter (mm)
A. parasiticus	B M (R)	9
	B M (S)	12
	G M (R)	17
	G M (S)	23
F. tricinctum	B M (R)	14
	B M (S)	18
	G M (R)	27
	G M (S)	35

B M: Ball Medium G M: Gregory Medium
(R): Resting culture (S): Shaking culture

cus and of the *F. tricinctum* were placed, even though the mycelia remained intact in all the situations studied.

The diameters corresponding to the inhibition zones obtained in test 4. b) are presented in Table 3.

TABLE 4 – Absorbance peaks presented by the filtrates of the *Streptomyces* sp. C/33-6 cultures and by the standards of antifungic substances

Filtrate OF	Wave Length (nm)
B M (R)	225-265
B M (S)	225-265
G M (R)	210
G M (S)	210
cicloheximide	230
amphotericin B	228

B M: Ball Medium G M: Gregory Medium
(R): Resting culture (S): Shaking culture

In the U.V. spectrum, fractions B of the filtrates of the culture media showed the absorbance peaks presented in Table 4. Fractions A presented absorption peaks in the same wave lengths as fractions B, but with less absorbance (which would indicate less concentration of the antifungic substance).

DISCUSSION

In the gravimetric estimation of the inhibition, when we compared the result obtained in culture nº 1 with that obtained in culture nº 2, and the result obtained in culture nº 3 with that obtained in culture nº 4, we may conclude that the addition of the *Streptomyces sp* C/33-6 inhibited the growth of the toxigenic fungi. It was a partial inhibition because the weights obtained in 1 and 3 were slightly higher than those found in 8 and 9 (corresponding to the development of a week's incubation). This would indicate that the effectiveness of the antagonistic effect of *Streptomyces sp* C/33-6 depends on the moment of its inoculation, which coincides with the results previously obtained. (2,3).

Instead, when the inoculation of the *Streptomyces sp* C/33-6 was simultaneous with that of the toxicogenic fungus, inhibition was stronger (practically no development of *A. parasiticus* was obtained in case 5, and practically no development of *F. tricinctum* was obtained in case 6).

In the cases in which the *Streptomyces* was sown before the toxicogenic fungi, inhibition was complete, which was also proved in previous works (2,3).

The results obtained through the turbidimetric estimation and in the inhibition tests on agar plates as well as in slides demonstrated that, even though

there was an antagonistic effect of the *Streptomyces sp* C/33-6 on the tested fungi, it was not due to lytic action.

The inhibition zones found in the plates where the filtrates were tested shown the presence of antifungic substances, more powerful against *F. tricinctum* than against *A. parasiticus*.

Besides, shaking stimulated the production of such substances, and the Gregory medium proved to be more suitable for the obtainment of the antifungic substance (which coincided with greater developoment in this medium).

The U.V. absorption spectra would indicate the existence of antifungic substances with absorption peaks close to the standards used, where the production of such substances would depend on the culture medium used.

Analysing all that has been previously stated, we may conclude that the *Streptomyces sp.* C/33-6 strain produced antibiotic substances which, when excreted to the medium were able to control biologically the toxigenic fungi, thus preventing contamination by mycotoxins.

RESUMO

Antagonismo entre fungos toxigênicos e uma raça de Streptomyces sp.

Foi objetivo deste trabalho determinar se um cepa de *Streptomyces sp.*, isolada do solo, antagonista de *Aspergillus parasiticus* NRRL 2999 (produtor de aflatoxinas) e de *Fusarium tricinctum* NRRL 3299 (produtor de trichotecenos), exercia o seu efeito inibidor por um mecanismo de lise ou de antibiose. Para isso foram efetuadas estimações turbidimétricas e gravimétricas de inibição em meio líquido, e estudos em um meio sólido defrontando os microorganismos em placas e lâminas de agar.

Além disso, para determinar a presença ou a ausência de substâncias antifúngicas, *Streptomyces sp* C/33-6 foi cultivado, em diferentes condições, em dois meios líquidos (MG e MB). Os filtrados de tais meios foram estudados frente aos fungos toxicogênicos em placas, e tentou-se caracterizar, por espectrofotometria de U.V., o antibiótico produzido.

Os resultados obtidos demonstraram que a inibição observada foi produzida por um mecanismo de antibiose.

Palavras chave: Fungos toxigênicos, *Streptomyces*, antagonismo.

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SCREENING, THERMAL PROPERTIES AND PRODUCTION IN YAM EXTRACT OF FUNGAL SUCROSE PHOSPHORYLASE

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SUMMARY

Screening of sucrose phosphorylase activity was performed in 16 fungi strains. Four strains were selected as the best enzyme producers.

Scytalidium sp. and *Colletotrichum gloesporioides* both displayed sucrose phosphorylase activity mainly intracellular while in *Sclerotinia sclerotiorum* and *Monilia sitophila,* the bulk of enzyme activity was released into the culture medium.

High optimal temperature values, 50°C and 40°C, were found respectively for the enzyme from *M. sitophila* and for the other three strains selected. The sucrose phosphorylase from *M. sitophila* was the most thermostable, retaining 90-95% of its initial phosphorolytic activity after incubation in the absence of substrate at 50°C-70°C for 30 minutes. This enzyme was selected for further studies.

The highest enzyme production (340 IU/L of culture or 0.336 IU/mg protein) occurred at the stationary growth phase, when *M. sitophila* was cultured at room temperature (28°C-30°C) with shaking, in sterile yam extract supplemented with 0.15% ammonium sulphate.

Preliminary studies of immobilization of the crude enzyme extract by adsorption on DEAE-cellulose or by covalent linkage with chitosan were performed. The best result was obtained with chitosan immobilized derivative, which retained 12% proteins and 217% of the enzyme initial specific activity.

Key words: Fungal sucrose phosphorylase, Yam extract, sucrose phosphorolysis, microbial enzyme, thermostable sucrose phosphorylase.

INTRODUCTION

Sucrose phosphorylase (disaccharide glycosyl transferase, EC 2. 4. 1. 7) catalyses the following reversible reaction:

sucrose + Pi $\rightleftharpoons$ fructose + glucose-1-phosphate

In recent review was analysed mainly from its economical feasibility (18) the potencial use of sucrose phosphorylase for sucrose synthesis starting from starch, using amyloglycosidase, phosphorylase and glucose isomerase as auxiliary enzymes (12), for fructose and glucose-1-phosphate production from sucrose (6) and in enzymatic electrode for sucrose determination (2). Considering the low specificity of sucrose phosphorylase towards the glycosyl acceptor, the enzyme also has been proposed for the synthesis of new disaccharides (21).

Sucrose phosphorylase has been found in cells of several strains of *Leuconostoc mesente-*

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roides (6, 13, 21, 12), *Pseudomonas saccharophila, P. putrefaciens* (6, 21), *Clostridium pasteurianum* (16), *Pullularia pullulans* (11) and *Acetobacter xylinum* (1). The growth of *L. mesenteroies* in a medium based in industrial subproducts (corn steep liquor and molasses) decreased nearly to a half the enzyme yield obtained in a defined medium (19).

The aim of this work was to select new microbial source capable to grow in a cheap medium such as yam or potato extract and to produce a high yield of thermostable sucrose phosphorylase.

MATERIAL AND METHODS

Screening – The following fungal strains were screened for sucrose phosphorylase activity: *Aspergillus niger* (019 — IPA-PE), *Kluyveromyces marxianus* (225 CBS-Holanda), were cultured in medium "a". *Sclerotinia sclerotiorum* (UFRPE-Lab. Fitossanidade), *Curvularia eragrotides* (2376 URM), *Curvularia lunata* (718 URM), *Monilia sitophila* (336 URM), *Colletotrichum gloesporioides* (2494 URM), *Penicilium sclerotiorum* (313 URM), *Sclerotium cafeiculum* (UFRPE-Lab. Fitossanidade), *Cercospora manihobae* (106-IPA-PE), were cultured in medium "b". *Macrophaminea phasiolina* (2667 URM), *Fusarium oxysporum* (2378 URM), *Scytalidium sp* (UFRPE-Lab. Fitossanidade), *Verticillum albo-atrum* (1906 URM), *Sclerotium rolfsii* (UFRPE-Lab. Fitossanidade), were cultured in both media. The fungi were kept on potato-dextrose slants at 4°C. Inoculum were initially prepared with a loopfull of the strain in 1 ml of liquid medium. After 24h growth at 28°-30°C with shaking, the inoculum (10% v/v) were respectively transfered to 25 ml Erlenmeyer flasks containing liquid medium (10 ml working volume). For the growth curve assay the growth was scalled up to 1 L.

Medium a: Potato extract (3-5% of starch/ml) + 2% (w/v) glucose

Medium b: Yam extract (15-20% of starch/ml) + 2% (w/v) glucose

Enzyme activity localization – After 48 hours growth, the cells were separated by filtration from the supernatant broth and both were used separately for assay of sucrose phosphorylase phosphorolytic activity.

Assay condition – The reaction mixture contained 100mM sucrose in 50mM sodium phosphate buffer pH 7.0 in a final volume of 1 ml, when the enzyme source was the supernatant broth or purified extract (0.5ml). A final volume of 5 ml of the reaction mixture was used for whole cells or immobilized enzyme (0.5g). Incubation was performed in a shaking bath at 40°C. Samples were withdrawn at time intervals, for determination of inorganic phosphate consumed, by the Fiske & Subbarow method (9). One unit of sucrose phosphorylase activity was defined as the amount of enzyme that catalyses the utilization of 1μmol of inorganic phosphate per min under the assay conditions.

The intracellular enzyme (from *Scytalidium sp* and *C. gloesporioides*) was extracted after the disruption of the cells by grinding with quartz sand (80 mesh) at the proportion of 1g cell/4g sand, followed by the addition of 20mN Tris-HCl buffer pH 7.0 (5ml/g of broken cells) and centrifugation at 7000xg.

The crude extracts as well the broth supernatant (from *M. sitophila* and *S. sclerotiorum)* were partially purified after nucleic acid precipitation with 50mM (final concentration) MnCL2 solution, follwed by enzyme precipitation with solid ammonium sulphate (30-100% saturation). The partially purified enzyme was suspended in 20mM Tris-HCl buffer pH 7.0 at 4°C and used for further experiments.

Determination of optimal temperature and thermal stability – Partially purified extracts prepared from supernatant broths of *M. sitophila, S. sclerotiorum* and from disrupted cells of *S. gloesporioides* and *Scytalidium sp* were used as enzyme source. The optimal temperature for the enzymes was determined under the assay conditions at the temperature range of 20°C-70°C.

The thermal stability was determined by preincubating the enzyme for 30 min in absence of its substrates, at temperatures between 20°-70°C. After rapid cooling, the residual phosphorolytic activity was tested under the assay condition. Effect of supplementation of yam extract for improving the production of sucrose phosphorylase by *M. sitophila* yam extract (1L) containing 1.5-2g/L of starch was used for *M. sitophila* growth after supplementation with 2% (w/v) glucose + 0.15-1.0% (w/v) ammonium sulphate; 0.15% (w/v) ammonium sulphate or 0.15% (w/v) yeast extract, both without glucose addition. Growth was carried out at room temperature (28°-30°C), with shaking, for 48h. At time intervals samples were withdrawn for determination of pH, starch, protein and sucrose phosphorylase activity.

Starch content was determined by iodine staining power (blue value). Blue values produced by the addition of 0.01% (w/v) iodine-potassium iodide solution (2ml) to starch samples (2ml), were determined at 610nm after dilution to 15ml with distilled water.

Proteins were determined by spectrophotometric method of Warburg & Cristian(20).

Immobilization – The supernatant from *M. sitophila* growth, was concentrated ten times under reduced pressure at 50°C, dyalized (with two changes) against 20mM Tris-HCl buffer pH 7.0 and used for immobilization by adsorption on DERE-cellullose and covalent linkage on chitosan.

Adsorption on DEAE-cellullose:

5ml of enzyme solution (2.57 mg total proteins) were mixtured with 0.5g of DEAE-cellulose 303C(Nutritional Biochemical Corporation) pretreated with 0.5M HCl and 0.5M NaOH. The mixture was kept under gentle stirring at 4°C during one hour, followed by filtration and washings with deionized water at 4°C. The absorbed enzyme, after activity determination, was stored in suspension in deionized water at 4°C, until use.

Immobilization by covalent linkage on chitosan:

Two samples of 5ml of enzyme solution (8.1mg total proteins) were respectively mixtured with chitosan (0.35g) prepared according to Darmon & Rudall(4) and activated with 2.5% (v/v) glutaraldehyde. The second sample was added of 5ml of substrate solution (100mM sucrose prepared in 50mM sodium phosphate buffer pH 7.0) before the coupling with chitosan-glutaraldehyde. The mixtures were kept under gentle stirring at 4°C during 2 and 48 hours. Next, the immobilized derivatives were washed according to Crook et al (3) and used for activity assay. The imobilized derivatives were stored in suspension in 20mM Tris-HCL buffer pH 7.0 at 4°C, until use.

Immobilized proteins were estimated by the method of Warburg & Christian(20) from the diference between the amount of proteins added and recovered after immobilization and washings.

RESULTS AND DISCUSSION

C. gloesporioides, Scytalidium sp, S. sclerotiorum and *M. sitophila* were selected among sixteen fungi grown in liquid medium, potato-glucose or yam-glucose, without the presence of inducers, for production of sucrose phosphorylase activity.

In *C. gloesporioides* and *Scytalidium sp*, the phosphorylase activity (0.29 and 0.15 IU.mg-1 proteins respectively) was mainly intracellular, while *S. sclerotiorum* and M. sitophila released the bulk of sucrose phosphorylase activity (0.085 and 0.13 IU.mg-1 proteins respectively) into the culture medium (Table 1).

TABLE 1 – Sucrose phosphorilase activity production by the fungi selected as compared with the production by *Leuconostoc mesenteroides*.

Fungi	Medium	Sucrose phosphorilase activity ($IU.ml^{-1}$)		Specific activity ($IU.mg^{-1}$ proteins)	Activity yield (%)
		Intracellular	Extracellular		
Colletotrichum gloesporiodes	Yam extract	0.275	ND	0.290	138
Monilia sitophila	Yam extract	ND	0.082	0.130	62
Sclerotinea escerotiorum	Yam extract	ND	0.115	0.085	40
Scytalidium sp	Potato extract	0.128	ND	0.150	70
Leuconostoc mesenteroides	Defined medium Doudoroff (6)	0.385	ND	0.210	100

ND = not delected

Intracellular activity = was determined at the assay conditions using as enzyme source the crude extract prepared after disruption of the cells and enzyme extraction with 20 mM Tris-HCL buffer pH 7.0.

Extracellular activity = was determined at the assay conditions using as enzyme source the supernatant broth.

Compared with *L. mesenteroides*, grown according to Doudoroff(6) in the presence of inducers (sucrose and phosphate), vitamins and salts, the activity vield of the four strains selected, was in the range of 40-138%. The activity yield from the other fungi tested was lower than 30%.

Being sucrose phosphorylase an enzyme with potential biotechnological application, optimal temperature and thermal stability are important parameters. Figure 1 shows the optimal temperature and thermal stability of sucrose phosphorylase from the sources selected. The highest optimal temperature (50°C) was determined for the enzyme from *M. sitophila* while for the enzyme from *Scytalidium sp, C. gloesporioides* and *S. sclerotiorum*, the optimal temperature value was 40°C. Until now, 40°C was the highest temperature for sucrose phosphorylase activity and has been found for the enzyme produced by *P. saccharophila* and *Clostridium pasterianum* (6, 8, 16). The optimal temperature reported for the enzyme from *L. mesenteroides* was 30°C (6, 13) and 35°-38°C (2,18).

The sucrose phosphorylase activity produced by the four strains selected, *C. gloesporioides, Scytalidium sp, S. sclerotiorum* and *M. sitophila*, was stable at its optimal temperature. After pre-treatment for 30 minutes at their respective optimal temperature (40°C or 50°C), the residual activity was in the range of 70-100%. The enzyme from *M. sitophila* was thermostable retaining 90-95% of activity even after pre-treatment at 70°C for 30 minutes (Figure 1).

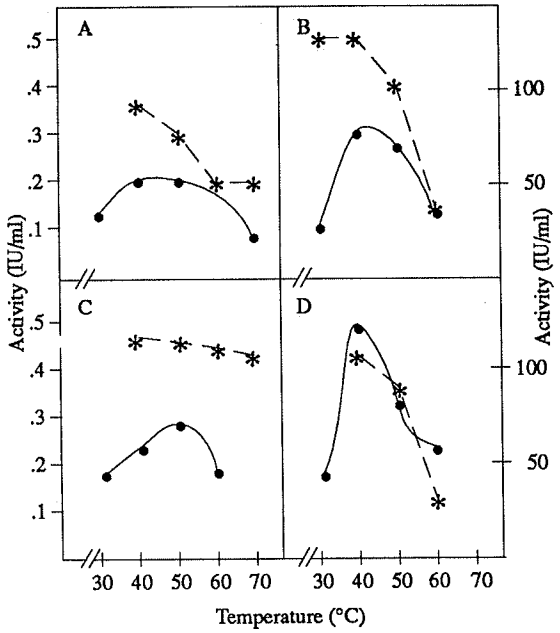

FIGURE 1 – Optimal temperature and thermal stability of sucrose phosphorila from fungal sources.
A - *Scytalidium sp.*; B - *Colletotrichum gloesporioides;*
C - *Monilia sitophila;* C - *Sclerotinia sclerotiorum;*
Optimal Temperatue (●—●); Thermal stability (✱—✱), after 30 min incubation in absence of substrate at pH 7.0 at the temperature indicated.

Few data are available about the thermal stability of sucrose phosphorylase. Has been reported that yhe enzyme from *L. mesenteroides* lost 70% of activity at 50°C for only four minutes, while the enzyme from *P. saccharophila* was denatured at temperature above 40°C (7). Because the enzyme high thermostability, *M. sitophila*, was selected for production of sucrose phosphorylase for further studies.

Table 2 shows the effect of media composition on the sucrose phosphorylase activity production by *M. sitophila*. There was about two folds increase of the enzyme specific activity yield, when growth proceeded in media made of yam extract (15-20% of starch, w/v), glucose (2% w/v) plus ammonium sulphate (0.15% w/v). Further increase of ammonium sulphate concentration (0.5 w/v). Further increase of ammonium sulphate concentration (0.5-1.0% w/v), reduced the enzyme production. The highest enzyme specific activity yield (0.33 IU.mg-1 proteins) was achieved with glu-

TABLE 2 – Effect of medium compositium on sucrose phos phorylase specific activity prodution by *Monilia sitophila*

Medium	Parameters at the enzyme maximal activity production			
	Time growth (h)	pH	Proteins ($mg.ml^{-1}$)	Specific activity ($IU.mg^{-1}$ proteins)
YE + 2%(w/v) glucose	36	4.0	4.62	0.14
YE + 2% (w/v) glucose + 0.15% (w/v) $(NH_4)2SO_4$	30	4.0	1.53	0.21
YE + 2% (w/v) glucose + 0.5% (w/v) $(NH_4)2SO_4$	24	4.0	2.38	0.16
YE + 2% (w/v) glucose + 1.0% (w/v) (NH_4) SO_4	30	4.0	2.76	0.16
YE	30	5.5	1.43	0.17
YE + 0.15% (w/v) $(NH_4)2SO_4$	30	4.5	1.01	0.33
YE + 0.15% (w/v) yeast extract	30	5.0	1.61	0.18

YE = yam extract (15 - 20% starch, w/v)

cose withdrawn in a medium made of yam extract plus ammonium sulphate (0.15% w/v), confirming the glucose repression on the production of microbial sucrose phosphorylase(21). The replacement of ammonium sulphate by yeast extract at the same concentration, repressed in nearly 50% the enzyme production. No inhibition has been reported with yeast extract. In fact, yeast extract together with triptone and vitamins mineral solution, was found necessary for *L. mesenteroides* growth (17).

The growth curve (Figure 2) of *M. sitophila* in yam extract plus ammonium sulphate (0.15% w/v) shows that the maximal specific activity production of 0.33 IU.mg-1 proteins (which correspond to 340 IU.L-1 media) was achieved with 30 hours growth, at the initial stationary phase. At this stage, almost all the substrate (assayed as starch) had been exausted and the media initial pH had changed from 5.0 to 4.5 reaching 4.0 at the end of fermentation, with decrease of approximately 21% of the specific activity yield.

Reports of sucrose phosphorylase production by *L. mesenteroides* showed that the activity yield was highest at pH 7.6 and 8.2 and decreased five to ten folds at pH 4.9 and 5.8 respectively (19). Moreover, in absence of pH control during of fermentation, the of maximum activity achieved with

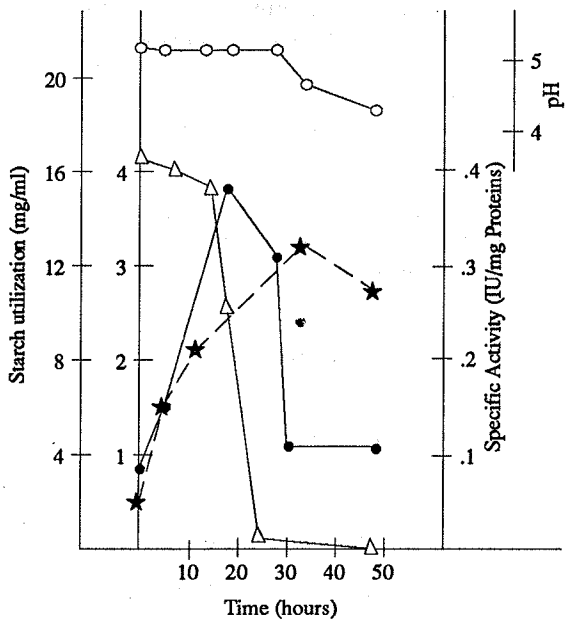

FIGURE 2 – Growth curve Monilia sitophila versus production of sucrose phosphylase activity.
The medium was made of yam extract (15-20% starch, w/v) plus ammonium sulphate (0.15%, w/v). Starch consumption (Δ—Δ); Protein (•—•); Specific activity (★- - -★); pH (o—o).

24h growth (12), at the begining of stationary phase (19) or end of log phase (17) decreased quickly. Maximal enzyme yield has been reported in *P. saccharophila* and *P. putrefaciens* at the early stationary phase (21), late log phase (14) or after 48h growth (15).

The maximal specific activity yield achieved with *M. sitophila* grown in a cheap medium compares well with the best yield (750-250 IU.L-1 medium, as calculated from author's data) reported for *L. mesenteroides* ATCC 12291, with 6h growth in defined media containing salts, vitamins and enzyme inducers. This media has been devised for rapid growth with high enzyme level production (17). When the growth of *L. mesenteroides* was carried out in a complex and cheap media based on corn steep liquor and molasses, added of vitamins-mineral solution, the maximum enzyme production was about half of that achieved in the defined media under optimized conditions of pH (7.3) and temperature (32°C) (19).

Because the surcrose phosphorylase produced by *M. sitophila* is an extracellular enzyme, its immobilization is necessary for the enzyme practical utilization.

Table 3 shows the preliminary results of immobliization of sucrose phosphorylase by adsorption on DEAE-cellulose and by covalent linkage to chitosan activated with glutaraldehyde (2.5% v/v). The specific activity retention, 65% for DEAE-cellulose enzyme and 97% for chitosan enzyme, was higher than the activity retention (18%, 4.6%-22% and 40%) reported respectively for the enzyme from *L. mesenteroides*, immobilized by absorption on DEAE-cellulose (13), by covalent linkage with aminopropylsilanylated glass beads, sepharose cyanogen bromide, porous ceramic beads (5, 15) and by entrappment in cellulose triacetate fibers (10). When the immobilization by covalent linkage to chitosan was carried out in presence of the enzyme substrate (100mM sucrose prepared in 50mM sodium phosphate buffer pH 7.0), the specific activity retention increase to approximately 217%. The increase of coupling time from 2 to 48h did not affect significatively the activity retention.

After and initial loss of specific activity retained by the DEAE-cellulose enzyme, the remaining activity (33%) was stable to storage at 4°C for 138 days. This initial activity loss due to proteins desorption, also has been reported for the enzyme from *L. mesenteroides* adsorbed on DEAE-cellulose (13). The chitosan-enzyme retained 58% of its initial activity after 74 days of storage at 4°C.

The author's results shows that sucrose phos-

TABLE 3 – Immobilization of sucrose phosphorilase

Suport	Immobilization	Initial total activity	Activity (IU.-1 suport)	Specific activity retention (%)	Stability (residual activity) Storage at 4°C days	(%)
DERE-cellulose	adsorption	1.83	1.64	65.2	01 138	33 32
Chitosan/ glutaraldehyde (2h coupling)	covalent	1.74	0.57	97.1	–	–
Chitosan/ glutaraldelhyde + substrate (2h coupling)	covalent	0.82	1.31	216.7	74	58
Chitosan/ glutaraldehyde + substrate (48h coupling	covalent	0.82	0.58	218.9	48	100

phorylase from *M. sitophila* may be used in biotechnological process considering that this thermostable and extracellular enzyme is produced in a cheap medium and after immobilization either by adsorption on DEAE-cellulose or by covalent linkage to chitosan, gave derivatives with good activity retention and stability.

RESUMO

Seleção de fungos produtores de sacarose fosforilase e sua produção a partir do inhame

Dentre 16 cepas de fungos testados, foram selecionadas 4 cepas como melhores produtoras da atividade sacarose fosforitase.

Scytalidium sp e Colletotrichum gloesporioides apresentaram atividade sacarose fosforitase predominantemente intracelular, enquanto que em *Sclerotinia sclerotiorum* e *Monilia sitophila*, a atividade foi predominantemente extracellular.

Altos valores de temperatura ótimas 50°C e 40°C foram encontrados respectivamente para a enzima de *M. sitophila* e para as outras 3 cepas selecionadas. Como sacarose fosforilase de *M. sitophila* foi a mais termoestável, retendo 90-95% de sua atividade fosforolítica inicial após prétratamento na ausência de substrato, a 50°C-70°C durante 30 minutos, ela foi selecionada para estudos posteriores.

A maior produção da enzima (340 UI/L de meio cultivado ou 0,336 UI/mg de proteínas) foi obtida no início da fase estacionária de crescimento da *M. sitophila* cultivada em extrato de inhame com 0,15% de sulfato de amônio, à temperatura ambiente (28°-30°C) e agitação continua.

Foram efetuados estudos preliminares de imobilização do extrato bruto da sacarose fosforilase, por adsorção em DEAE-celulose e ligação covalente em quitosana. O melhor resultado foi obtido com o derivado imobilizado em quitosana o qual reteve 12 % de proteínas e 216% da atividade específica inicial.

Palavras-chave: Sacarose fosforilase de fungos, extrato de inhame, enzima microbiana, sacarose fosforilase termoestável.

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ANALISYS OF BOVINE NORMAL SERA SUBSTITUTES IN THE "CARD-TEST" REACTION TO ANAPLASMA MARGINALE

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SHORT-COMMUNICATION

SUMMARY

A survey was developed to analyze the possibility of using guinea pig serum as a complement source associated with a bovine serum negative to *Anaplasma marginale* in the "card-test" reaction. The substitution seemed to be valid and the results obtained were similar to those where fresh bovine serum was utilized.

Key words: *Anaplasma marginale*, "card-test", complement.

It has an been known that the complement's proteins and conglutinins are essential in the search of antibodies against *Anaplasma marginale* in the "card-test" reaction (1, 4). Despite this, complement lability is a limiting factor to carry out the test, since it needs fresh bovine normal serum (BNS) serologically negative to *A. marginale*, as a source of these proteins. Thereby, a substitute of BNS had to be found, because in endemic areas this serum is not easy to obtain and this makes anaplasmosis diagnostic difficult.

The alternatives used are listed as follows:

1) BNSL– Bovine normal serum lyophilized and stored at 4°C;

2) BNSS– Bovine normal serum stored at -20°C for seven days;

3) BNSD– Bovine normal serum submitted to dialysis and ressuspended into saline solution until the initial volume;

4) FC– Fresh complement from guinea pig, collected in the day of usage;

5) SC- Pool of guinea pig sera mantained at -20°C for aproximately fifteen days.

The methods used are described in prior work (2). Fifteen sera were analyzed, where seven sera were serologicaly positive to *A. marginale* and eight were negative.

Tests were accomplished in this manner:

a) Controls:

Positive serum	+	Antigen	+	BNS	=	Positive control
Negative serum	+	Antigen	+	BNS	=	Negative control
Negative serum	+	Antigen	=	Nonspecific agglutination control		
Positive serum	=	Antigen	=	Nonspecific agglutination control		

B) Assay

							Result
Positive serum	+	Antigen	+	BNSL			Positive(+)
Positive serum	+	Antigen	+	BNSS	+	FC	Positive(+)
Positive serum	+	Antigen	+	BNSS	+	SC	Positive(+)
Negative serum	+	Antigen	+	BNSS	+	FC	Negative(-)
Negative serum	+	Antigen	+	BNSS	+	SC	Negative(-)
Positive serum	+	Antigen	+	BNSD	+	FC	Positive(+)
Positive serum	+	Antigen	+	BNSD	+	SC	Positive(+)
Negative serum	+	Antigen	+	BNSD	+	FC	Negative(-)
Negative serum	+	Antigen	+	BNSD	+	SC	Negative(-)

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Despite of the small number of analyzed sera, the substitution of BNS by guinea pig's fresh serum or stored in freezer added by aged BNS (BNSS) or BNSD (dialyzed) as a conglutinin source was showed to be viable, since the results obtained were reproduced with the analyzed sera.

In the performed tests, nonspecific agglutination was not observed among the components when aged BNS (BNSS) was added as a conglutinin source and a complement source (3rd and 4th controls), since a negative result was expected, because there shouldn't be enough complement's proteins to developed the reaction.

These preliminary results make possible to substitute BNS collected in the day of usage by BNS stored at -20°C and negative to *A. marginale* added of guinea pig complement. This is valuable where the prevalence of serologically positive animals is high, because one may substitute the BNS by a pool of guinea pig sera.

The conglutinin source can be bovine serum negative to the hematozoan, and doesn't need to be collected in the day of usage.

The dialysis of BNS was employed to demonstrate that the conglutinin activity was in the euglobulinic fraction(3). This fact could be observed when BNSD was used as conglutinin source. BNSD remained stable at 4°C during 14 days. It's only necessary to add guinea pig serum as a complement source.

Thus, it also avoids the lyophilization, that's not always available at all laboratories or the mainteinance of negative animals to *A. marginale*. It isn't also necessary to hurry for the test because bovine serum has few and very labile complement. Conglutinin, however, is a very stable(3) and can be found in bovine sera stored at -20°C(2).

RESUMO

Substituto do soro bovino normal na reação de "card-test" para anaplasma marginale

Analisou-se a possibilidade de empregar soro de cobaio como fonte de complemento adicionado de um soro bovino sorologicamente negativo para *Anaplasma marginale* na reação de "card-test". A substituição mostrou-se válida e os resultados foram equivalentes aos obtidos com o emprego de soro normal bovino fresco.

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AGAR MEDIUM FOR HUMAN UREAPLASMA UREALYTICUM

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SHORT-COMMUNICATION

SUMMARY

The authors wish to report a simple medium for the isolation and cultivation of *Ureaplasma urealyticum* starting from clinical materials. This medium induced growth in eleven of the twelve different serotypes of *Ureaplasma urealyticum* studied.

Key words: *Ureaplasma urealyticum*, Ureaplasma culture medium.

There is a increasing interest in the isolation of *Ureaplasma urealyticum* from clinical materials. Many types of media have been described. Differences among them include type and sources of peptones, agars, types of yeast, antibiotics and supplements such as VX or CVA enrichment.

The U-9 urease color test fluid medium was described in 1970 (Shepard, M.C.; Lunceford, C.D. *Appl. Microbiol.*, **20:** 539-43, 1970) for the detection and identification of Ureaplasma in primary or secondary Mycoplasma cultures.

A modification of U-9 for the cultivation of ureaplasmas was tested in order to investigate if different serotypes grow in media without supplements. The basal medium is made up of tryptic soy agar (Difco) 26,3g; $MnSO_4.7H_2O$ (J.T. Baker) 0,15g; yeast extract (Difco) 2,5g; deionized water 820 ml (the ingredients are dissolved and the pH adjusted to 5.5 with 1N HCl) and phenol red (solution 0,4% - Merck) 5.0 ml. The sterile base medium, after cooling to 55°C, is enriched with the following sterile supplements: unheated normal horse serum 200ml; urea (10% solution-Difco) 10ml and penicillin G potassium (100.000 U/ml solution - Fontoura-Wyeth) 10ml. Mixing is done carefully and dispensed aseptically into 12 x 60mm petri plates. The final pH is 6.0-6.2.

The medium yields the growth strains of *U.urealyticum* My 10643 (serotype 1), My 9410 (serotype 2), My 11237 (serotype 3), My 9411 (serotype 5), My 11861 (serotype 6), My 8330 (serotype 7), ATCC 27618 (serotype 8), Vancouver type 9 (serotype 9), Taylor-Robinson (serotype 10), JsL-U24-Cx3 (serotype 12) and JsL-U26-Cx3 (serotype 14). The JsL-U38-Cx3 strain (serotype 13) was shown to be a more exacting ureaplasma and did not grow in this medium.

The colonies are a golden to brown color under the microscope, between 10 to 50 um in diameter, round, coarsely granular with rough edges. As the agar is supplemented with manganese sulfate, the brown pigment indicates strong urealytic activity and is specific for *Ureaplasma urealyticum.*

Serotype frequencies may vary depending on geographical areas and as serotypes 4 and 11 were

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not tested, further investigation is needed to indicate the above described medium to be used for the cultivation of *Ureaplasma urealyticum* in clinical laboratories.

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RESUMO

Meio sólido para isolamento de *ureaplasma urealyticum*

Um meio simples é apresentado para o isolamento e cultivo de *Ureaplasma urealyticum* a partir de material clínico. Este meio permitiu o crescimento de onze dos doze sorotipos de *U. urealyticum* testados.

Palavras-chave: *Ureaplasma urealyticum*, meio de cultura para *Ureaplasma.*

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