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VIROLOGY

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INHIBITION OF FUNGAL GROWTH BY EXTRACTS OF *VERNONIA SCORPIOIDES* (LAM.) PERS.

Márcia de Fátima Inácio Freire[1]*
Heber dos Santos Abreu[1]
Luiz Celso Hygino da Cruz[2]
Ronald Bastos Freire[2]

ABSTRACT

Extracts from stalks and leaves of *Vernonia scorpioides* (Lam.) Pers. were evaluated for their ability to restrict the development of *Penicillium citrinum*. Tests of relative agar-diffusion with different concentrations of active stalk extracts showed that 1.0mg of the compounds was sufficient to produce an inhibition halo of 40.0mm in diameter. Extract concentrations of 1.0mg, 3.0mg and 5.0mg per 100 µl of diluent generated inhibition halos of 40,0, 50.0 and 80.0mm in diameter, respectively. Relatively good results were also obtained with crude derivatives of aqueous green leafs, which gave inhibition halos of 10 - 15mm and 15 - 20mm in diameter for *Aspergillus alutaceus (ochraceus)* and *Penicillium citrinum*, respectively. When aliquots of medium from treated cultures were transferred to nutritive Czapeck-Dox medium without antibiotics or extracts, no fungal growth was detected for all the pre-treated samples. It is concluded that *V. scorpioides* stalk extracts exert a fungicide action on at least *Penicillium citrinum*. Although leaf extracts were less active than the obtained from stalks, in both cases it was possible to demonstrate antibiosis and inhibition of hyphal growth. Infrared spectroscopy of the extracts showed characteristic absorption between 1750cm^{-1} and 1770cm^{-1}, suggesting the presence of sesquiterpene lactones as main bioactive antifungal compounds.

Key words: sesquiterpene lactones, *Vernonia scorpioides*, Asteraceae, antifungal activity, *Aspergillus alutaceus (ochraceus)*, *Penicillium citrinum*, mycotoxin.

INTRODUCTION

Mycotoxins are secondary metabolites of low molecular weight that are harmful to many organisms and especially vertebrates, including man. Because the presence of mycotoxins in food and feeds is potentially hazardous, environmental exposure to such compounds in agriculture is a recognized problem to be solved. Mycotoxins are produced by pathogenic fungi responsible for important diseases that reduce crop yield and quality and which can also produce unacceptable levels of toxins in corn and wheat grains. Although it is difficult to remove mycotoxins from human and animal diets, it is nonetheless possible to reduce the risk of exposure through a rigorous control program for mycotoxins in foods and feeds (12, 15, 22). Neutralizing substances should not only

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provide healthier diets for animals but should also be safe, preventing any toxic carryover in animals for human consumption. The level of contamination could be reduced substantially by using environmentally safe naturally occurring neutralizants. The major advantages in using natural materials are that they have less impact on the environment (10, 12, 19) and that their production by plants for large scale isolation may be achieved by genetic manipulation. Tropical countries have the biggest stock of potentially bioactive plants in the world. In such regions, biodiversity assumes a very important role, not only from an ecological perspective but also as a true source of future medicines. Nowadays, approximately 50% of the Brazilian territory still has representatives of the original Atlantic rain forest (5, 7, 14). Among the numerous families that are part of such extensive plant arsenal, the Asteraceae (Compositae) family of plants produces characteristic compounds, the sesquiterpene lactones, with diverse reported biological activities (1, 2, 3, 6, 8, 9, 11, 13, 16, 18, 24). Among the various members of the Asteraceae with medicinal activity, the Vernonieae tribe are a taxon to which belongs the *Vernonia* genus (5). The potential antifungal activity of some species of this genus becomes a fascinating topic when related to the biodiversity of microorganisms, particularly when taking on a harmful role to animals and man in the environment.

The aim of the present study was to examine for the first time the effects of naturally occurring compounds from stalks and leaves of *Vernonia scorpioides* (Lam.) Pers. on the hyphal growth of *Penicillium citrinum* and *Aspergillus alutaceus (ochraceus)* isolates.

MATERIALS AND METHODS

Extracts from *Vernonia scorpioides*

A previously described methodology was used to obtain the extracts (2, 7, 9). In brief, stalks and leaves were collected at the UFRRJ campus during springtime (September, 1993), dried and ground to give standardized 40 to 80 mesh diameter particles. The extracts were obtained by sequential derivation in organic solvents of increasing polarity (hexane, chloroform and 70% ethanol, respectively) followed by addition of distilled water and heating at 60°C for 60 minutes. The plant preparations were concentrated, freeze-thawed and stored until use. The extracts from leaves (l) and stalks (s) were identified as 1 (aqueous), 2 (hexane), 3 (chloroform), 4 (ethanol) and 5 (final aqueous). The l and s extracts, were solubilized in dimethylsulphoxide (DMSO, Vetec,SP, Brazil), methanol, chloroform or hexane (P.A. reagents, Merk, Brazil) depending on their obtention, and added with an equal volume of phosphate buffered saline, (PBS, 8,00 g/l NaCl; 0,20 g/l KCl; 1,15 g/l Na_2HPO_4; 0,20g/l de NaH_2PO_4, pH 7.2). Each extract was spectrophotometrically characterized using an infrared apparatus (Perking Elmer-USA). The spectra were registered in KBr and in NaCl tablets.

Fungal cultures

Isolates of *Penicillium citrinum* (NRRL5907) and *Aspergillus alutaceus (ochraceus)* (NRRL410) were obtained from the Center of Mycology and Mycotoxicology - UFRRJ, Brazil.. Small blocks (approximately 1mm) of the original cultures were transferred to Erlenmeyer flasks containing liquid 0.2% Czapeck-Dox medium (Difco Laboratories, MI) and recultured to attain a constant concentration of active organisms. The cultures were maintained at 28°C for one week and then stored at 5°C, whereas subcultures were incubated for four days prior to their use in hyphal growth and antibiosis tests. The quantity of spora was standardized spectrophotometrically (540 nm) according to a previously described methodology (6). A pour-plate technique containing 48 hours growth and a homogeneous nug covering the bottom of the culture flask could be seen in the selected suspensions.

Antibiotic activity assays

In order to detect the antibiotic activity of the different *V. scorpioides* extracts, two three-dimensional diffusion methods were tested: the paper-disk agar-diffusion assay and the method of cylindrical holes placed on the surface of a Czapeck-Dox agar layer. In both cases, petri dishes were previously inoculated with fungal spores, as described elsewhere (17). The assay systems utilized a single-layer plate containing 10 ml of

inoculated agar medium. In the paper method, eight plates were analyzed, each containing four discs impregnated with different plant extracts. These discs were applied with extracts exponentially diluted in PBS at concentrations of 10°, 10^{-1}, 10^{-2} and 10^{-3}. Two alternate discs were used for the samples and the other two for controls. The solutions were applied to discs with the aid of a capillary pippet calibrated to deliver 0.08 ml. The cylindrical-hole method was performed with 1.0; 3.0 and 5.0mg per each 0.1 ml of diluent applied to the agar-hole (one hole per petri dish). After incubation for 72 hours, the inhibition zones were measured and standard dose-response curves were constructed by plotting the zone values in millimeters. All the results are obtained from four repetitions. The control system contained the diluent and (or) the extractor (water, hexane, chloroform or ethanol). All the results were compared with 1000 international units (IU) of Nistatin (Difco Laboratories, MII) as standard for antifungal activity.

Hyphal growth tests

The methodology used has been described elsewhere (12, 19). Solubilized samples of *V. scorpioides* extracts were incorporated into previously melted Czapeck-Dox-agar and maintained at 48°C. The test samples were added to the medium at a ratio of 2.5mg of extract per 5.0 ml of melted agar medium. Fungal colonies developed after 72 hours of incubation with respect to the control system. In the other assay, 200 ml Erlenmeyer flasks containing 20 ml of liquid Czapeck-Dox medium were inoculated with 1 ml of a fungal suspension (1:100) and added with 40mg per ml of each extract. After 48, 72, 96 or 120 hours of incubation, fungal growth was interrupted by addition of 0.01 µg of NaN3. The material was then filtered trough a Büchner device containing a previously weighted Whatman number 1 filter paper, dried and weighted again in order to determine hyphal development related to dose and exposure time for each plant extract. After 72 hours of exposure to the extracts, aliquots of the differently treated fungal cultures were transferred to Czapeck-Dox-agar devoid of inhibitors or antibiotics to determine the fungicidal activity. Four replicates were made for each extract concentration. The tubes were subcultured during at least 20 days.

RESULTS AND DISCUSSION

The modalities of microbial control based on plant products will be of great practical utility in protecting the environment against mycotoxins and fungi. In India, a governmental program developed for this purpose selected extracts from *Andrografhis peniculata* (Acantaceae) that had the ability to restrain the development of *Aspergillus flavus*, an aflatoxin producer (12). Using the same approach, the Canadian Center of Plant Research has undertaken intensive research work on special metabolites from Asteraceae to control different phytopathogenic fungi (19, 20, 21). The present study shows that, within the Brazilian diversity of natural compounds, *Vernonia scorpioides* may be an important source of such natural products. Fungal inhibition diameters for the fungal isolates are given in FIGURE 1. Although the effect varied among the compounds tested, *V. scorpioides* stalk and leaf extracts significantly reduced mycelial growth (at a 5% level by the

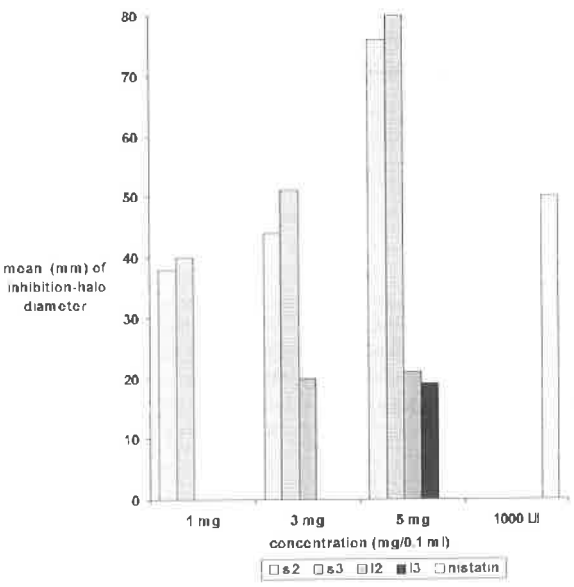

FIGURE 1. Effect of extracts from stalks and leaves of *Vernonia scorpioides* on spore development of *Penicillium citrinum*. Extracts that did not significativcly affect colony growth are not included. Data presented are significantly different from controls at 5% level (Tuckey's test). For further explanation see material and methods.

Tukey's test). The time at which significant inhibition was first observed is given in FIGURE 2. The most active extract was s3, which at a very low concentration (less than 10mg.ml^{-1}) inhibited growth of the *Penicillium citrinum* isolates. Hexane and chloroform stalk extracts (s2 and s3) were the most efficient, giving rise to inhibition halos of 41.0, 50.7 and 80.0mm in diameter at concentrations of 10, 30 and 50mg.ml^{-1}, respectively. The hexane extract of leaves, which generated a halo of approximately 20mm in diameter at concentrations of 30 and 50mg.ml-1, was also considered active when compared with 1000 IU Nistatin, whose halo did not exceed 50mm in size. Mycelial growth in the presence of stalk extracts was highly affected, since pre-treated fungi where unable to develop when subcultured in Czapeck-Dox medium without interfering substances (FIGURE 2).

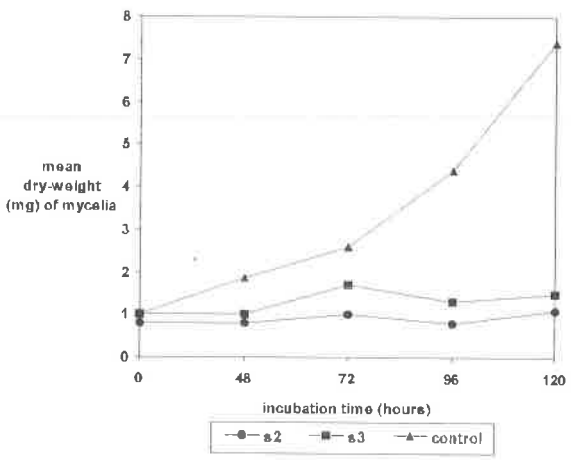

FIGURE 2. Effect of stalk-extracts of *Vernonia scorpioides* on mycelial growth of *Penicillium citrinum* and time of incubation when significant inhibition was first observed. Both, s2 and s3 were added at a final concentration of 2mg/ml. The rate of mycelial growth for each extract was compared with the control (diluent) using the Tuckey's test at a 5% confidence level. Further details are described in material and methods.

The highly polar extracts l4, l5, s4 and s5 showed substantial differences when compared to hexane or chloroform extracts, causing stimulation of growth instead of inhibition of the isolates assayed. The massive presence of glycosides in the ethanol extracts (l4 and s4) might explain this fact (6). In this study, the low concentrations of extracts s2 and s3 needed for significant inhibition are comparable to those recorded for l2 and l3. These extracts may contain similar compounds with properties that enable them to penetrate the hyphal cells and affect the same metabolic targets. The low aqueous solubility of these active extracts might play an important role in their membrane permeability and lead to microbial death. The infrared analysis of l2, l3, s2 and s3 indicates the possible presence of sesquiterpene lactones, which varied in concentration according to the intensity of action of each assayed extract. Thus, s3, the most potent in inhibiting hyphal growth when compared with all the other extracts examined, showed the highest absorption values, between 1750cm^{-1} and 1770cm^{-1}, that could correlate with the presence of sesquiterpene lactones as agents of its broad-spectrum antifungal activity (4, 14, 15, 23, 25). In addition, the susceptibility of the pathogens to *V. scorpioides* extracts appears to be quite consistent, since the growth responses of the isolates were comparable. Although the more virulent isolate *Aspergillus alutaceus* was less susceptible to these extracts than *Penicillium citrinum*, the antibiotic action of *s3* was much more pronounced than the obtained with Nistatin (FIGURE 1). Furthermore, a crude aqueous extract of grean leaves gave inhibition halos of 10 - 15 and 15 - 20mm in diameter for *Aspergillus alutaceus (ochraceus)* and *Penicillium citrinum*, respectively, confirming the potential antibiotic activity of *V. scorpioides*.

The present findings on antifungal activity indicate that *Vernonia scorpioides* extracts contain compounds such as sesquiterpene lactones which should be further tested and eventually used to develop an environmentally safe fungicide and mycotoxin-neutralizing agent. These studies are currently in progress in our laboratory.

ACKNOWLEDGMENTS

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RESUMO

Inibição do crescimento fúngico por extratos de *Vernonia scorpioides*(Lam.) Pers.

As propriedades antifúngicas de extratos obtidos a partir de folhas e caules de *Vernonia scorpioides* (Lam.) Pers., foram testadas para *Penicillium citrinum*. Os extratos foram testados pelo método de difusão em agar, nas concentrações de 1mg, 3mg e 5mg para cada 100 µl de diluente, em poços escavados no centro de placas de Petri. Extratos clorofórmicos e hexânicos obtidos a partir de caules foram extremamente eficientes, originando halos de inibição com 40,0, 50,0 e 80,0mm, respectivamente. O desenvolvimento de hifas, na presença desses extratos mostrou-se incipiente. Os extratos de folhas verdes também apresentaram efeitos semelhantes, embora de menor intensidade, uma vez que originaram halos de inibição entre 10 e 15mm de diâmetro para *Aspergillus alutaceus (ochraceus)* e entre 15 e 20mm de diâmetro para *Penicillium citrinum*. Concluiu-se que os extratos ativos de *V. scorpioides* exercem uma ação fungicida, uma vez que não se conseguiu, a partir dos tratamentos, realizar subcultivos em meio Czapeck-Dox isento de inibidores. Através dos espectros no infravermelho dos extratos ativos, observou-se a existência de sinais, característicos do estiramento de carbonilas lactônicas (com absorção entre 1750cm^{-1} e 1770cm^{-1}) ativas, indicando a presença provável de lactonas sesquiterpênicas, possíveis responsáveis pela atividade observada.

Palavras-chave: lactonas sesquiterpênicas, *Vernonia scorpioides*, Asteraceae, atividade antifúngica, *Aspergillus alutaceus (ochraceus)*, *Penicillium citrinum*, micotoxinas.

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CELLULOLYTIC FUNGI ISOLATED FROM PROCESSED OATS

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ABSTRACT

Fourty-eight filamentous fungi isolated from processed oats were analyzed regarding their cellulolytic capacity. All samples were positive for cellulase; *Aspergillus janus* var. *brevis* and *Penicillium bilaii* revealed the highest enzymatic levels. Species with a smaller colony diameter had higher cellulolytic activity, while those with a higher colony diameter presented a lower enzymatic level.

Key words: fungi, processed oat; cellulolytic fungi.

INTRODUCTION

Oat (*Avena sativa* L.), a cereal originated from Scotland, is now produced worldwide because of its great nutritive and dietetic value. It is used 'in natura" and as processed food in the form of oat flour or unrefined oat bran (5). It contains water, carbohydrates, fatty acids, fibers, ashes, proteins, growth and tissue renewing aminoacids, vitamins A, B_1, B_2, pantothenic acid and niacin, as well as phosphorus, calcium and small amounts of iron, copper, sodium, zinc, and manganese, a composition that makes it a quality food product (13). Fungi are among the microorganisms responsible for food contamination (2, 12). The diversity of enzymes produced by microorganisms, including fungi, have wide applicability. Among them, cellulase acts in fermentative processes for production of liquid fuel, for cellulose degradation, as an auxiliary of digestion, and is also used in other biotechnological processes (14).

The aim of this work was to analyze filamentous fungi isolated from processed oats and characterize the samples that produce cellulase.

MATERIALS AND METHODS

After identification, the fungi isolated from processed oats were inoculated onto potato dextrose agar and Czapek agar (8). Species of the genera *Aspergillus*, *Penicillium*, and other Hyphomycetes were incubated for 5 and 7 days at room temperature (28°C±2°C), while species of *Rhizopus* and *Syncephalastrum* were incubated for only 3 and 5 days due to their faster rate of growth. Inocula from all these cultures were transferred to Petri dishes containing 20ml of culture media for cellulolytic fungi (1,3). The Petri dishes were incubated at room temperature for 3, 5, or 7 days and subsequently submitted to thermal shock at 50° C for 16 hours. A staining technique was employed using Congo red, as follows: 10 ml of Congo red solution (0,025%) in 0,1M HCl Tris buffer (pH 8,0) were placed on each Petri dish tested; after 30 minutes, the solution was discarded and the cultures washed for 5 minutes with 5-6 ml of 0,5M NaCl in the same buffer. Colony size was then measured and the halos visualized. In order to evaluate the cellulolytic activity of each species, an enzymatic index was established by measuring colony diameter plus the halo formed and dividing the resulting value by colony diameter (6).

RESULTS AND DISCUSSION

On analyzing the cellulolytic activity of the cultures, the formation of a diaphanous halo around the colonies and a strong pigmentation of mycelia

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was observed, indicating production of cellulase by all samples. The enzymatic indexes were evaluated during time periods of either 3 and 5 days or 5 and 7 days. It was observed that species with smaller colony diameter revealed higher cellulolytic activity, while those with a larger colony diameter showed a lower enzymatic index.

Aspergillus janus var. *brevis* and *Penicillium bilaii* had the highest levels of cellulolytic activity on the basis of their enzymatic indexes. Other species such as *Penicillium sclerotiorum, P. islandicum, Trithirachium oryzae, Aspergillus sydowii, A. caesiellus, A. janus,* and *Cladosporium tenuissimum* showed regular levels of enzymatic activity while the other species tested presented medium and/or low enzymatic activity (TABLE 1). Lower levels of cellulolytic activity were also observed for the species *Rhizopus microsporus, R. stolonifer,* and *Syncephalastrum racemosum*, which were analyzed during the 3 and 5 days' period (TABLE 2).

Tischler *et al.* (16) reported that high levels of cellulase were produced by strains of *Penicillium janthinellum* used for ethanol production. These strains were grown on media with cellulose as the sole source of carbon and in the presence of wheat and rye grains as substrate for induction. The methods used to verify the cellulolytic activity of a microorganism depend upon its growth rate, which may cause fast digestion of cellulolytic substrates. Incubation of cultures grown at room temperature for 16 hours at 50° C promotes acceleration of extracellular cellulase activity and the rapid development of a clear zone around the colonies. The increase in temperature allows fast hydrolysis of cellulose and ensures the selection of species that produce thermostable cellulolytic enzymes. However, a temperature of 50° C may cause arrest of growth in some species, as is the case with *Trichoderma reesei* (9). The formation of a clear halo of hydrolysis results from the effect of the cellulolytic activity together with the increased inhibition of fungal growth. This is probably due to the liberation of cellulase by thermal autolysis of hyphae that are at the borders of the colonies. According to Neirotti and Azevedo (11), this effect is not observed with *T. reesei*, since the strains were still alive after the heating process.

The halo formed by the 48 taxa tested was probably seen as a function of higher enzyme activity in the medium when the fungus was submitted to a suitable temperature. Hagerdal *et al.* (7) observed that the enzyme found during the exponential growth phase of *Thermoactinomyces*

TABLE 1. Cellulolytic activity of fungi from processed oats based on the enzymatic indexes obtained after 5 and 7 days of culture.

	Time period	
Genera/species	5 Days	7 Days
Acremonium fusidioides (Nicot) W. Gams	++	+
A. griseoviride (Onions & Barron) W. Gams	++	+
Alternaria alternata (Fr.) Keissi	+	+
Aphyllophorales Donk	+	+
Aspergillus aureolatus Munt	+	+
A. brevipes Smith	+	+
A. caesiellus Saito	+++	++
A. duricaulis Rapper e Fennell	+	+
A. flavus Link	+	+
A. fumigatus Fres.	+	+
A. janus Rapper e Thom	+++	++
A. janus var. *brevis* Rapper e Thom	++++	+++
A. niveus Blochwitz	+	+
A. parasiticus Speare	+	+
A. sydowii Bain. e Sarf.	+++	++
A. terreus Thom	+	+
A. variabilis Gasperine	+	+
Cladosporium cladosporoides (Fres.) de Vries	++	+
C. oxysporum Berk. & Curt.	+	+
C. sphaerospermum Pens.	++	+
C. tenuissimum Cooke	+++	++
Curvularia pallescens Boedjin	+	+
Eupenicillium sp. Ludwig	++	++
Eurotium chevaliere Mangin	++	+
Nigrospora sphaerica (Sacc.) Mason	++	+
Oidiodendron griseum Robak	+	+
Paecilomyces lilacinus (Thom) Samson	+	+
Penicillium aurantio-griseum Diercky	++	+
P. bilaii Chalabuda	++++	++
P. citrinum Thom	++	+
P. corylophillum Durckx	++	+
P. decumbens Thom	+	+
P. expansum Link	+	+
P. funiculosum Thom	+	+
P. implicatum Biourge	+	+
P. islandicum Sopp	+++	++
P. janthinellum Biourge	+	+
P. melinii Thom	++	+
P. oxalicum Currie e Thom	+	+
P. pinophillum Hedgcock	++	+
P. sclerotiorum Van Beyma	+++	++
P. waksmanii Zaleski	++	+
Rhinocladiella atrovirens Nannf.	++	++
Sporothrix cyanescens (de Hoog) de Vries	+	+
Tritirachium oryzae Vicens (de Hoog)	+++	++

Activity halo (cm): from 0 - 0,99 (-); 1,0 - 1,10 (+); 1,11 - 1,20 (++); 1,21 - 1,30(+++); 1,31 - 1,40 (++++).

TABLE 2. Cellulolytic activity of fungi from processed oats based on the enzymatic indexes obtained after 3 and 5 days of culture.

	Time period	
Genera/species	3 Days	5 Days
Rhizopus microsporus V. Tieghem	+	+
Rhizopus stolonifer (Ehrem ex. Link) Lind.	+	+
Syncephalastrum racemosum Cohn ex. Schrot	+	+

Activity halo (cm): from 0 to 0,99 (-); 1,0-1,10 (+); 1,11-1,20 (++); 1,21-1,30 (+++); 1,31-1,40 (++++).

was associated with the mycelium; when almost all the substrate had been degraded, the cellulolytic activity was not detected by halo formation but by intense mycelial pigmentation.

During the analysis of enzymatic activity under various conditions, mechanisms of synthesis and/or excretion may be involved. Moscoso (10) states that small colonies with a dense mycelial mass could possess a higher number of nuclei per unit area, a feature that results in higher enzymatic production proportional to the size of the colony. The same author mentions that *Aspergillus nidulans* strains with slower growth presented higher enzymatic indexes for lipase, amylase, and protease.

Teather and Wood (15), examining zones of hydrolysis of carboxymethylcellulase activity for cellulolytic bacteria, observed formation of a diaphanous halo around the colonies that measured 1 to 2 cm. Some of the strains revealed high activity, presenting a halo of 1,5 cm. The results obtained in the present work are similar to those reported by Teather and Wood (15) regarding halo size.

RESUMO

Fungos celulolítocos isolados de aveia industrializada

Foram procedidas análises de 48 fungos filamentosos isolados de aveia industrializada, quanto à capacidade de ação celulolítica. Todas as amostras testadas apresentaram resultados positivos, destacando-se *Aspergillus janus* var. *brevis* e *Penicillium bilaii* por apresentarem maiores níveis enzimáticos. As espécies que exibiram menor diâmetro da colônia apresentaram valores de maior atividade celulolítica, em relação àquelas que mostraram maior diâmetro da colônia, as quais apresentaram menor índice enzimático.

Palavras-chave: fungos isolados de aveia industrializada; fungos celulolíticos.

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PRODUCTION OF GLUCOAMYLASE BY FED- BATCH CULTURE OF *ASPERGILLUS AWAMORI* NRRL3112

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ABSTRACT

Glucoamylase production by *Aspergillus awamori* NRRL3112 was 92% higher in fed-batch culture relative to the traditional batch fermentation for runs with 20g/l of total carbon source from cassava flour. An analysis of the specific production rate shows that this was due to a reduction in the repressive effect of glucose, as the inductive effect of the polysaccharide concentration was also reduced.

Key words: induction, repression, enzyme synthesis, process.

INTRODUCTION

Glucoamylase synthesis by *Aspergillus sp.* is regulated by catabolic induction and repression. Although this enzyme is produced in the presence of glucose and fructose as carbon source, polysaccharides lead to higher yields. The production of this enzyme by *Aspergillus awamori* NRRL3112 in culture medium containing cassava starch or maltose was shown to be 60% higher than in medium containing glucose (2, 3, 9).

The source of nitrogen also influences enzyme synthesis. When using peptone instead of NH4Cl, microbial growth was strongly enhanced and higher production levels of glucoamylase were obtained, although a reduction in specific production relative to cells was also reported (12).

Many other factors, like pH, specific respiration rate, type of culture, type of microorganism and its method of preservation are also important to enzyme synthesis (5, 6, 8).

Since polysaccharides are converted to glucose during culture, production strategies must be introduced in order to maintain low glucose concentrations and a certain amount of polysaccharides, which ensure a low repressive effect and a mild inductive effect, respectively (4).

In batch cultures with cassava starch, glucoamylase production is strongly induced at the start of cultivation but becomes repressed after complete hydrolysis of the polysaccharide to glucose. It was possible to increase productivity by means of a pulse fed batch process, though the final glucoamylase activity remained the same as with the conventional batch fermentation (6).

This work shows some results on glucoamylase synthesis by *Aspergillus awamori* NRRL3112 using a fed-batch approach that was compared to the traditional batch process. The latter presents higher glucose concentration levels and therefore a greater inhibitory effect on enzyme synthesis.

MATERIALS AND METHODS

Microorganism and inoculum

Spores of *Aspergillus awamori* NRRL3112 stored in tubes with sterile soil were used throughout this study (7). The strain was cultured in an Erlenmeyer flask (1L capacity) containing 200 ml of the culture medium described below. Incubation was carried out for 24 h at 35°C in a rotative shaker (250 min^{-1}). The culture obtained

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had 1.8g dry cell/l and was used as inoculum for the one batch and two fed-batch runs analyzed in the present study.

Culture medium

The medium of the inoculum and batch run contained an initial total reducing sugars concentration (TRSo) of 20 g/l. The TRS concentration was adjusted adding an adequate volume of cassava flour syrup (1). For fed-batch runs, the TRSo was 2g/l; 18 g/l were then transferred through the feed medium to give a total concentration (TRSt) of 20g/l. Since the TRS concentration of the feed medium was high (370 g/l), the total volume fed to the system was small (only about 5% of the culture broth volume) and this allowed all the results to be expressed as concentrations. The culture medium also contained the following nutrients (g/l): yeast extract 0.1; $(NH_4)_2SO_4$ 5.0; $Na_2HPO_4.12H_2O$ 3.78; KH_2PO_4 3.5; $MgSO_4.7H_2O$ 0.5.

Culture conditions

The batch and fed-batch runs were performed in a 15-l bioreactor LSL Biolafitte SA France. The operation conditions were as follows: reaction volume=10 L; inoculum fraction (volume of the shaker culture / volume of culture medium in the reactor) =0.1; agitation rate=700 m^{-1}; air supply =10 L/min; head pressure=0.2atm; pH=4.0; temperature=35°C.

Runs

One batch run (run 1) and two fed batch runs (runs 2 and 3) with feeding rates (f_S;gTRS/h) of 16.4 and 22.5, respectively, were performed.

Analytical techniques

Samples collected periodically from the fermenter were evaluated for: dry cell mass (X); glucose concentration (G) by the glucose-oxidase method; total reducing sugars (TRS), determined as glucose after enzymatic hydrolysis of the polysaccharide in the sample (10); glucoamylase activity (A), (11). One glucoamylase activity (U) was defined as the quantity of enzyme that releases 1 g of glucose per hour in the presence of 4% (w/v) soluble starch solution, at 60°C and pH 4.2. The concentration of polysaccharide (PS) corresponds to TRS minus G.

RESULTS AND DISCUSSION

The characteristics of the batch and fed-batch runs are specified in TABLE 1 and the results are summarized in TABLE 2. FIGURE 1 shows the typical time course of the variables measured and the specific production rate of glucoamylase (μ_a) for the batch culture (run 1). The high μ_a values at the beginning of culture result from the high PS concentration present. However, after 10 h, the μ_a value strongly decreased because by then the carbon source was almost exclusively glucose.

The low feeding rate in run 2 led to glucose concentrations lower than those obtained for the batch run. As can be observed in FIGURE 2, the maximum glucose concentration in run 2 was only 5 g/l whereas in run 1 it reached 15 g/l . On the other hand, positive PS levels were observed for run 1 until 8 h of culture, yet almost no PS was detected in the fed-batch culture due to immediate hydrolysis of added polysaccharide by glucoamylase. The PS profiles are mirrored in the μ_a values, which reached a maximum of 24 U/gh and of only 13 U/gh in the batch and fed-batch runs, respectively. However, as shown in TABLE 2, this maximum μ_a for the batch run occurred at the start of fermentation when X was low, decreasing thereafter to a μ'_a/μ_{amax} value of 0.33 when X was high (80% of Xmax). The fed-batch maximum μ_a was constant during 14 h of culture, with X corresponding to 80% of maximum X. This value is higher than the one recorded for the batch run during the same time period. Maximum activity (Amax) during fed-batch culture (840 U/l) was 68% higher and productivity was 60% higher than the corresponding values for the batch culture. This increased production using the fed-batch approach is related to a reduction in glucose levels, since the concentration of polysaccharide was lower than that in the batch culture.

Growth was not influenced by glucose profiles. For all the runs, maximum cellular concentration and cell productivity were about 8.5 g/l and 0.5 g/l.h, respectively.

Run 3 differed from run 2 in its higher f_s value and the fact that substrate feeding was initiated at the start of culture, as described in TABLE 1. These two distinct features of run 3 led to higher glucose values relative to run 2 but still lower than those reached during batch fermentation. The maximum glucose concentration for run 3 was 12 g/l and its level of PS was higher than that for run 2. The combination of both higher glucose and PS concentrations in run 3 resulted in a net positive gain in microbial production capacity, with an Amax value of 960 U/l corresponding to a 92% increase relative to the batch process. The μ_a profile (FIGURE 3) illustrates these points.

Although the greatest production of enzyme was attained with run 3, the low glucose concentration observed in run 2 is an interesting finding concerning cultures with higher TRSt values, since high levels of X and A indicate fast polysaccharide hydrolysis and thus glucose as the sole source of carbon in the medium.

The data obtained show that glucoamylase production can be enhanced in fed-batch fermentation relative to the traditional batch fermentation due to a reduction in the inhibitory effect of glucose.

RESUMO

O processo descontínuo alimentado na produção de amiloglicosidase por *Aspergillus awamori* NRRL3112

A produção de amiloglicosidase por *Aspergillus awamori* NRRL3112 em processo descontínuo alimentado é 92% superior em relação ao processo descontínuo em cultivos com 20 g/l de concentração total de fonte de carbono oriunda de farinha de mandioca. Uma análise da velocidade específica de produção mostra ser este resultado decorrente da redução do efeito repressivo causado pela glicose, já que o efeito indutivo, relacionado à concentração de polissacarídeo, também foi reduzido.

Palavras-chave: indução, repressão, síntese de enzimas, processo.

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INFLUENCE OF STORAGE TEMPERATURE ON THE PERFORMANCE OF PRESSED YEAST AS INOCULUM FOR ETHANOL FERMENTATION

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SHORT COMMUNICATION

ABSTRACT

The activity of pressed yeast stored at 5°C, 20°C or 30°C and used as agent of ethanol fermentation was practically unaffected when the storage times were not greater than 133, 6 and 2 days, respectively.

Key words: ethanol fermentation, storage of pressed yeast, pressed yeast performance.

The influence of storage conditions on the performance of pressed yeast in the production of bread is well known (6). Under storage at 5°C, the percentage of pressed yeast dead cells increased from 2% to 35% after 15 weeks (2), while the gassing power decreased to zero after 12 weeks (3). Despite the fact that pressed yeast is frequently used as inoculum for ethanol fermentation both in industrial plants (4) and in laboratory-scale tests (5), no information has been reported on the influence of storage temperature on pressed yeast activity during the fermentation process. An analysis of this latter point is the main purpose of the present communication.

Pressed yeast (*Saccharomyces cerevisiae*), recently produced by Produtos Alimentícios Fleischmann Royal Ltd., was used in all the experiments. Disaggregated pressed yeast was stored in tightly closed 750-ml glass bottles (to avoid water evaporation) at (5±1)°C, (20±1)°C or (30±1)°C.

The composition of the inoculated medium was (g/l): glucose, 121.0; KH_2PO_4, 4.4; urea, 1.7; yeast extract, 1.7; $MgSO_4.7H_20$, 0.80; yeast cells, 14 (dry matter). The medium was not sterilized.

Fermentation tests were carried out in 250-ml Erlenmeyer flasks containing 100 ml of inoculated medium and one drop of antifoam (FLUENT - Dow Produtos Químicos Ltda). The flasks were incubated at 30°C in a rotary shaker (150 rev/min). CO_2 production was measured by weighing the flasks (1). To evaluate evaporation losses, a flask containing non-inoculated medium was also submitted to incubation. The duration of each test was 6h, enough to complete fermentation when recently produced pressed yeast was used as inoculum. The data presented herein corresponds to the average values obtained from three simultaneous experiments. The fermentation tests were carried out with pressed yeast samples stored during 2, 6, 14, 20, 28, 25, 42, 56, 63, 69, 77, 84, 92, 105, 119 and 133 days.

The evaporation loss after 6h of incubation was measured in 17 tests and gave an average value of 0.26 g (standard deviation = 0.07 g), which corresponds to only about 5% of mean total

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weight loss (5.3 g) due to fermentation.

FIGURE 1 shows the results obtained using pressed yeast stored at 5°C, 20°C or 30°C for 6 days and at 20°C for 14 days. The data clearly illustrates the influence of storage conditions on yeast activity. The influence of storage time at 5°C on yeast performance is presented in FIGURE 2.

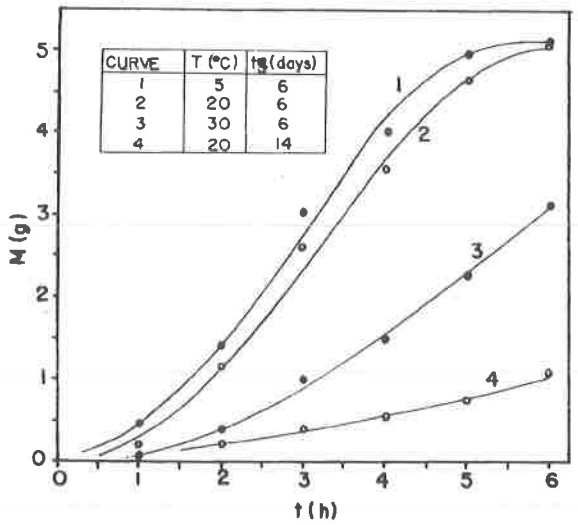

FIGURE 1. Influence of the storage conditions (T = storage temperature; ts = storage time) of pressed yeast on weight loss (M) due to fermentation.

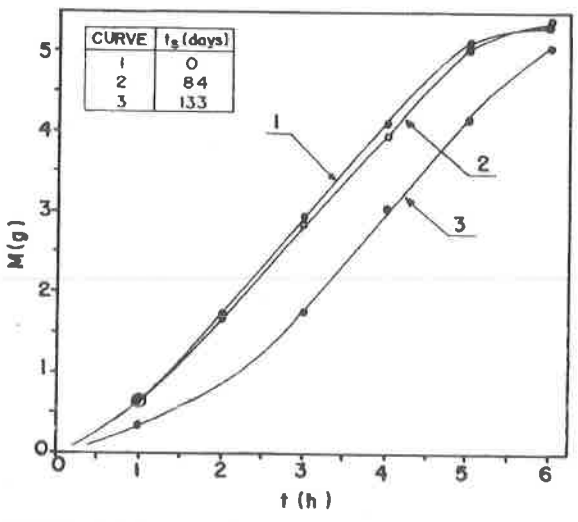

FIGURE 2. Influence of the storage time (ts) of pressed yeast on weight loss (M) due to fermentation. Storage temperature = 5°C.

When T = 20°C and T = 30°C, the pressed yeast performance was practically unaffected if the storage time was not greater than 6 days and 2 days, respectively.

When T = 5°C, on the other hand, the pressed yeast activity remained practically unchanged even after long term storage, although for this latter period (133 days) the performance of the yeast was negatively affected at the start of the fermentation test (FIGURE 2). In this case, the new yeast cells produced during fermentation probably compensated for the reduced initial activity of stored cells, leading to practically the same final result.

RESUMO

Influência da temperatura de armazenamento do fermento prensado em seu desempenho como inóculo de fermentação alcoólica

A atividade de fermento prensado, armazenado a 5°C, 20°C e 30°C, como agente da fermentação alcoólica, manteve-se praticamente inalterada decorridos não mais de 133, 6 e 2 dias de armazenamento, respectivamente.

Palavras-chave: fermentação alcoólica, armazenamento de fermento prensado, desempenho do fermento prensado.

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ISOLATION OF PECTINASE HYPERPRODUCING MUTANTS OF *PENICILLIUM EXPANSUM*

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SHORT COMMUNICATION

ABSTRACT

In order to obtain pectinase from hyperproducing strains, *Penicillium expansum* was mutated with UV and NTG. The selected mutants were analyzed for pectinolytic enzyme production and compared with the parental line. Mutants with up to a two-fold increase in pectin lyase or polygalacturonase activity were obtained. Mutant P462 presented a two-fold increase in activity of both enzymes.

Key words: *Penicillium expansum,* polygalacturonase, pectin lyase, mutant.

The enzymes of the pectinolytic complex produced by fungi are of commercial interest to the food and textile industries. These enzymes are also involved in phytopathogenic processes (10). In most cases, *Aspergillus* species are utilized for the industrial production of pectinases (9). However, over the last few years, research on the fungus *Penicillium* has demonstrated that some species of this genus are promising in terms of pectinase production. Brumano *et al.* (3) and Baracat *et al.* (2) detected significant pectin lyase (PL) activity in *P. griseoroseum* cultured in medium containing sucrose and yeast extract. The specific activity of the partially purified PL produced by *P. expansum* was found to be within the range reported for PL from other fungi (11). *P. expansum* has also proved to be a good polygalacturonase (PG) producer when cultured on medium containing citric pectin (6). Considering that the production of microbial enzymes at the industrial level requires high producer strains, the aim of the present study was to isolate pectinase from higher production mutants of *P. expansum* obtained by mutagenesis with physical and chemical agents.

Penicillium expansum was isolated from forest seeds in the Department of Phytopathology, Federal University of Viçosa, and selected by Baracat *et al.* (2) as one of the best pectinase producers among the isolates investigated. *P. expansum* parental line and selected mutants were grown on slanted agar-oatmeal medium for five days at 25°C and them used to produce a spore suspension. Ultraviolet (UV) light or N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was used as mutagen. A 2 ml spore suspension (5 x 10^3 spores/ml) on a Petri dish was exposed to UV light at a distance of 15 cm for 5 min (5% survival). NTG (1 mg/ml) was added to 20 ml of a spore suspension containing 5 x 10^6 to 1 x 10^7 spores/ml in 0.1 M phosphate buffer, pH 7.0. The mixture was incubated at room temperature for 30 min with moderate shaking, with a 5% survival rate. For mutant screening, 0.1 ml of UV-irradiated or NTG-treated spore suspensions were spread on medium containing

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2.0 g/l KH_2PO_4, 7.0 g/l K_2HPO_4, 0.1 g/l $MgSO_4$ $7H_2O$, 1.0 g/l $(NH_4)_2SO_4$, pH 7.0, supplemented with 3.0 g/l citrus pectin (Eskisa) and 1.3 g/l agar. The colonies grown after 5 days incubation at 25°C were cut out with a cork borer (7 mm), placed in petri dishes containing solid medium (MacIlvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid, pH 6.0), 0.25% citrus pectin (Eskisa) and 1.5% agar) and incubated for 48h at 40°C. After this time, iodine-potassium iodide solution (1.0 g iodine, 5.0 g potassium iodide and 330 ml H_2O) was added to detect clearance zones. Colonies with the largest clearing zones were transferred to enzyme production media. For enzyme production, cultures were grown in a rotary shaker (120 rpm) in 125 ml Erlenmeyer flasks with 50 ml of liquid medium. To each flask, 10^6 spores/ml were added as inoculum. The medium used for PL production contained 3.4 g/l KH_2PO_4, 6.8 g/l K_2HPO_4, 0.1 g/l $MgSO_4$ $7H_2O$, 1.0 g/l $(NH_4)_2SO_4$, pH 7.0, supplemented with 3.0 g/l citrus pectin (Eskisa). The medium used for PG production contained 2.0 g/l KH_2PO_4, 0.62 g/l K_2HPO_4, 0.1 g/l $MgSO_4$ $7H_2O$, 1.0 g/l $(NH_4)_2SO_4$, pH 6.3, supplemented with 3.0 g/l citrus pectin (Eskisa) was used for PG production. After 36h of culture in medium for PL production and 72h in medium for PG production the mycelium was separated by filtration and dried at 105°C (4). The filtrate was used to evaluate PL or PG activity. PL activity was determined spectrophotometrically by measuring the increase in absorbance at 235 nm (1). The reaction mixture, containing 0.6 ml of culture supernatant and 0.4 ml of 1% citrus pectin (SIGMA) in 50 mM phosphate buffer, pH 7.0, was incubated at 40°C for 20 min. The reaction was stopped by addition of 9.0 ml of 0.01 N HCl. One unit of enzyme activity (U) was defined as µmol unsaturated products released per minute. The concentration of unsaturated products was calculated using the molar extinction coefficient for unsaturated products (5500 M^{-1} cm^{-1}). PG activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent DNS assay (9) and galacturonic acid as standard. The reaction mixture, containing 0.5 ml of culture supernatant and 1.5 ml of 0.3% polygalacturonic acid (SIGMA) in 50 mM sodium-acetate buffer, pH 4.5, and 0.1 M NaCl, was incubated at 40°C for 20 min. One unit of enzymatic activity (U) was defined as µmol galacturonic acid released per minute. All the experiments were carried out three times with triplicate samples.

The genealogy of the selected mutants is presented in FIGURE 1. To obtain mutants, the spores were treated separately with UV light or NTG, with a survival rate of 5%. For each treatment, an average of 300 colonies were isolated and tested for pectinolytic enzyme production in solid medium. After halo measurement, for each treatment, 50 colonies were selected and stored for subsequent evaluation of mutation stability and pectinase production in liquid medium.

The PG and PL activity of some mutants presented a two-fold increase when compared with the wild isolate (TABLE 1). Similar improved rates of pectinase synthesis have been reported for *Aspergillus niger* mutants. Zhang (13) obtained a mutant with 930 U/g after mutagenesis with UV light and 1400 U/g after δ-treatment when compared with the control (600 U/g). Fiedurek and Ilczuk (5) detected increase in PG activity with rates between 20 and 125% by mutagenesis with UV light and NMU. Leuctenberger and Mayer (7) obtained a mutant of *A. niger* (M 1348/126) with marked endopolygalacturonase activity (3060 U/g) compared with the parental line (1120 U/g) and also with marked endo and exopolygalacturonase activity (3250 U/g) compared with the parental line (1590 U/g). The PL activity of mutant M 1348/126 was 1.25 U/g while that of the parental line was 0.65 U/g. Solis *et al.* (12) isolated a mutant of *Aspergillus* sp CH-Y-1043 with a four-fold increase in endopolygalacturonase production using an industrial subproduct (lemon peel) as substrate. Mutant P462 presented a two-fold increase in PL and PG activity compared with the wild isolate (TABLE 1). After treatment of mutant P462 with NTG, revertants for PL production (P462/54, P462/77 and P462/96) were obtained, which presented lower activity but at a level similar to that of the wild strain (FIGURE 1 and TABLE 1).

In general, the increase in PG activity was not accompanied by an increase in PL activity, suggesting different control mechanisms for the expression of genes coding for these two enzymes.

Mutants P257 and P462 showed a high stability as judged from a series of transfers and analyses of PG and PL activity carried out for about one year.

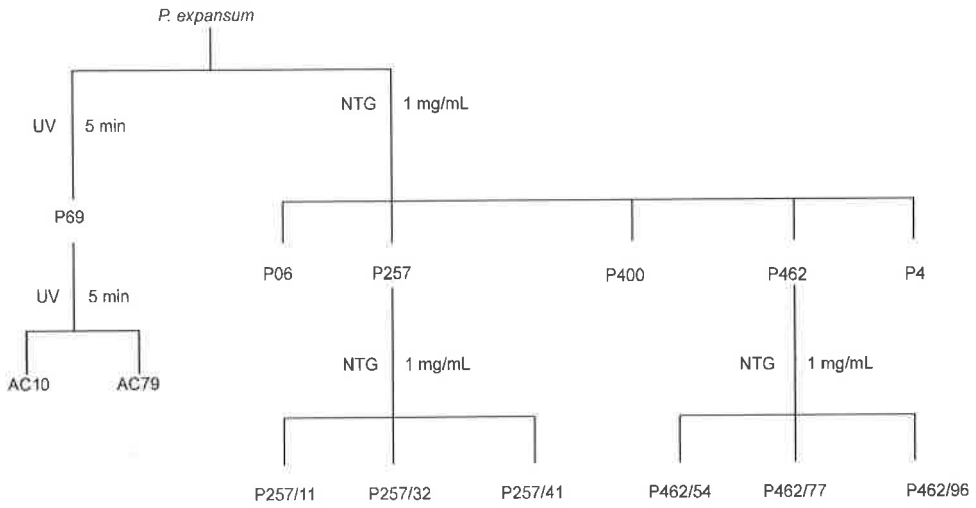

FIGURE 1. Genealogy of *P. expansum* mutants with pectinolytic activity after multiple mutagenesis **steps**

TABLE 1. Mycelial dry weight (DW) and pectin lyase (PL) and polygalacturonase (PG) Activity of *P. Expansum* mutants obtained after exposure to UV light or NTG

Strains	mutagenesis		PL Production			PG Production		
	Strains	mutagen	DW (g mL^{-1}) x 10^{-3}	U g^{-1}	%	DW (g mL^{-1}) x 10^{-3}	U g^{-1}	%
Wild	Parent	-	0.94	26.00	100	0.82	503	100
P69	Wild	1 X UV	0.42	13.10	58	0.34	847	168
AC10	P69	2 X UV	0.64	4.30	16	0.62	596	118
AC79	P69	2 X UV	0.38	7.23	28	0.46	724	144
P06	Wild	1 X NTG	1.23	28.39	109	1.06	665	132
P257	Wild	1 X NTG	1.19	15.37	59	1.05	994	198
P400	Wild	1 X NTG	1.12	16.51	63	1.04	415	83
P462	Wild	1 X NTG	1.11	51.08	196	1.02	984	196
P480	Wild	1 X NTG	1.35	31.24	121	1.03	643	128
P257/11	P257	2 X NTG	1.19	26.36	101	1.08	1010	201
P257/41	P257	2 X NTG	1.40	14.67	56	1.03	919	183
P257/32	P257	2 X NTG	1.19	18.37	71	1.05	919	183
P462/54	P462	2 X NTG	1.08	26.78	103	0.56	1026	204
P462/77	P462	2 X NTG	1.18	24.22	93	0.43	1166	232
P462/96	P462	2 X NTG	1.15	28.98	111	0.55	1107	220

U: Unit of enzyme activity.

Experiments are currently under way in our laboratory to optimize the culture conditions for these mutants in fermenters.

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RESUMO

Isolamento de mutantes de *Penicillium expansum* superprodutores de pectinases

Com o objetivo de obter-se linhagens com maior produção de pectinases, *Penicillium expansum* foi mutagenizado com UV and NTG. Os mutantes selecionados foram analisados quanto à

produção de enzimas pectinolíticas e comparados com a linhagem parental. Foram obtidos mutantes com aumento de até duas vezes na atividade de Pectina liase ou de Poligalacturonase. O mutante P462 apresenta aumento de duas vezes na atividade de ambas enzimas.

Palavras-chave: *Penicillium expansum*, poligalacturonase, pectina liase, mutante.

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NATURAL BIOREMEDIATION OF AQUIFER MATERIAL CONTAMINATED WITH GASOLINE-ETHANOL MIXTURES

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ABSTRACT

Natural bioremediation, the use of indigenous microorganisms to degrade hazardous substances within aquifers without engineered stimulation, is a promising cost-effective approach to hydrocarbon plume management. This technique requires thorough site characterization and monitoring in order to verify that the natural attenuation processes continue to provide adequate risk protection. Significant progress has been made towards understanding the hydrogeochemical and microbiological factors that influence the feasibility of natural bioremediation of fuel-contaminated aquifers in North America and Europe. Nevertheless, this experience should be extrapolated with care to contaminated sites in Brazil, where gasoline contains about 22% of ethanol. Preliminary laboratory studies show that ethanol can enhance the solubilization of benzene, toluene and xylene (BTX) in water, and it might exert diauxic effects during BTX biodegradation. A better understanding of the biochemical, physical, and ecological effects of ethanol is needed to develop a rational basis for the selection, mathematical modeling and monitoring of appropriate natural bioremediation systems in Brazil.

Key words: aquifer, biodegradation, BTX, ethanol, intrinsic bioremediation.

INTRODUCTION

Groundwater contamination by petroleum hydrocarbons is a widespread occurrence. To put the magnitude of this problem in perspective, it is estimated that 600,000 out of 2 million underground tanks storing gasoline in the USA are leaking (14). In Brazil, it is not known how many of about 100,000 underground tanks are leaking. Up to now, the main concerns are focused on the State of São Paulo, where groundwater is used by about 70% of the population. In the 1970's economic boom, during the so called ''Brazilan miracle'', there was a large increase in the number of gas stations in the country. Considering that the mean life time of underground storage tanks is about 20 years, Brazil will probably be dealing soon with soil and groundwater contamination problems similar to those currently faced by the USA and Europe. A major concern with leaking gasoline tanks is groundwater contamination by toxic and water soluble components such as benzene, toluene, and xylenes (BTX). These hydrocarbons have relatively high pollution potential because of their significant concentration in gasoline, relatively high water solubility and chronic toxicity. All BTX compounds are powerful depressants to the central nervous system and benzene can cause leukemia in humans. Consequently, BTX are priority pollutants and their common occurrence in aquifer drinking water

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supplies has lead to the development of several physical, chemical, and biological methods for their removal. Many of these technologies, however, are difficult to implement in developing countries such as Brazil because of economical and technical limitations. A need exists for an easy and cost-effective method that will mitigate the risk to public health associated with BTX contaminated groundwater.

Developing countries usually base their selection of environmental restoration technologies on the extensive experience accumulated in North America and Europe. An extrapolation of this experience to Brazil, however, will have to take into account differences in the formulation of Brazilian gasoline, which contains 22% of ethanol. Furthermore, ethanol is widely used as fuel for cars in Brazil and is usually stored in neighboring tanks which may also be leaking. Therefore, ethanol is likely to be present in BTX plumes. This paper presents a perspective on natural bioremediation as an approach to BTX pollution control in Brazil, with emphasis on the implications of the presence of ethanol in BTX plumes.

Why natural bioremediation?

Traditional processes for removal of BTX from aquifers involve pumping the contaminated groundwater for above ground treatment with activated carbon or air strippers. Nevertheless, BTX compounds are moderately hydrophobic and tend to sorb to the aquifer material. This makes them difficult to withdraw by pumping and constitutes a slow-release mechanism for sustained groundwater contamination. Pump-and-treat technologies alone can result in prohibitively long time periods for the removal of residual contamination and are often economically unfeasible (13). In addition, treatment with activated carbon or air strippers merely transfers the contaminants from one phase to another rather than transforming them into less harmful compounds. In situ bioremediation, which involves the use of indigenous microorganisms to degrade target compounds within the aquifer, is receiving increasing attention due to its potential cost-effectiveness. Furthermore, bioremediation includes minimum land disturbance, is a treatment that moves with the groundwater, does not dewater the aquifer due to excessive pumping and is environmentally sound because it ultimately transforms the target BTX into harmless products such as carbon dioxide and water (19).

The common approach to in situ bioremediation is to engineer the environment in order to overcome limitations to natural degradative processes. For example, fertilizers and oxygen can be injected into contaminated aquifers to overcome the insufficient supply of nutrients and electron acceptors. This approach is called active or engineered bioremediation. In some cases, however, natural conditions at contaminated sites meet all the essential environmental requirements so that bioremediation can occur without human intervention to stimulate microbial activity. This process is called natural or intrinsic bioremediation and differs from no-action alternatives in that it requires thorough documentation of the role of microorganisms in eliminating the target contaminants. This is accomplished via tests and monitoring at field sites or on-site derived samples of soil, sediment, or water to ensure that the natural attenuation process continues to provide adequate risk protection. Natural bioremediation is becoming increasingly popular with liable parties because it is the least expensive approach to control BTX plumes. It should be emphasized, however, that this technique is not a panacea applicable to all situations. Indeed, its successful application is site-specific and requires fulfillment of the conditions discussed next.

MATERIALS AND METHODS

Serum bottles (120 ml) with Teflon-coated silicone septa and aluminum crimp caps were used for incubations. The sandy aquifer material used in the biodegradation studies was obtained from Jurerê beach, Florianópolis, Santa Catarina. Samples were collected at a depth of 2 meters in a non-contaminated area. Aquifer slurries were prepared by adding 20 grams of the sand and 50 ml of mineral medium into the serum bottles. A synthetic nutrient solution was used to provide environmental conditions favorable to the growth of indigenous microorganisms as described in Corseuil and Weber, 1994. Sodium azide (2000 mg/l) was used in the controls to aid the inhibition

of potential microbial activity. Fifty ml of distilled water plus 5 ml of o-xylene were added to the serum bottles for experiments to test the cosolvency effect. Following ethanol addition at various concentrations, the bottles were shaken periodically and stored inverted at 25°C for five days. A 100-µl gastight syringe was used to withdraw gas samples from each bottle, which were directly injected in a gas chromatograph. The limit of detection of this procedure was approximately 200 µg/l.

RESULTS AND DISCUSSION

Requirements for the success of natural bioremediation:

1. Presence of microorganisms with potential to biodegrade the target compounds. Hydrocarbons have a natural pyrolytic origin and have been in contact with microorganisms throughout evolutionary periods of time. Thus, it is not surprising that many bacteria have acquired the ability to utilize hydrocarbons as food. The ability of microorganisms to degrade BTX has been known since 1908, when Stormer isolated the bacterium Bacillus hexabovorum by virtue of its ability to grow aerobically on toluene and xylene (15). An early review (27) identified over 100 microbial species from 30 genera that could degrade hydrocarbons. The existence of BTX degraders is a widely accepted fact. Furrthermore, these microorganisms are widely distributed. The ubiquity of soil bacteria capable of degrading BTX was first demonstrated in 1928 by Gray and Thornton, who reported that 146 out of 245 uncontaminated soil samples contained bacteria capable of metabolizing hydrocarbons (15). Therefore, this requirement is easily met.

2. Accessibility of target pollutants to the microorganisms. A common limitation of natural degradative process is the lack of adequate contact between pollutants and microorganisms. The target pollutants must be accessible in various aspects, including physicochemically (e.g,. desorption from aquifer solids to enhance bioavailability), structurally (e.g., bonds requiring cleavage must be exposed and not be sterically blocked by large atoms such as chlorine) and biochemically (e.g., the target pollutant must be able to pass through the cellular membrane). With respect to BTX, these requirements are generally met. It is not known, however, whether the presence of ethanol in a hydrocarbon plume can exert a sufficient cosolvent effect to enhance BTX bioavailability.

3. Induction of appropriate degradative enzymes. This process involves activation of specific regions of the bacterial genome. When some target substrates are present, they initiate a cascade of biochemical reactions that result in the transcription of genes coding for the synthesis of the necessary degradative enzymes. With respect to BTX degradation, many enzymes require induction, and the inducer (e.g., toluene) must be present at a higher concentration than the minimum threshold for induction (20). In general, this threshold is very low and enzyme induction is rarely a limiting factor in BTX bioremediation. Furthermore, BTX contamination is often discovered several years after the fact, and significant microbial acclimation and enzyme induction can occur during this time (8). Nevertheless, the presence of easily degradable substrates could exert diauxic effects (i.e., preferential substrate utilization) which would hinder the induction of BTX degrading enzymes. This may be an important factor in Brazil where gasoline contains 22% of ethanol, an easily degradable substrate. In this case, a lag period may be observed during which ethanol is degraded before any significant BTX degradation occurs (FIGURE 1).

4. Availability of electron acceptors. Hydrocarbons are in a reduced state, and their oxidation is thermodynamically very feasible. Microorganisms mediate their oxidation using electron acceptors during natural respiratory processes. The following preferential utilization has been observed, and reflects a decreasing oxidation potential of the potential electron acceptor: oxygen > nitrate > ferric iron > sulfate > carbon dioxide. In general, the kinetics of hydrocarbon oxidation is faster for electron acceptors with higher oxidation potential.

i) *Biodegradation using molecular oxygen.* Aerobic BTX degradation is usually the fastest. Using

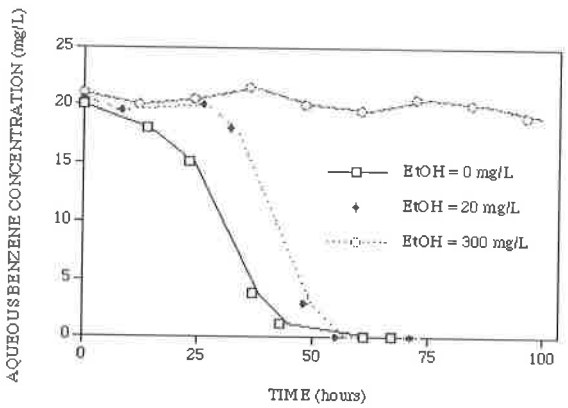

FIGURE 1. Effect of ethanol concentration on aerobic benzene degradation in aquifer microcosms. Batch microcosms were prepared as described by Alvarez and Vogel (1991). The lag period for benzene degradation increased with the initial ethanol concentration, possibly due to diauxy. The microcosm that was fed 300 mg/l of ethanol had a greater oxygen demand than available oxygen and became anoxic before the onset of benzene degradation.

toluene as an example, half lives under aerobic conditions can vary between 1 and 20 days, depending on the active microbial concentration (1; 9; 10; 25). Aerobic toluene degradation can be represented by the following stoichiometry:

$$C_7H_8 + 9\ O_2 \rightarrow 7\ CO_2 + 4\ H_2O$$

Therefore, the complete mineralization of toluene requires about 3 mg/l of oxygen for each mg/l of toluene (or many other hydrocarbons). The extent of aerobic BTX degradation is controlled by the amount of contamination released, the rate of oxygen transfer into the subsurface, the background oxygen concentration of the groundwater (usually 6 to 12 mg/l), and the occurrence of alternate substrates (2; 7). The presence of ethanol in Brazilian gasoline represents a significant additional oxygen demand by the soluble components, and is likely to decrease the extent of aerobic BTX degradation in oxygen limited aquifers (FIGURE 1).

ii) *Biodegradation using nitrate.* Once oxygen is depleted, some facultative denitrifiers can replace oxygen with nitrate as the terminal electron acceptor during BTX degradation. Half lives for toluene degradation under denitrifying conditions are a little longer, typically ranging from 20 to 50 days (2; 17), but could be much shorter if the initial denitrifiers concentration exceeds 1 mg/l (FIGURE 2). The mineralization of toluene coupled to the complete nitrate reduction to nitrogen gas is represented by the following reaction:

$$C_7H_8 + 7.2\ NO_3^- + 7.2\ H^+ \rightarrow 7\ CO_2 + 7.6\ H_2O + 3.6\ N_2$$

Numerous laboratory and field studies have shown that toluene, *m*-, *p*-, and *o*-xylene, ethylbenzene, and naphthalene can be degraded under strictly anaerobic denitrifying conditions. However, benzene, which is the most toxic of the BTX, is recalcitrant and requires microaerophilic conditions for its degradation with nitrate as electron acceptor (4; 5; 18).

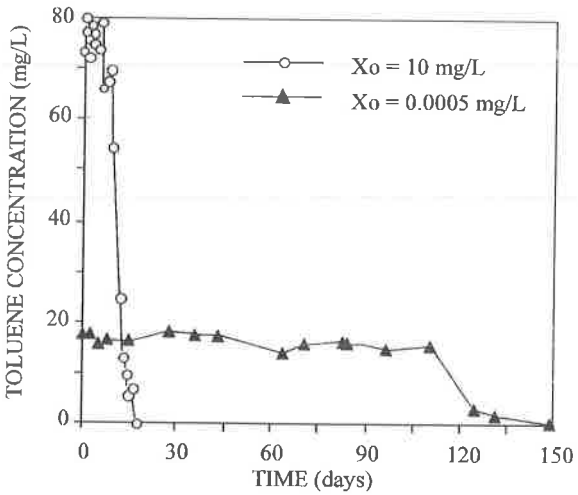

FIGURE 2. Effect of initial denitrifiers concentration (Xo) on toluene degradation time in aquifer microcosms. Denitrifying microcosms were prepared as described by Alvarez *et al.* (1994). A longer lag period was observed with the lower initial concentration of denitrifiers, even though the initial toluene concentration was lower. This lag reflects the time required to grow a sufficient concentration of microorganisms capable of exerting measurable biodegradation rates.

iii) *Biodegradation using ferric iron.* When oxygen and nitrate have been used up, some indigenous microorganisms can use ferric iron [Fe(III)] as the electron acceptor during BTX degradation (21; 22). Large amounts of ferric iron are present in mineral forms in most aquifers, which constitute a large potential electron acceptor pool for

hydrocarbon oxidation. For example, the mineralization of toluene coupled to the reduction of ferric hydroxide can be represented as follows:

$$C_7H_8 + 36\ Fe(OH)_3 + 72\ H^+ \rightarrow 7\ CO_2 + 36\ Fe^{+2} + 94\ H_2O$$

Although the mechanisms of hydrocarbon degradation under iron-reducing conditions are not fully understood, field evidence suggests that this is an important mechanism in the subsurface biodegradation of dissolved BTX (21). Similar to other anaerobic processes, however, degradation by iron reducers is greatly underestimated by current mathematical bioremediation models (7).

iv) *Biodegradation using sulfate.* Laboratory studies have shown that BTX can be degraded under sulfate reducing conditions (6; 12). Anaerobic toluene mineralization under sulfidogenic conditions is described by the following stoichiometric equation:

$$C_7H_8 + 4.5\ SO_4^{-2} + 3\ H_2O \rightarrow 7\ HCO_3^- + 2.25\ H_2S + 2.25\ HS^- + 0.25\ H^+$$

This process is relatively slow, and its extent and significance in aquifers has not yet been quantified. When it occurs, it is usually near the center of the plume, farthest from the surrounding uncontaminated (aerobic) groundwater.

v) *Biodegradation using carbon dioxide.* Laboratory studies have also shown that BTX can be degraded under methanogenic conditions (16; 26). The reaction for toluene can be represented as:

$$C_7H_8 + 5\ H_2O \rightarrow 2.5\ CO_2 + 4.5\ CH_4$$

Similarly to BTX degradation under sulfate reducing conditions, this process is relatively slow and its significance as an attenuation mechanism in aquifers has not been proven. This may be due, in part, to the high sensitivity of methanogens and sulfate reducers to a wide variety of environmental conditions, including pH, temperature, and toxicants (including oxygen in this case). An imbalance in any of these factors could easily inhibit these anaerobic BTX degradation processes.

5. Availability of inorganic nutrients. Microorganisms need macronutrients to synthesize cellular components, such as nitrogen for aminoacids and enzymes, phosphorus for ATP and DNA, sulfur for some coenzymes, calcium for stabilizing the cell wall, and magnesium for stabilizing ribosomes. A C:N:P ratio of 30:5:1 is generally sufficient to ensure unrestricted growth in aquifers (24). Microbes also need micronutrients to perform certain metabolic functions. For example, trace metals such as Fe, Ni, Co, Mo, and Zn are needed for some enzymatic activities. In general, aquifer minerals contain sufficient nutrients to support microbial activity. Nevertheless, geochemical analyses and laboratory biodegradation assays should be performed to verify that the presence of inorganic nutrients is sufficient for the success of natural bioremediation.

6. Adequate pH. Enzymes are polymers of aminoacids and their activity requires the proper degree of aminoacid protonation. This is controlled by the pH. Optimum groundwater pH is usually near the neutral value of 7.0, but most aquifer microorganisms can perform well between pH values of 5 and 9. Groundwater is typically well buffered within this range, so that the microbial physiological requirement for adequate pH is generally met in aquifers (8). Nevertheless, aquifers contaminated by municipal landfill leachates may contain elevated concentrations of organic acids and pH's as low as 3.0. In these cases, pH may represent a significant environmental problem to the indigenous bacteria.

7. Adequate temperature. Temperature is one of the most important environmental factors influencing the activity and survival of microorganisms. Low temperatures reduce the fluidity and permeability of the cellular membrane, which hinders nutrient (and contaminant) uptake. Higher temperatures are associated with higher enzymatic activity and faster biodegradation rates, up to an optimum value which is species specific. In this range, BTX degradation rates can triple as a result of a temperature increase of 10°C (11). If the temperature rises much beyond the optimum

value, proteins, enzymes and nucleic acids become denatured and inactive. The temperature of the upper 10 m of the subsurface may vary seasonally; however, that between 10 and 100 m approximates the mean annual air temperature of a particular region (19). Groundwater temperatures in Brazil typically vary between 20 to 25°C, which is a very favorable range for natural bioremediation.

8. Absence of toxic substances. Some contaminants can be present in aquifers at sufficiently high concentrations that inhibit microbial activity. For example, it is not uncommon for aquifer microorganisms to encounter potentially toxic heavy metals such as Pb, Hg, Cd, and Cr. While heavy metals are required in trace quantities for nutritional purposes, they can be bactericidal if present in soluble form at concentrations greater than about 1 mg/l. Although Brazilian gasoline has no lead, geochemical analyses and laboratory degradation assays with representative aquifer samples should be performed to verify the absence of inhibitory substances.

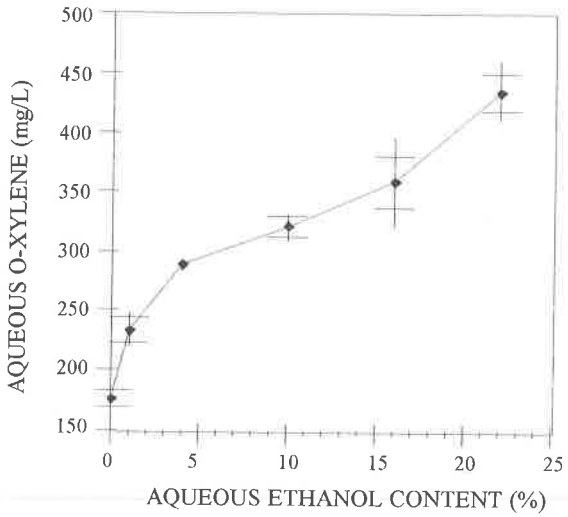

FIGURE 3. Effect of ethanol concentration on the water solubility of *o*-xylene. Fifty ml of distilled water plus 5 ml of *o*-xylene were added to 100-ml bottles. Following ethanol addition at various concentrations, the bottles were shaken periodically and stored inverted at 25¡C for 5 days. Emulsification of *o*-xylene was observed in bottles amended with 10% ethanol or more, but not with 1% ethanol or less. Error bars depict the standard deviation from 3 bottles.

9. Faster biodegradation than migration rate. This condition is necessary to ensure that the hydrocarbon plume will recess rather than expand and reach potential groundwater users. These relative rates depend on the type and concentration of the contaminants, the indigenous microbial community and the subsurface hydrogeochemical conditions. BTX compounds enter groundwater primarily by solubilization from the gasoline that reaches the water table. In Brazil, the solubility of BTX in groundwater can be enhanced by the cosolvent effect of ethanol, as illustrated for *o*-xylene in FIGURE 3. Therefore, higher BTX concentrations should result in groundwater that is in equilibrium with Brazilian gasoline compared to North American or European fuel. Once BTX are dissolved, they are transported by the moving groundwater. Their transport can be retarded by sorption onto aquifer materials, especially in aquifers with a high organic carbon content. It is not known, however, whether the presence of ethanol in the plume can decrease BTX retardation and facilitate their transport.

The primary mechanisms that limit BTX migration are biodegradation and, to a lesser extent, volatilization (10). Abiotic reactions such as hydrolysis are not significant because BTX are stable under environmental conditions. In natural BTX bioremediation, the rate-limiting attenuation mechanism is frequently the influx of oxygen, which in turn limits aerobic BTX degradation kinetics (23). Nevertheless, the presence of a free phase is a critical factor influencing whether a plume will expand or recess. For example, a fluctuating water table can continue to flush BTX into the plume, which would hinder its recession. Consequently, the removal of the free-phase and the sorbed hydrocarbons from the source area is an important prerequisite for the successful implementation of natural bioremediation.

CONCLUSIONS

At present, there are only a few well documented, full-scale demonstrations of unaided natural bioremediation. Indeed, this emerging technique has not yet achieved pedagogical maturity. Nevertheless, natural bioremediation shows great promise as a practical and cost-effective alternative for managing low-risk, BTX contaminated aquifers in Brazil and elsewhere,

provided that it is implemented with adequate site characterization, analysis and monitoring.

While some progress has been made towards understanding the hydrogeochemical and microbiological factors that influence the feasibility of natural BTX bioremediation in North America and Europe, this experience should be extrapolated with care to contaminated sites in Brazil, where gasoline contains about 22% of ethanol. To take full advantage of natural bioremediation in Brazil will require further basic and applied research. In particular, research is needed to gain a better understanding of (a) How does the presence of ethanol affect BTX solubilization, transport, microbial community structure, enzyme induction and biodegradation kinetics under different electron acceptor conditions; (b) When and where is natural bioremediation the appropriate choice?, (c) How well can we predict whether a given plume will expand or recess?, and (d) What is the critical hydrogeological, geochemical, and microbiological information needed to address these questions? A better understanding of these issues should lead to the development of a rational basis for the selection, mathematical modeling, and monitoring of appropriate natural bioremediation systems.

ACKNOWLEDGMENTS

We thank Centro de Pesquisas e Desenvolvimento Leopoldo Américo Miguez de Mello (CENPES/ Petrobrás) and the National Science Foundation for their financial support, and Martha Montenegro, Marilda Fernandes, and Ruy Carlos Ferreira dos Santos for their technical assistance.

RESUMO

Biorremediação natural de aqüíferos contaminados com misturas de gasolina e etanol

A biorremediação natural, ou seja, o uso de microorganismos nativos para degradar substâncias perigosas sem nenhuma estimulação tecnológica, mostra-se como uma técnica promissora efetiva e de baixo custo para ser aplicada no manejo de plumas de hidrocarbonetos de petróleo. Esta técnica exige que se faça uma total caracterização e monitoramento do local contaminado para se verificar se os processos de atenuação natural continuam proporcionando uma adequada proteção de riscos. Um grande progresso tem sido feito na Europa e Estados Unidos para entender os fatores hidrogeológicos e microbiológicos que influenciam a biorremediação natural de aquíferos contaminados por combustíveis. No entanto, esta experiência deve ser extrapolada com cuidado em locais contaminados no Brasil, onde a gasolina contém 22% de etanol. Estudos preliminares de laboratório mostram que o etanol pode aumentar a solubilização dos compostos BTX (Benzeno, Tolueno, e Xileno) na água, e pode interferir na degradação biológica dos BTX. É necessário que se conheça os efeitos bioquímicos, físicos e ecológicos do etanol para que se desenvolva no Brasil uma base racional para seleção, modelagem matemática e monitoramento de sistemas apropriadas de biorremediação natural.

Palavras-chave: biorremediação natural, compostos BTX, gasolina, etanol, aquíferos.

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Rev. Microbiol., São Paulo, 27(1): 27-32, 1996

UTILIZATION OF EXTRACELLULAR POLYSACCHARIDES FROM *ANKISTRODESMUS DENSUS* KORS (CHLOROPHYCEAE) BY HETEROTROPHIC BACTERIA

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Armando A. H. Vieira[2]

ABSTRACT

Compounds of high molecular weight excreted by *Ankistrodesmus densus* could be separated into two polysaccharide fractions, "A" (MW close or larger than $2x10^6$ Da) and "B" (MW of about 10^4 Da), using Gel Permeation Chromatography.

The results show that both fractions were separately utilized as substrate by heterotrophic bacteria, which were able to degrade them into compounds of lower molecular weight.

The present work is in agreement with results from the literature on the existence of an algal excretion-heterotrophic relationship and gives a further contribution to already available data by showing that high molecular weight polysaccharides can definitely be used as substrate and degraded by heterotrophic bacteria.

Key words: excretion, *Ankistrodesmus densus*, extracellular polysaccharides, bacteria, heterotrophy.

INTRODUCTION

Extracellular organic carbon (EOC) released by phytoplankton can be an important carbon and energy source for heterotrophic bacteria. Many studies have revealed that EOC supplies a substantial part of the bacterial production (2, 3, 5, 7, 8, 13, 18).

Some researches have studied EOC by measuring its molecular weight distribution and subsequent uptake by bacteria. The findings have suggested that, in general, low molecular weight components of EOC are quantitatively much more important to bacterioplankton than the high molecular weight components. Small molecules (<500 Da) are fast and efficiently assimilated and metabolized by bacterioplankton and, as a consequence, high molecular weight compounds accumulate and are found to be dominant in the aquatic environment (4, 12, 19).

An understanding of the real importance of high molecular weight compounds to bacterioplankton is still contradictory. Some researchers have demonstrated that more complex compounds are utilized by bacteria too, though often at lower rates than the small ones (6, 14, 16, 17). Other workers (25) have suggested that these polymers cannot be easily used by bacteria and probably contribute to the build-up of a refractory pool of dissolved high molecular weight organic carbon in aquatic ecosystems.

The aim of the present work was to investigate the utilization and transformation of polysaccharide compounds excreted by the alga *Ankistrodesmus*

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densus by heterotrophic bacteria under laboratory conditions.

MATERIALS AND METHODS

Organism and Culture Conditions

Ankistrodesmus densus (Kors) was isolated from Broa Reservoir, S.P., Brazil and has been maintained in the freshwater microalgae culture collection at the Federal University of São Carlos as strain 003CH-UFSCar. The cells were grown in WC medium (11) under axenic conditions at 20-22°C. The culture was continuously aerated by gentle bubbling with filtered and moist air. Illumination was provided by 40W fluorescent tubes (664 $\mu E.m^{-2}.s^{-1}$) and with light/dark cycle of 13:11 hours.

Obtention of high molecular weight EOC

After 30-40 days of growth, the original medium containing the extracellular polysaccharides was filtered through different porous Millipore membranes (AP-20, 5.0 and 1.2-µm) and concentrated under reduced pressure at 40°C. The medium with the extracellular polysaccharide was dialyzed against distilled water in a dialysis tube with a 12,000-14,000 Daltons (Da) molecular weight cut off and freeze-dried. The freeze-dried material was dissolved in distilled water and separated in subfractions using Gel Permeation Chromatography and Sephacryl S-400 (10^4 to $2X10^6$ Da). The gel filtration experiment was performed under the following conditions: bed dimensions = 2.6 x 95 cm, flow rate - 1.67ml/min.; eluent = NaCl 0.05M + Na H_2PO_4 0.05M, pH 7.0 at environmental temperature. The column was calibrated using the pattern dextrans of 10^4 and $2X10^6$ Da. The sample size of total EOC applied in the column was 5% of bed volume (30 ml). Successive 5ml fractions were collected for determination of carbohydrate content using the phenol-acid sulfuric method (10). The fractions isolated with Sephacryl were used to determine the contents of carbohydrate by the phenol-acid sulfuric method (10), of protein by Lowry et al. (20), of sulfate according to Antonopoulos (1), and also to determine the monosaccharide composition by methanolysis of the samples, using gas-chromatography according to the methodology of Reinhold (22) .

Experiments of bacterial utilization of extracellular polysaccharide fractions

The experiments of bacterial utilization of the excreted polysaccharides were done with fractions isolated by Gel Permeation Chromatography and bacteria collected from a Marginal Lagoon of the Mogi-Guaçu River, SP, Brazil. The lagoon water with bacteria was prefiltered through Millipore membranes (AP-20 and 5.0 µm) in order to eliminate larger organisms and grazers. The isolated fractions dissolved in distilled water and the filtered water with bacteria were placed in Erlenmeyer flasks (2 l capacity). The proportion of this mixture was 1 volume of fraction water to 2 volumes of water with bacteria, making up a total volume of 700 ml (Experiment I) or 600 ml (Experiment II). The final carbohydrate concentrations used were: 0.10 mg/ml of Fraction A and 0.04 mg/ml of Fraction B in Experiment I; 0.06 mg/ml of Fraction A and 0.03 mg/ml of Fraction B in Experiment II. The flasks were maintained in the dark at room temperature for 30 or 40 days. Sampling was performed at regular intervals to determine bacterial transformations of the carbohydrate fractions by Gel Permeation Chromatography, using different gels and using as eluant a solution of NaCl 0.05M and Na H_2PO_4 0.05M, pH 7,0 at environmental temperature. The experiments with bacteria were carried out in aseptic conditions to avoid contamination with other microorganisms. The lagoon water with bacteria and devoid of excreted compounds was used as control culture. The carbohydrate content of peaks was measured using the phenol-sulfuric acid method (10). Bacterial counts were done to monitor microbial growth. Two experiments were performed: Experiment I, where transformation of fractions "A" and "B" by bacteria was analyzed with Gel-Sepharose CL6B after 30 days; Experiment II, where transformation of fractions "A" and "B" by bacteria was analyzed using different gel types (Sephacryl S-500 and Sephadex G-50) after 40 days of incubation. A diagram of the experimental procedure is shown in FIGURE 1.

RESULTS AND DISCUSSION

The EOC produced by *Ankistrodesmus densus* microalgae could be separated in two fractions of different molecular weight (MW), namely fractions "A" (76,5%) and "B" (23,5%), using Gel Permeation Chromatography. Fraction "A" had a MW close to or larger than $2X10^6$ Da, while fraction "B " had a MW of approximately 10^4 Da (FIGURE 2). Both fractions contained a larger portion of sulfated polysaccharide (75,63%) and a minor portion of protein (24,37%).

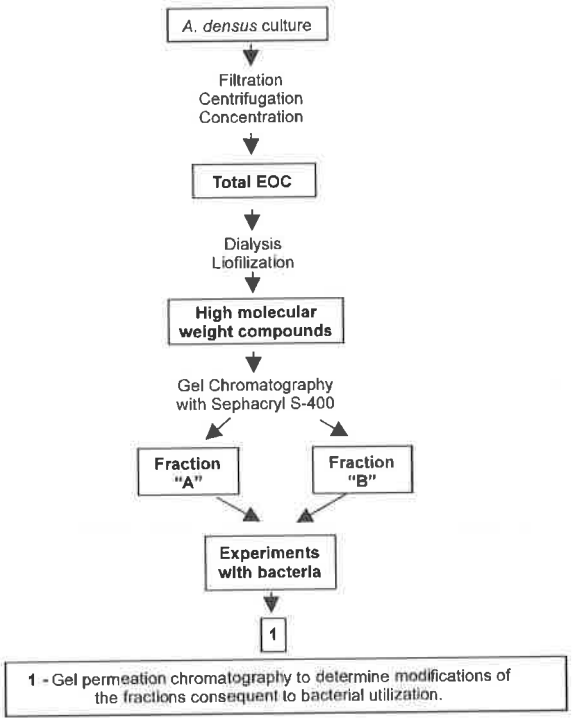

FIGURE 1. Overall scheme of the experimental procedure used to estimate the release of high molecular weight EOC by *Ankistrodesmus densus* and its utilization by heterotrophic bacteria.

The monosaccharide composition of the polysaccharide in fraction "A" contained fucose (40%), 3-O-methyl galactose (37%), glucose (11%), rhamnose (6%), mannose (4%), 3-O-methyl -rhamnase (tr) and galactose (2%). Fraction "B" presented a similar composition, namely: mannose (46%), glucose (34%), rhamnose (11%), 3-O-methyl-galactose (6%), but did not contain any galactose or 3-O-me rhamnose. As shown in FIGURES 3 and 4 (Experiment I), a reduction in

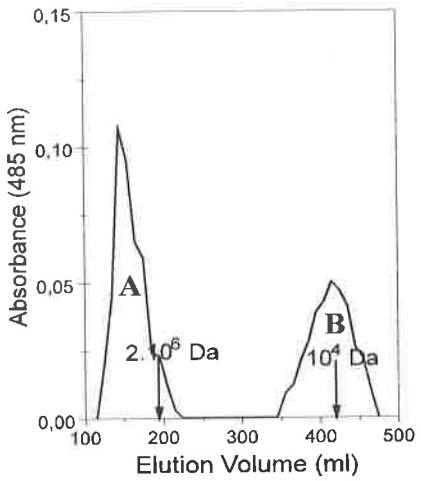

FIGURE 2. Gel-filtration (Sephacryl S-400) of high molecular weight EOC. **A** and **B** are the two fractions separated. Arrows show the position of 2.10^6 and 10^4 Da pattern dextrans. Absorbance at 485 nm is used with phenol-sulfuric acid reaction.

the peaks of both fractions was observed as a consequence of bacterial metabolism. However, the results obtained in this experiment, where Sepharose CL6B was used, did not make clear that the substrates were being degraded into molecules of lower MW. In Experiment II, the use of different gel types (Sephacryl S-500 for fraction A and Sephadex G-50 for fraction B) made it possible to clearly visualize that the bacteria metabolized both fractions into lower MW components (FIGURES 5 and 6). The data on bacterial cell counts show that the microorganisms were able to grow in the presence of both fractions (manuscript in preparation).

One of the problems associated with the study of bacterial utilization of high MW compounds has been the length of the incubation time. Since some works use the C^{14} methodology and a short incubation time, an overestimation of small molecules can occur. This happens because the complex and larger molecules of photosynthetic origin are labeled later than the smaller ones in the metabolic sequence (23). Another point that has been demonstrated by some researches is that the bacterial turnover time for high MW weight compounds is much longer than that for low MW components and thus cannot be estimated during short incubation periods (12, 15, 17, 25). However, these researchers, except Sundh (25), did not de-

termine the biochemical composition of the material investigated.

The results of the present work using longer incubation periods (30 and 40 days) show that the high MW EOC in the form of complex polysaccharides can be utilized as carbon source by bacteria (FIGURES 3, 4, 5 and 6). Ogura (21) also observed the utilization of larger molecules while studying the decomposition of released organic carbon during 30 to 120 days. Nevertheless, he did not determine the biochemical or MW characteristics of the released organic carbon. The EOC fraction not subject to rapid uptake by bacteria was studied by Coveney and Wetzel (9), who found that this residual EOC could be metabolized by lake heterotrophs but that such slow carbon flux would not be noticed in conventional assays for bacterial utilization of algal EOC. These authors also demonstrate that complex molecules can be used by heterotrophic bacteria, however they did not separate the residual EOC into fractions of different MW nor characterized their biochemical composition.

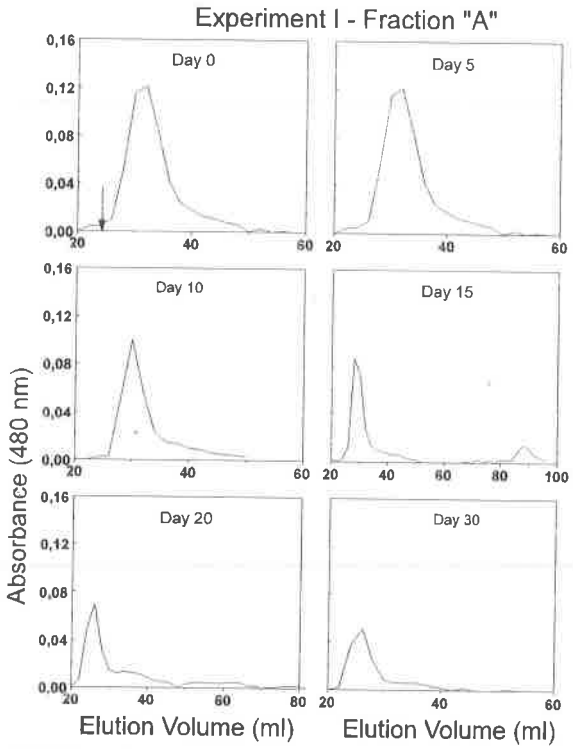

FIGURE 3. Experiment I: Sepharose CL6B chromatograms of **Fraction "A"** during incubations with heterotrophic bacteria. The elution position of 2.10^6 Da pattern dextran is indicated by the arrow. Absorbance at 485 nm is used with the phenol-sulfuric acid reaction. Conditions of gel filtration: Bed Dimensions - 56,0x1.3cm; Flow rate- 1ml/min.

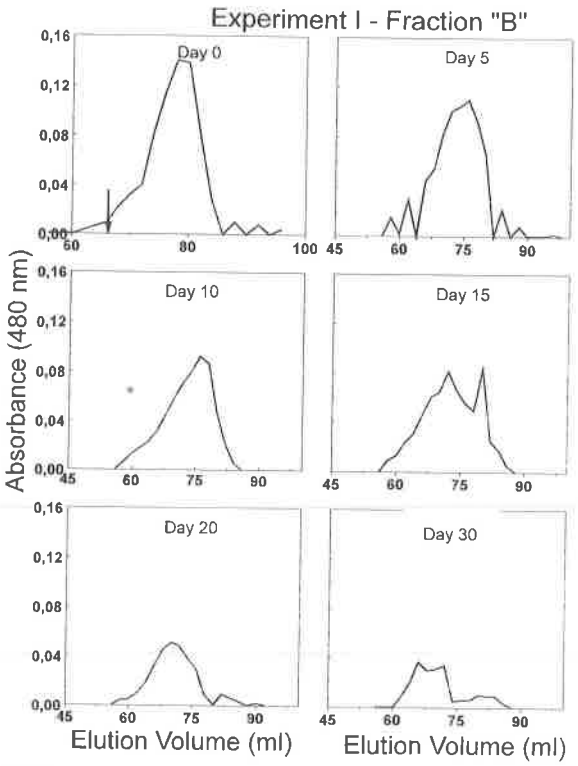

FIGURE 4. Experiment I: Sepharose CL6B chromatograms of **Fraction "B"** during incubations with heterotrophic bacteria. The elution position of 10^4 Da pattern dextran is indicated by the arrow. Absorbance at 485 nm is used with the phenol-sulfuric acid reaction. Conditions of gel filtration: Bed Dimensions - 56,0x1.3cm; Flow rate- 1ml/min

Investigations that emphasize the use of polysaccharides by bacteria are thus uncommon and most of them work with the total excreted material separated into low and high MW fractions.

Different MW ranges are adopted in fractionation studies. Most workers consider that high MW substances range between 1500 and 10.000 Da (12, 16, 23, 24, 25, 26). A small number of approaches (7, 21) expand this range to values higher than 10.000 Da. Chröst and Faust (6) considered the MW of their macromolecule fraction higher than 300.000 Da. Thus fraction "B", with a MW of about 10.000 Da, falls within the conventional size range established for large compounds in the relevant literature. The results obtained with fraction "A", with a MW close or larger than 2×10^6, definitely reinforce the observation that bacteria can metabolize high MW compounds.

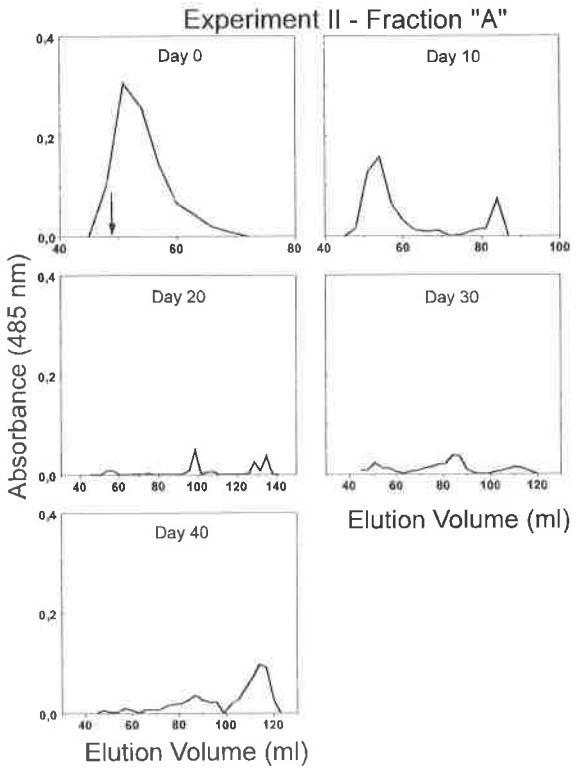

FIGURE 5. Experiment II: Sephacryl S-500 chromatograms of **Fraction "A"** during incubations with heterotrophic bacteria. The elution position of 10^4 Da pattern dextran is indicated by the arrow. Absorbance at 485 nm is used with the phenol-sulfuric acid reaction. Conditions of gel filtration: Bed Dimensions - 62,5x2,6cm; Flow - 1ml/min.

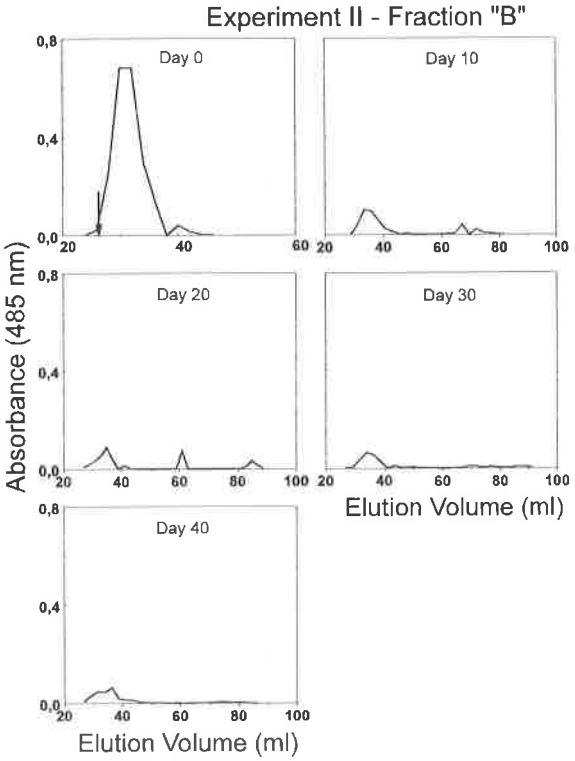

FIGURE 6. Experiment II: Sephadex G-50 chromatograms of **Fraction "B"** during incubations with heterotrophic bacteria. The elution position of 10^4 Da pattern dextran is indicated by the arrow. Absorbance at 485 nm is used with the phenol-sulfuric acid Conditions of gel filtration: Bed Dimensions - 49,0x2,6cm; Flow rate - 0,5ml/min.

To summarize, the results presented in this study make it possible to certify that very complex molecules like extracellular polysaccharides can be utilized as carbon source by heterotrophic bacteria and are thus not refractory to metabolization, as some researches have previously concluded. Further studies are necessary to improve our knowledge of this subject and to better understand the actual role of complex molecules as bacterial substrates in nature.

RESUMO

Utilização dos polissacarídeos extracelulares de *Ankistrodesmus densus* Kors (Chlorophyceae), por bactérias heterotróficas

Compostos polissacarídicos de elevado peso molecular excretados pela alga *Ankistrodesmus densus* foram separados em duas frações com pesos moleculares diferentes, através de cromatografia de filtração gélica. As frações assim isoladas foram utilizadas nos experimentos de decomposição por bactérias.

Os resultados mostraram que as bactérias utilizam os polissacarídeos de elevado peso molecular de ambas as frações e os transformam em compostos com baixo peso molecular.

Este trabalho concorda com os dados da literatura sobre a existência da relação excretado algal-bactérias heterotróficas. Além desse aspecto, demonstra também que compostos de elevado peso molecular podem ser utilizados como fonte de carbono por bactérias hetrotróficas.

Palavras-chave: excreção, *Ankistrodesmus densus*, polissacarídeos, bactérias, heterotrofia.

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YEASTS ASSOCIATED WITH FLOWERS AND FRUITS FROM A SEMI-ARID REGION OF NORTHEASTERN BRAZIL

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ABSTRACT

Yeast species on flowers and fruits from 4 trees (cashew, *Anacardium occidentalis*; cajá, *Spondia lutea;* umbu, *Spondia* sp.; and mango, *Mangifera indica*) of the Northeastern region of Brazil were studied in field and market samples. Vigorous shaking removed most yeasts from fruit surfaces, and blending suspended and disaggregated the more firmly attached ones. Ripe fruits were dissected asseptically to separate the surface and internal communities. Isolations were made on Glucose-Yeast Extract-Peptone medium and Vitamin Free Yeast Base, both adjusted to pH 3.7 and containing 200 mg/l of chloramphenicol. The yeast communities of flowers and green fruits were dominated by basidiomycetous anamorphs (especially *Cryptococcus laurentii*). and black yeasts, but there was a sucession on ripe fruits to a community dominated by diverse ascomycctous species and their anamorphs. *Metschnikowia pulcherrima* was frequently isolated from cashew flowers and associated bees and a *Candida entomaea* -like species was prevalent on Cajá fruits in the field. The ascomycetous yeast community of ripe fruits consisted preferentially of fermentative species with low physiological profiles, including *Issatchenkia orientalis* and *Kloeckera javanica* as frequent isolates. A sequence of samples from different stages of flowering and fructification, vigorous pre-isolation treatments and different isolation media should be used to obtain an adequate description of these yeast communities.

Key words: yeasts, fruits, flowers, isolation methods, cashew, mango, cajá, umbu.

INTRODUCTION

Flowers and fruits are traditionally considered excellent habitats for yeasts, offering a succession of ephemeral microhabitats during their development and deterioration. Insects are among the most important yeast vectors, and their specialization for certain niches promotes unequal distribution and barriers to genetic exchange between yeast species of different substrates and microhabitats. Yeast communities on most plant surfaces are dominated by basidiomycetous forms and their anamorphs, especially *Cryptococcus* and *Rhodotorula* species, and also by black yeasts such as the ascomycetous yeast-like organism *Aureobasidium pullulans* (6, 13, 26, 30). Studies carried out mostly in temperate climates have shown that yeast communities of flowers are

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dominated by *Cryptococcus*, *Rhodotorula* and *Sporobolomyces* species that are typical of plant surfaces, and often also include some animal vectored yeasts like the terrestrial *Metschnikowia* species, that can grow in flower nectar (4, 24, 25, 30). When fruits are ripe they become an ephemeral habitat with high simple sugar levels that can be invaded by a succession of yeasts and their insect vectors (21). Ripe and rotting fruits often have communities dominated by fermentative yeasts, such as the apiculate yeasts *Hanseniaspora* spp (and their anamorphs of the genus *Kloeckera*), *Pichia kluyveri*, *Pichia fermentans*, and *Issatchenkia orientalis* (and its anamorph *Candida krusei*) which are typically vectored by insects like drosophilids (3, 7, 22, 32). Few studies have been made on yeasts of tropical fruits and flowers, and in general, the results agree with data from temperate areas, but revealing the presence of some unique biotypes (12, 18, 26, 33). Apiculate yeasts, especially of the genera *Hanseniaspora* and *Kloeckera*, are often associated with ripe or spoiling fruits and were found to be more diverse in a tropical forest of Rio de Janeiro than reported in studies from temperate climates (16, 17). In regions with cooler climates there are typically fewer *Issatchenkia* and *Hanseniaspora* species and more *Saccharomyces* and *Kluyveromyces* species than in tropical regions (5, 24).

Yeasts that colonize seasonally occurring fruits and flowers, like those in the present study, must be vectored from other substrates. The species *C. guilliermondii*, *C. krusei* and *C. parapsilosis* are opportunistic pathogens found to be prevalent yeasts associated with ripe fruits in some studies (2, 9, 15, 23, 26, 33). There are diverse ecosystems in Brazil that could serve as sources of yeasts colonizing flowers and fruits in the field and ripe and spoiling fruits in the markets. The Atlantic Forest, sandy coastal plains ("restingas") and mangroves, typical ecosystems of most of the Brazilian coast, are possible sources. Probable vectors in the tropics can include animals involved in pollination such as hummingbirds, bats and insects like bees and lepidoptera that visit open flowers. *Drosophila* are among the most important yeast vectors of ripe and rotting fruits. The yeasts associated with drosophilids of Brazilian forests include a large variety of ascomycetous species and their anamorphs and are most diverse in the least disturbed areas (19, 20, 24). In São Paulo, yeasts on grapes and in musts at the beginning of wine fermentation were found to be dominated by *Kloeckera apiculata* and *Pichia fermentans*, although *Saccharomyces* spp. predominated at the end of fermentation (35). Fermenting sugar cane juice in São Paulo had only five yeast species but fresh juice showed a greater diversity, with prevalence of the species *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Pichia membranaefaciens*, *Candida krusei*, *Candida stellata*, *Candida guilliermondii*, *Candida intermedia*, and *Schizosaccharomyces pombe* (31). Pineapple flowers and developing fruits from a plantation in Rio de Janeiro were found to have a yeast community dominated by black yeasts, *Cryptococcus* and *Rhodotorula* spp. However, the ripe fruits had more ascomycetous species, especially *Candida guilliermondii*, and the spoiling fruits contained mostly *C. guilliermondii*, *C. krusei*, and *Kloeckera* spp. associated with bacteria of the genus *Erwinia* (26). Water from the tanks of wild bromeliads in Rio de Janeiro had mostly basidiomycetous anamorphs in *Neoregelia cruenta* from a sunny restinga habitat, whereas a balance between these forms and ascomycetous yeasts was found in *Quesnelia quesneliana* of a more shaded habitat in a mangrove ecosystem (10).

Cacti are common in the semiarid regions of Brazil and *Opuntia* cacti are grown as crop for cattle feed in times of drought. Cactophilic yeasts, like those associated with ripe fruits, are mostly ascomycetes and their anamorphs. They are, however, mostly restricted to specific cactus habitats, and community identities are maintained to a large degree by specific insect vectors. These yeasts have been studied extensively on a worldwide basis. Cactus yeast communities in Brazil are similar to those reported for other regions, although a number of new biotypes have been found (19, 27, 28, 29, 34). Although cactophylic yeasts dominate cactus rot microhabitats, cactus fruits have less specialized communities which are more typical of other fruits.

The fruits studied here are economically important as a regional food source at the beginning of the summer and cashew nuts are also an important product for export. The shelf life of the fresh fruits is very limited, resulting in considerable

loss due to spoilage, and they are frequently consumed as juice or conserved in dried or candied form. Yeasts are among the microbes expected to be involved in spoilage of these fruits. Traditional isolation methods have been criticized for not detecting the true level of yeast populations in plant substrates or their diversity (14). In this study we applied aggressive pretreatments and different media in the isolation procedures to describe yeast communities associated with some economically important fruits typical of the Northeastern region of Brazil.

MATERIALS AND METHODS

Ripe cashew (*Anacardium occidentalis)* and mango (*Mangifera indica*) fruits were collected asseptically in sterile plastic bags in markets of Copacabana in the city of Rio de Janeiro. Collections of flowers, green fruits and ripe fruits of cashew, cajá, (*Spondia lutea*), and umbu (*Spondia* sp.) were also made in the field near Campina Grande and João Pessoa, state of Paraíba, Brazil. The material was transported on ice to the laboratory and processed within 5 hr. Whole fruits were surface washed by shaking (120 RPM; 20 min) in 100 ml of sterile 0.05% Tween detergent in distilled water or of 0.85% NaCl + 0.05% Tween 40 (saline-Tween) solution, and the wash suspension was then blended for 1 min. After this procedure, market fruits had their skins removed asseptically and samples of skin and pulp were blended separately in 10 volumes of sterile saline-Tween solution. Yeasts were isolated on GYP medium containing 2% glucose, 1% peptone, 0.5% yeast extract, 2% agar medium and 200 mg/l of chloramphenicol, pH 3.7, and Vitamin Free Yeast Base (VFYB) with 2% agar and 200 mg/l chloramphenicol. Incubation was carried out at 26 ± 3°C for 3 to 5 days. Representative colonies were selected and streaked out on GYP agar to obtain pure cultures. These were grown on GYMP agar (Glucose 0.5%, Yeast Extract 0.5%, Malt Extract 2%, monobasic Sodium Phosphate 0.2%, Agar 2%) at 26± 3°C for about 3 days and stored under sterile mineral oil at 8± 4°C. Yeasts were characterized and identified according to conventional methods, using a multityped inoculating device for most of the growth tests (1, 8, 11).

RESULTS

Yeast counts continued to increase with up to 8 min of shaking by hand, and stable counts were reached only after 20 min of shaking on a rotatory (120 RPM) or reciprocal (250 RPM) shaker. The detergent Tween apparently facilitated dispersion of the yeasts and maintained them dispersed. Blending the washings after removal of the fruits increased counts by 2 to 461%. Blending of asseptically removed skin and pulp after surface washing demonstrated the presence of yeasts that were well attached to the fruit surface or had penetrated through the skin. Maximum yeast counts from surface washings were obtained by processing in sterile 0.05% Tween 40 in water or 0.85% NaCl, with shaking on a rotatory shaker (120 RPM; 20 min) followed by blending of washings for 1 min. VFYB did not support growth of *Kloeckera* and *Hanseniaspora* spp which facilitated the recovery of other yeasts and generally resulted in higher total yeast counts than those obtained on GYP medium. Yeast species and the number of cultures isolated from different fruits and flowers of field and market sources of the state of Paraíba are presented in TABLE 1. Yeast counts for the collections of cashew, cajá and umbu fruits in Paraíba are listed in TABLE 2. Yeast species and their percentage of the counts for surface washings, skin, and pulp of cashew and mango fruits from markets of Rio de Janeiro are noted in TABLE 3.

The yeast communities of flowers were dominated by black yeasts and basidiomycetous anamorphs, especially of *Rhodotorula spp.* and *Cryptococcus laurentii*. These were apparently generalist species associated with normal plant surfaces and do not necessarily need to be vectored from other sources. The Ascomycete *Metschnikowia pulcherrima* also prevailed in cashew flowers and was probably vectored by the bees from which it was also isolated. Yeasts associated with green fruits were mostly the same species present on flowers. A notable exception was the prevalence of a *Candida entomaea* -like species on green cajá fruits. There was a dramatic change in the yeast community of ripe cajá fruits compared to that of green fruits and flowers. The prevalent yeast species changed to mostly ascomycetous species, including *Candida entomaea* -like, *Issatchenkia orientalis*,

TABLE 1. Yeast isolates of field flowers, green and ripe fruits and marketed fruits of Cashew, Caja, and Umbu. The samples studied were collected in the state of Paraíba, Brazil.

		Cashew			Caja			Umbu		
		flower	fruit		flower	fruit		flower	fruit	
Yeast species	DBB[1]		green	ripe		green	ripe		green	ripe
Candida spp	+	[1][2]	(1)[3]		4				1	
Cryptococus laurentii	+	12 [2]	5 (24)	3	12	25	1	11	2	2
Cryptococcus spp.	+	2	2 (5)		1	1	2			1
Haltermannia corniformus	+				3					
Rhodotorula aurantiaca	+		(1)					1	1	
Rhodotorula glutinis	+	2	(3)		2			3	1	
Rhodotorula graminis	+	1								
Rhodotorula minuta	+	2	3 (4)	1	7	1	1		4	1
Rhodotorula rubra	+	1								
Rhodotorula spp	+	1			1					
Sporobolomyces roseus	+	1								
Tremella spp.	+	2	(1)		3					1
Black yeasts	-	12	16 (25)	6	2	4		6	6	2
Candida albicans	-								1	
Candida entomaea -like	-					17	12			
Candida guilliermondii	-					2	2			
Candida krusei	-						5			
Candida parapsilosis	-								1	
Candida shehatae	-								1	
Candida sorbosa	-						1			
Candida torresii -like	-					2				
Candida tropicalis	-						1			
Candida spp	-		(1)	1	2	4	3	6	4	
Debaryomyces sp	-								1	
Geotrichum sp	-					2				
Hanseniaspora occidentalis	-									2
Issatchenkia orientalis	-		(4)	1		2	14			2
Issatchenkia terricola	-					2	8		1	
Kloeckera apiculata	-						6			
Kloeckera javanica	-					1	15			
Kloeckera sp.	-						4			
Pichia etchellsii	-								1	
Pichia membranaefaciens	-			1						
Metschnikowia pulcherrima	-	7 [4]		1		2	5		2	
Metschnikowia sp	-								2	
Torulaspora delbruckii	-						1			
yeast like fungus	-						1			

1. DBB = Diazonium blue B test result, + indicatesthat the species is a basidiomycete, - indicates that the species is an ascomycete.
2. number in parenthesiss indicates isolates from bees associated with substrate
3. number in parenthesis indicates isolates from nut

Issatchenkia terricola, Kloeckera apiculata, Kloeckera javanica, and Metschnikowia pulcherrima. The proportion of basidiomycetous yeasts and black yeasts was also reduced relative to ascomycetous yeasts on ripe cashew and umbu fruits. Ripe and overripe cashew fruits from markets of Rio de Janeiro had yeast communities dominated by *Kloeckera apiculata*. This species prevailed in surface washings of the fruits and corresponded to an even greater proportion of the skin and pulp communities. Yeasts were not found within the pulp of sound ripe fruits collected in the field. Ripe mango fruits from markets of Rio de Janeiro showed a prevalence of *Candida krusei*, *Candida* spp., *Issatchenkia terricola* and *Kloeckera apiculata* in surface washings. The prevalent yeast

TABLE 2. Yeast counts from three tropical fruits of semi-arid regions of Northeastern Brazil

Substrate	Cashew	Caja	Umbu
Flower bud	$3.9x10^3$	ND	ND
Flower open	$6.0x10^6$	$2.4x10^3$	ND
Green fruit (early)	$1.8x10^4$	ND	ND
Green fruit (late)	$4.5x10^2$	$1.2x10^4$	$8.7x10^3$
Semiripe fruit	$8.3x10^2$	$7.2x10^4$	$3.0x10^3$
Ripe fruit (field)	$4.3x10^3$	$1.9x10^6$	ND
Ripe fruit (market)	$1.0x10^7$	$8.7x10^5$	$6.7x10^5$
green nut	$1.9x10^3$	ND	ND
semiripe nut	$2.4x10^4$	ND	ND
Bark of tree trunk	$1.7x10^3$	ND	ND
Soil under tree	$1.5x10^2$	ND	ND

Counts in colony forming units per g wet wt. determined on Glucose 2%, peptone 1%, yeast extract 0.5%, agar 2%, chloramphenicol 100 mg/ml, and pH 4.0 medium incubated for 5 to 7 days at 28°C

on the skin of washed mango fruits and in their pulp was *Candida krusei*.

DISCUSSION

Our results confirm the importance of aggressive pretreatment procedures for the isolation of yeasts from fruits and flowers, as recommended by Martini *et al.* (14). Counts were increased by blending, probably as a result of dispersing the yeasts aggregated in microcolonies or attached to particles in the suspension. Dissection separated some of the distinct fruit microhabitats and demonstrated that they contained different yeasts. The use of more than one isolation medium also helped in recovering a greater diversity of yeasts. Rich complex media like GYP or Y-M agar favored isolation of fast growing and more fermentative ascomycetous species and the synthetic medium VFYB facilitated the recovery of slower growing and more aerobic yeasts.

The succession whithin the yeast communities was similar to that noted previously for pineapples (26), passing from mostly oxidative basidiomycetous species and black yeasts typical of healthy plant surfaces and found on the flowers and green fruits to fermentative ascomycetous species on very ripe and degrading fruits. Yeast communities of healthy plant surfaces are apparently more stable and able to use a broader spectrum of resources compared with those of the more ephemeral, and rich in simple sugars, flower

TABLE 3. Yeasts from the surface wash, skin and pulp of cashew and mango fruits from markets of Rio de Janeiro, Brazil.

	Cashew			Mango		
Yeast	Surface	skin	pulp	Surface	skin	pulp
Brettanomyces custersianus -like	1.5[a]					
Black yeasts	4.5	0.1	0.4			
Candida berthetii -like	2.7					
Candida diddensii				5.4		
Candida krusei	0.6	0.8	1.1	23.1	76.5	52.3
Candida guilliermondii	7.6	1.3		3.8		4.0
Candida lusitaniae				6.4	13.4	6.3
Candida sorbosa				1.2		
Candida tropicalis	0.1	0.1	0.7			
Candida spp	3.2	7.2		18.9	4.1	8.0
Issatchenkia terricola	0.1			12.1		
Kloeckera apiculata	77.9	90.3	95.5	24.1	3.6	23.8
Lipomyces -like	0.6					
Rhodotorula rubra	0.3				1.0	1.6
Pichia burtonii -like	0.1					
Pichia membranaefaciens				3.2		
Pichia ohmeri	0.1					
Not identified	0.4			1.7	1.9	4.0
mean count in CFU/g	$1.5x10^6$	$6.4x10^6$	$8.3x10^5$	$2.4x10^5$	$1.3x10^3$	$4.0x10^2$

a = % of species population in yeast community

nectar and ripe fruit microhabitats. The yeasts of ripe and deteriorating fruits apparently corresponded to a typical succession of fast growing species with more limited physiological profiles, as noted by Morais *et al.* (21) for the Amapá fruit. The unicellular forms of these yeasts make them practically cultures of blastospores essentially oriented toward reproduction for rapid opportunistic colonization of ephemeral habitats. After these opportunist species have established and used the more readily available resources, then more competitive species with broader assimilation profiles and other competitive features like production of killer factors or resistance to ethanol can become established and replace them (21). The ascomycetous yeasts of flowers and fruits were probably vectored by insects. Pollinating insects like bees can vector associated yeasts to flowers as evidenced by the *M. pulcherrima* isolates from flowers and bees. Similar associations may exist between flowers and other pollinating animals such as bats and hummingbirds and they deserve further investigation. Senescent flowers can attract another set of insects that include the drosophilids vectoring associated yeasts, as observed by Lachance *et al.* (12) for tropical *Ipomea flowers* in Hawaii. At least the initial stage of fruit spoilage seemed to be dominated by yeast species that can be vectored by drosophilids, as was observed for the Amapá fruit and Brazilian forest ecosystems (17, 20, 21). In order to make a good description of the yeast communities of flowers and fruits, it is important to consider that the substrates can include a number of different ephemeral microhabitats, each having its own succession of species. A few samples taken at one stage of the succession will not necessarily give a good representation of the yeast communities associated with such habitats.

Rapid deterioration of tropical fruits is one of the factors that limit their commercial importance, especially outside the areas in which they grow. Pathogenic yeasts did not make up a major part of the populations associated with these fruits, making the problem, more one of esthetic factors that discourage purchasing rather than one of health hazard. Preventing access of insect vectors to the ripe fruits should help control deterioration by yeasts since the spoilage yeast community appeared to be of insect vectored species and was distinct from that present on green and sound ripe fruits.

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RESUMO

Leveduras associadas a flores e frutos na região do semiárido no nordeste do Brasil

As espécies de leveduras associadas a flores e frutos de cajueiro (*Anacardium occidentale)*; cajazeiro (*Spondia lutea);* umbuzeiro (*Spondia* sp).; e mangueira (*Mangifera indica*) da região nordeste do Brasil, foram determinadas no campo e no mercado. A metodologia usada para remoção das leveduras da superfície dos frutos, utilizando a agitação e homogenização em liquidificador, foi eficaz para a desagregação das células nesses substratos. Os frutos maduros foram dissecados assepticamente para permitir a separação entre as células presentes na superfície e as situadas no interior dos frutos. Para o isolamento das leveduras foram usados os meios de cultura: Glicose-Extrato de Levedura-Peptona e Meio Básico sem Vitaminas (Vitamin Free Yeast Base), ajustados a pH 3.7 e adicionados de cloranfenicol (200 mg/l). As comunidades de leveduras presentes nas flores e nos frutos (verdes e maduros) foram dominadas pelas "leveduras pretas" e anamorfos de basidiomicetos, especialmente da espécie *Cryptococcus laurentii*, que no entanto sofrem uma sucessão à medida que os frutos amadurecem, passando a predominar as espécies de ascomicetos e seus anamorfos. A espécie *Metschnikowia pulcherrima* foi frequentemente isolada em flores de caju e nas abelhas associadas a esse substrato. Uma espécie fenotipicamente semelhante à *Candida entomaea* foi prevalente em frutos de cajá coletados no campo. Nos frutos maduros foram prevalentes as espécies fermentativas de ascomicetos como *Issatchenkia orientalis* e *Kloeckera javanica* que utilizam poucos substratos. As amostragens efetuadas durante os diferentes estágios de floração e frutificação, o tratamento vigoroso no isolamento e a utilização de meios de cultura diferentes permitiram a determinação efi-

ciente da composição das comunidades de leveduras associadas às plantas.

Palavras-chave: leveduras, flores, frutos, métodos de isolamento, caju, cajá, manga, umbu.

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SURVEY OF MYCOTOXINS IN WHEAT AND WHEAT PRODUCTS SOLD IN HEALTH FOOD STORES OF THE CITY OF CAMPINAS, STATE OF SÃO PAULO

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ABSTRACT

Thirty-eight samples of whole wheat and its products (whole wheat: 4, whole wheat flakes: 1, wheat bran: 3, wheat germ: 4, gluten flour: 2, cracked wheat: 7, whole wheat flour: 5, whole wheat spaghetti: 11, semolina: 1) were purchased in health food stores of the city of Campinas, S.P., during 1991. The samples were analyzed for deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol (DAS), toxins T-2 and HT-2, T-2 triol and T-2 tetraol by a gas chromatographic method. A thin layer multitoxin method was employed to screen for aflatoxins B_1, B_2, G_1, G_2, ochratoxin A (OCHRA A), zearalenone (ZEN), and sterigmatocystin. The results were negative for the 14 toxins investigated.

Key words: wheat, aflatoxins, trichothecenes, ochratoxin A, zearalenone.

INTRODUCTION

Molds are, in many instances, useful to mankind. However, they can also cause food spoilage and produce secondary metabolites named "mycotoxins", which may bring about serious diseases in humans and animals. To date, a few hundreds of these compounds have been identified. Among them, aflatoxins have been by far the most studied and the most frequently detected mycotoxins in surveys around the world. These are highly toxic compounds that have the liver as main target organ and exhibit teratogenic, mutagenic and carcinogenic activity. Aflatoxins are produced by *Aspergillus flavus* Link and *A. parasiticus* Speare (1).

In general, the so called "health foods" receive less or no processing when compared to other food items. Their keeping qualities, thus, are expected to suffer. On the other hand, weather and storage conditions in the tropics are believed to be conducive to mycotoxin contamination. Cereals have been the subject of surveys in many countries, involving mostly trichothecenes (5, 7, 9, 17, 18, 19, 21). To our knowledge, only one of such surveys focused on health foods (16). In Brazil, seventy samples of wheat and wheat products were examined for deoxynivalenol by a thin layer chromatographic method (8). Wheat freshly harvested in the State of São Paulo (20 samples) (4) and wheat stored in silos in the State of Rio Grande do Sul (18 samples) (3) were analyzed for 7 trichothecenes, aflatoxins, ochratoxin A and zearalenone. Besides the mentioned surveys, further studies on wheat products have not been carried out in our country concernig mycotoxin contamination and products sold in health food stores have not been examined so far. Trying to fill part of this gap, the present work was undertaken to verify the incidence of mycotoxins in wheat and wheat products sold in health food stores of the city of Campinas, State of São Paulo, during the year of 1991.

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MATERIALS AND METHODS

Sampling and sample treatment

Thirty-eight samples of whole wheat and its products, corresponding to 14 different brands, were purchased in health food stores of the city of Campinas, São Paulo (TABLE 1). Each sample weighed at least one kilogram.

Determination of trichothecenes

The samples were analyzed for deoxynivalenol, nivalenol, diacetoxyscirpenol, toxins T-2 and HT-2, T-2 triol and T-2 tetraol by gas chromatography. Samples were extracted with methanol/4% KCl (9 + 1). The cleanup involved clarification with ammonium sulfate, partition to chloroform, and passage through a charcoal/celite/alumina column, as described by Furlong and Soares (2). Some modifications were introduced for derivatization of the extract. Briefly, 200 ml of a pyridine-acetic anhydride mixture (1:1) were added to a 4 ml amber vial containing the dried extract. The flask was closed and left in a 60°C water bath for 2 hours. The flask was then brought to room temperature, uncapped, and the mixture taken to dryness under a nitrogen stream. Next, 100 µl of hexane were added and the mixture was sonicated for 30 seconds. Standards were submitted to a similar procedure. Samples and standards were run in a gas chromatograph using a JW Scientific DB-225 (cyanopropylmethyl-phenylpolysiloxane, 50:50), 0.25 mm film thickness, 15m x 0.33 mm i.d. capillary column on a Varian instrument, model 3300, with a flame ionization detector, oven temperature program 100°C(1 minute), 6°C/min to 220°C, 220°C (20 minutes). Detection limits were 0.1 mg/g for DAS, T-2 and NIV, 0.2, 0.3, 0.4 and 0.5 mg/g for DON, T-2 triol, T-2 tetraol, and HT-2 toxin, respectively.

Determination of aflatoxins, ochratoxin A, zearalenone, and sterigmatocystin

A thin layer chromatographic method (13, 14) was employed for simultaneous detection of aflatoxins B_1, B_2, G_1, G_2, ochratoxin A, zearalenone, and sterigmatocystin. It involved extraction with methanol/4% KCl (9+1,) followed by clarification with ammonium sulfate, and partition to chloroform. Detection limits were 2, 5, 15 and 35 ng/g for aflatoxin B1, ochratoxin A, zearalenone and sterigmatocystin, respectively.

RESULTS AND DISCUSSION

The results were negative for the 14 compounds investigated in all the commodities examined (TABLE 1), possibly indicating that contamination by mycotoxins may not be so widespread in the tropics as believed. FIGURES 1 and 2 show gas chromatograms of an uncontaminated sample and of an acetylated T-2 triol standard. A great amount of work remains to be done before a picture of mycotoxin contamination of Brazilian foods may be drawn. The data we have at present indicate that our wheat and its products are equally or less contaminated than samples from other parts of the world where wheat destined for human consumption has already been studied in terms of trichothecenes and zearalenone incidence. For instance, retail cereal flours marketed in Japan had 26 out of the 36 samples of wheat flour contaminated with DON (2 - 29 ng/g), and 12 with NIV (4 - 84 ng/g). Twenty seven of the wheat flour samples were also examined for ZEN and 3 were positive (1 - 6 ng/g) (16). In China, 10 samples of whole wheat were analyzed and 10 contained DON (73 - 1051 ng/g), 8 NIV (8 - 373 ng/g) and 2 ZEN (5 - 25 ng/g) (5). In the U.S., 123 samples of wheat were examined

TABLE 1. Wheat and wheat products sold in health food stores and analyzed for mycotoxins[a] during the year of 1991 in the city of Campinas, S.P.

Type of product	number of samples
Whole wheat	4
whole wheat flakes	1
wheat bran	3
wheat germ	4
gluten flour	2
cracked wheat	7
whole wheat flour	5
whole wheat spaghetti	11
semolina	1
TOTAL	38

[a] - deoxynivalenol, nivalenol, diacetoxyscirpenol, toxins T-2 and HT-2, T-2 triol and T-2 tetraol, aflatoxins B_1, B_2, G_1, G_2, ochratoxin A, zearalenone, and sterigmatocystin.

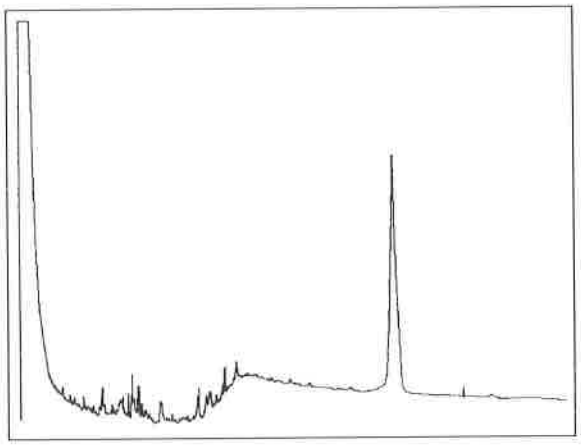

FIGURE 1. Uncontaminated sample of wheat after acetylation, examined for trichothenecenes by gas chromatography on a JW Scientific DB-225 (cyanopropylmethyl- methylphenylpolysiloxane, 50:50), 0.25 mm film thickness, 15m x 0.33 mm i.d. capillary column on a Varian instrument, model 3300, with a flame ionization detector, oven temperature program 100°C(1 minute), 6°C/min to 220°C, 220°C (20 minutes).

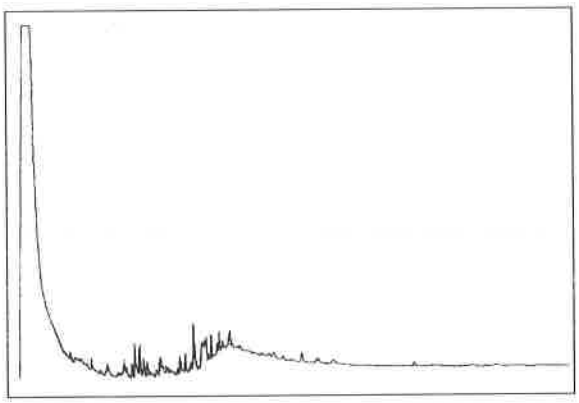

FIGURE 2. Gas chromatogram of an acetylated T-2 triol standard run under the same conditions described in FIGURE 1.

for DON and the toxin was detected in 75 of them (100 - 2.290 ng/g) (21). In Germany, 5 out of 80 samples and 7 out of 81 samples analyzed contained DON (10 - 1300 ng/g) (18) and ZEN (15 - 2000 ng/g), respectively. Tanaka *et al.* (17) examined samples of cereals from 19 countries and found that out of the 222 samples of wheat analyzed 87 were contaminated with DON (average of 488 ng/g DON). They also found that 69 of the samples contained ZEN (mean of 23 ng/g) and 111 contained NIV (mean of 127 ng/g). In the former U.S.S.R., where 81,3% of the wheat analyzed contained DON (150 - 13.900 ng/g) (19), the situation may be considered particularly serious. In contrast, the present work detected none of the seven trichothecenes nor zearalenone in the samples studied. A previous survey conducted in our country showed that two samples of wheat bran were contaminated with 183 ng/g DON out of 70 samples of wheat products examined (8). Freshly harvested wheat from São Paulo (20 samples), produced during an unusually wet and cold growing season, was analyzed in our laboratory and six samples were found to be contaminated with trichothecenes, four with DON (470 - 590 ng/g), three with NIV (160 - 400 ng/g), two with T-2 toxin (400 - 800 ng/g), one with DAS (600 ng/g) and three with ZEN (40 - 210 ng/g) (4). These results show that contamination may occur under unusual weather conditions but that it may not be the rule in our generous tropical climate. Still in another survey conducted in our laboratory, DON was found in one sample of Brazilian wheat (400 ng/g) collected from silos of the state of Rio Grande do Sul. DAS (300 ng/g, one sample), toxin T-2 (350 - 360 ng/g, two samples) and T-2 tetraol (1.680 ng/g, one sample) were found in imported wheat stored in silos of that state (3). Few countries have enacted regulations for these toxins in foods. The former U.S.S.R. had a maximum tolerated level of 1000 ng/g for DON. Canada has a guideline for DON of 2000 ng/g for uncleaned soft wheat (20). Brazil has no regulations on trichothecenes levels in foods or feeds.

No aflatoxins and ochratoxin A were detected in 102 samples of wheat analyzed in the U.S. but zearalenone was found in 19 of them (360 - 11.050 ng/g) (10). In another study which took place in the State of Virginia, U.S., during a 5-year period, 100 samples of wheat were analyzed and no aflatoxins, ochratoxin or zearalenone were found (11). Recently, a review by Pohland *et al.* (6) reported a 1.3% rate of incidence of ochratoxin A in wheat from the U.S., based on data from 848 samples (15 - 115 ng/g). Speijers and Van Egmond (15) reviewed the worldwide incidence of ochratoxin A and state that contamination of wheat in European countries ranges from 1.6% in Germany (0.4 ng/g) to 72% (>1 ng/g) in former Czechoslovakia. In India, a survey conducted over a three-year period showed 54 samples of wheat flour to be contaminated with aflatoxins (20 - 2000 ng/g) out of 185 analyzed (12). In another study also conducted in India, 114 samples of wheat and its products were examined

for aflatoxins and trichothecenes. No aflatoxins were detected but 24 of the samples were contaminated with either DON, Acetyl-DON or T-2 toxin (7). In Brazil, only one sample so far has been found contaminated with ochratoxin A (40 ng/g) and no aflatoxins were detected in the 32 samples of wheat analyzed (3,4) in previous studies and in the 38 samples of wheat and its products examined in the present work.

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RESUMO

Levantamento da incidência de micotoxinas em trigo e produtos de trigo comercializados em lojas de produtos naturais na cidade de Campinas, Estado de São Paulo

Trinta e oito amostras de trigo integral e produtos derivados (trigo intergral 4, trigo integral laminado 1, farelo de trigo 3, germe de trigo 4, farinha de glúten 2, trigo para quibe 7, farinha de trigo integral 5, macarrão de trigo integral 11, semolina 1) foram adquiridos em lojas de produtos naturais na cidade de Campinas, S.P., durante o ano de 1991 e foram analisadas para deoxinivalenol (DON), nivalenol (NIV), diacetoxiscirpenol (DAS), toxinas T-2 and HT-2, T-2 triol and T-2 tetraol por cromatografia gasosa. Foram também analisadas para aflatoxinas B_1, B_2, G_1, G_2, ocratoxina A (OCHRA A), zearalenona (ZEN), e esterigmatocistina por cromatografia em camada delgada. Os resultados foram negativos para as quatorze micotoxinas procuradas.

Palavras-chave: trigo, aflatoxinas, tricotecenos, ocratoxina A, zearalenona.

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CANDIDA ALBICANS BYOTIPES ISOLATED FROM THE ORAL CAVITY OF HIV-POSITIVE PATIENTS

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ABSTRACT

Candida albicans has been frequently isolated from several infectious processes among patients with immunosuppressive illnesses, patients submitted to transplants and/or treatment with antibiotics and HIV positive individuals. The virulence mechanisms by which this yeast expresses its pathogenicity include adherence patterns, production of extracellular enzymes and ability to form pseudomycelia, among others. In this work, the behavior of 50 samples of *Candida albicans* isolated from oral lesions of HIV positive patients were studied with respect to serotype, production of proteinase and phospholipase and sensitivity to killer toxins. Serotyping was performed by the agglutination method using a monospecific serum (serotype A). The proteolytic and phospholipase activities were determined using bovine serum albumin (Fraction V) medium and Sabouraud Dextrose Agar with egg yolk. The killer system with 9 standard strains was utilized.

Most of the samples studied belonged to serotype A (70%). Production of proteinase and phospholipase was detected in 96% and 90% of the samples, respectively. Concerning susceptibility to killer toxins, 10 different types were observed, with prevalence of biotype 211 (64.5%).

Key words: *Candida albicans*, oral cavity, HIV+ patients, mechanisms of virulence.

INTRODUCTION

Human immunodeficiency virus-infected individuals (HIV positive) are predisposed to a great number of fungal infections due to the profound functional alterations that take place in their T cell immune compartment.

The microbiota of the oral cavity comprises, among other microorganisms, yeasts of the genus *Candida* that are typically opportunistic and can be found in 10 to 50% of healthy individuals. Favorable conditions can transform these yeasts into pathogens. (8).

Among the various species of the genus, *Candida albicans* plays a relevant role as a causative agent of mycotic diseases, of which the mucosal forms are the commonest and earliest. Oral candidiasis develops in 90 to 95% of symptomatic HIV-infected individuals and its prevalence increases in parallel with the severity of the immune dysfunction. The occurrence of

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oral candidiasis was described since the first cases of Acquired Immunodecifiency Syndrome (AIDS) were reported. It constitutes an important clinical sign for diagnosis as well as an indicator of the evolution of immunodeficiency among HIV carriers (8).

Candida albicans has been divided into two distinct serotypes (A and B) according to agglutination reactions with specific antisera. It can produce two types of enzymes, a proteinase and a phospholipase, that have been correlated with virulence in recent years. This yeast can also be susceptible to "killer" toxins, a feature which is used as an important epidemiological marker (21, 24, 25, 27, 34, 35). Over the last years, several authors have been demonstrating the need to include additional characterizations such as morphotyping, evaluation of other enzymes (hyaluronidase and chondroitin-sulphatase) and chromosomal DNA analysis (5, 16, 28, 33) for sub-species differentiation and possible correlation with high and low virulence of the yeast.

The aim of the present study was to isolate yeasts from the oral cavity of HIV-positive patients presenting oral lesions. Fifty strains of *C. albicans* were then serotyped and their virulence studied through the production of proteinase and phospholipase as well as susceptibility to "Killer" toxins.

MATERIALS AND METHODS

Samples were collected from HIV positive patients, including users of endovenously injected drugs, homosexuals and heterosexuals, all being attended at the Centro de Referência e Treinamento de AIDS - São Paulo, SP, Brazil.

Oral yeast isolates were obtained from various sites using sterile swabs previously wetted in saline solution, predominantly from regions were the oral mucosa presented alterations clinically compatible with candidiasis (jugal mucosa, lateral edge and dorsum of tongue). Specimens were received on Sabouraud-dextrose agar containing 200mg/L chloranphenicol and kept at 25°C for 10 days. After growth, yeast identification was done according to routine mycological techniques (14).

C. albicans samples were streaked onto Sabouraud-dextrose agar, incubated for 10 days at 25°C and the fringe characters (presence or absence) and streak surface features (smooth or wrinkled) of the colonies subsequently recorded.

Fifty samples positive for *C. albicans* isolated from 46 HIV positive patients were analyzed with respect to serotype, production of proteinase and phospholipase and susceptibility to "killer" toxins.

Type A monospecific antiserum was provided by the Mycology Section of the Microbiology Department, Instituto de Ciências Biomédicas, Universidade de São Paulo. The test was carried out according to the agglutination technique on glass slides and direct reading with the naked eye.

Production of the exoenzymes proteinase and phospholipase was detected as described by Ruchell *et al.* (30) and Price *et al.* (27), respectively. The enzymes were classified according to Silveira *et al.* (36).

Strains of *C. albicans* were biotyped and differentiated according to the "killer" system described by Polonelli *et al.* (25).

RESULTS

On macroscopic examination, 68% of the colonies were smooth with no fringes, 18% were wrinkled with no fringes, 8% were wrinkled with fringes and 6% were smooth with fringes.

The frequencies of *C. albicans* serotypes isolated from the oral lesions were 70% (35 out of 50) for serotype A and 30% (15 out of 50) for serotype B (FIGURE 1).

With respect to exoenzyme production, 96%

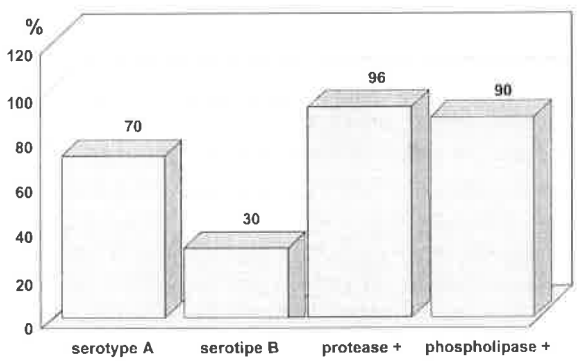

FIGURE 1. Frequencies of serotypes and proteinase and phospholipase production by oral *C.albicans* isolates from HIV + patients. (Total of samples = 50)

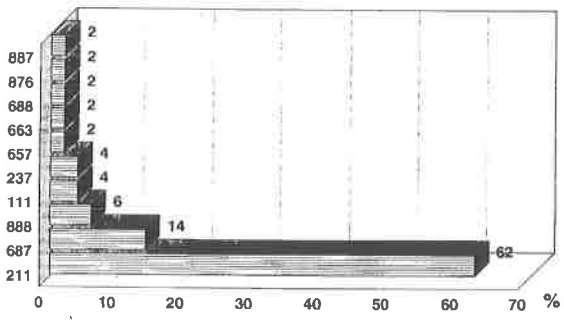

FIGURE 2. Distribution of killer strain types among oral isolates of *C.albicans* (Total of isolates = 50)

of the sample secreted proteinase (of which 84% were intermediate producers, 8% were high producers and 4% were low producers) and 90% secreted phospholipase (of which 22% were high producers, 66% were intermediate producers and 2% were low producers) (FIGURE 1).

No secretion of proteinase and phospholipase was detected in 4% and 10% of the samples, respectively.

Evaluation of yeast susceptibility to "killer" toxins revealed the following 10 biotypes, with frequencies included in parenthesis: 211 (62%), 678 (14%), 888 (6%), 111 (4%), 237 (4%) and also 663, 688, 876, 657 and 887 (each corresponding to 2% of the samples), (FIGURE 2).

DISCUSSION

Within the genus *Candida*, the yeast *Candida albicans* is the most frequently associated with lesion development. This species is part of the normal microbiota of man, found in de buccal cavity of half of the healthy carriers and in association with a wide array of microorganisms, including bacterial species. The coexistence of such microbial population with an individual stars at birth. It involves the action of immunological mechanisms in a continuous process of adaptation and readaptation that ensures the saprophyte condition of the microorganism and a state of balance between it and the human carrier (29).

The morphological variability of *C. albicans* was demonstrated by Phongpaichit *et al.* (23) and Hunter *et al.* (13) based on colony morphology. In the present work, morphotyping was not accomplished according to the techniques described by these authors, but the smooth or wrinkled surface appearance as well as presence or absence of fringes was recorded for each colony. Of the samples examined, 68% exhibited fringes. This result does not agree with the finding of a high incidence of fringeless colonies reported by Oliveira *et al.* (20), which was, however, observed in clinically healthy individuals. Hunter *et al.* (13), and Oliveira *et al.* (20) correlated highly fringed morphotypes with virulence of the isolates, a feature that should facilitate adherence of the yeast to epithelial cells. It is worth noting that, in 1968, the work of Brown - Thomsen (2) demonstrated that colony morphology depends on the composition of the culture medium as well as the partial pressure of oxygen.

The mophological characteristics observed were more evident in cultures kept at 25°C.

Since *C. albicans* is the most important species of the genus, a study of its biotypes is a requirement that allows more detailed characterizations of isolated strains.

C. albicans has been divided into serotypes A and B. The distinction relates to differences in the mannan structure of the yeast's cell wall. In this work, 70% of the samples studied were identified as type A, in agreement with results described by others (21). On the other hand, Martins and Lamb and Angel *et al.* (17) consider that the occurrence of this serotype is a consequence of its selection against serotype B *in vivo*.

The finding that 30% of the samples examined belonged to serotype B matches with or is similar to data on HIV positive and immunodeficient patients from other parts of the world. For example, Odds *et al.* (19), Drohuet *et al.* (7), and Dupont *et al.* (9) described frequencies of 31.2%, 43% and 50%, respectively. According to Poulain *et al.* (26), a possible explanations for the increase in frequency of serotype B in the latter studies could be related to the fact that strains of this serotype can be induced to express, in vivo, antigenic determinants belonging of type A.

The production of extracellular enzymes can reflect *C.albicans* degree of pathogenicity, taking into account the clinical evaluation of the patient and laboratorial findings.

In this study, 96% of the samples analyzed produced the exoenzyme proteinase. Other studies carried out in Brazil showed that this activity was present in about 53 to 100% of *C. albicans* cultures

(20, 22, 28, 34, 35, 36). According to international data, proteinase activity was detected in 16 to 100% of the samples studied (6, 11, 38).

Of the proteinase-secreting samples, 84% were intermediate producers according to the classification of Silveira *et al.* (36). Ghannoum and Radwan (12) maintain that a strain's ability to secrete this enzyme does not ensure its virulence. Our data are in agreement with these results. Chakrabanti *et al.* (6) have associated a high production of proteinase *in vitro* with the occurrence of candidiasis and emphasize the importance of the kind of proteinase detected rather than the amount of enzyme produced, a feature that can be essential to the characterization of a pathogen.

Germaine *et al.* (10), based on the denaturing of *Candida albicans* proteins at high pH values (above 6), have suggested that proteinases do not contribute significantly to the pathogenesis of candidiases, especially the oral ones. Evidence presented by Germaine *et al.* (10) and Samaranayake *et al.* (31), on the other hand, shows that the human buccal cavity can offer appropriate conditions for the secretion, activity and stability of proteases from *C. albicans*. Additionally, the saliva of some patients suffering from immunodeficiency can become more acid under certain circumstances thus leading to increased growth of yeast populations as well as providing conditions of susceptibility to enzyme activity. Another important aspect of the pathogenic potential of this type of enzyme has been observed with neutral proteinases secreted by *C. albicans*, when proteolysis occurred at near neutral pH values (15).

The enzyme phospholipase acts on membrane phospholipids of the host and thus can cause the invasion and damage of susceptible tissues, thereby allowing the penetration of yeasts and their hyphae. In the present study, phospholipase activity was detected in 88% of the samples examined, whereas values ranging from 46 to 100% were described by Price *et al.* (27), Samaranayake *et al.* (32), Williamson *et al.* (37), Shimizu *et al.* (34), Quirino *et al.* (28), Silveira *et al.* (36), Candido *et al.* (3) and Oliveira *et al.* (20). Our results show that 66% of the yeasts were intermediate producers of this enzyme. According to Ghannoum and Radwam (12), strains which are high producers of this phospholipase have an increased power of colonization. Odds (18), in 1988, concluded as undoubtedly true that distinct types of *C. albicans* associated with human infections differ greatly in their ability to cause disease.

Recently, "killer" toxins (toxins produced by *Saccharomyces cereviseae* which are lethal to some yeasts) have proved to have good applicability as epidemiological markers. Our biotyping identified 10 different biotypes, 4 of which were detected in Brazil by Candido (3) and by Oliveira *et al.* (20). Polonelli *et al.*, Caprilli *et al.* (4, 25) and Zaror *et al.* (38) recorded, respectively, 25, 7 and 48 different biotypes of *C. albicans* isolated from the human buccal cavity that were susceptible to "killer" toxins.

Biotype 211 was not found to be common in studies carried out by Polonelli *et al.* (25) and Zaror *et al.* (38) yet it has been detected more frequently in recent investigations (3, 20).

Oliveira *et al.* (20) state that the 211 *C. albicans* biotype can coexhist in harmony within the oral cavity of healthy carriers yet under conditions that bring about an unbalance of the normal local microbiota. Due to either physiopathological or iatropharmacogenic causes, *C. albicans* populations can become opportunistic and promote oral candidiasis. Therefore, the definition of biotypes is also very important to our understanding of the pathogenesis of oral candidiasis. Such characterization bring about conditions for adequately geared treatments, with the obtention of good responses to drugs and consequent cure of the infection. This procedure should be emphasized considering the relative susceptibility of HIV carriers to *C. albicans* infections, that may vary in pathogenicity depending -on the degree of immunodeficiency of each individual. This complex relationship can also be evaluated by other factors such as adherence, DNA expression patterns and RNA formation.

Finally, is worth remembering that other factors related to yeasts and/or hosts must be simultaneously taken into account.

RESUMO

Biotipos de *candida albicans* isoladas da mucosa bucal de pacientes HIV positivos

Candida albicans tem sido freqüentemente isolada de processos infecciosos de pacientes com

doenças imunossupressoras, como a AIDS, e em indivíduos transplantados e/ou submetidos a antibioticoterapia prolongada. Os mecanismos de virulência pelos quais esta levedura expressa sua patogenicidade incluem: aderência, produção de enzimas extracelulares e habilidade de formar pseudomicélio, entre outras. A sorotipagem foi realizada pelo método de aglutinação com soro momoespecífico (sorotipo A). A pesquisa das enzimas proteinase e fosfolipase foi realizada utilizando meio com soro albumina bovina (Fração V) e Agar Sabouraud Dextrose acrescido de gema de ovo. No sistema Killer foram utilizadas 9 cepas padrões. A grande maioria das cepas estudadas foi sorotipo A (70%). A produção das enzimas proteinase e fosfolipase foi positiva em 96% e 90% das amostras, respectivamente. Em relação às toxinas Killer, 10 diferentes tipos foram observados, sendo o biotipo 211 prevalente em 64,5% das amostras estudadas.

Palavras-chave: *Candida albicans*, mucosa bucal, HIV positivos, mecanismos de virulência.

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PURIFICATION OF STAPHYLOCOCCAL ENTEROTOXINS B AND C_2 BY DYE LIGAND CHROMATOGRAPHY - PRODUCTION OF ANTISERA

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ABSTRACT

Enterotoxins B and C_2 were purified from sac culture supernatants by a combination of Amberlite CG-50 (cation exchange) chromatography and Red A ligand chromatography. One-hundred milliliters of initial supernatant fluid containing 270 µg per milliliter of SEB and 150 ml of supernatant fluid containing 93 µg per milliliter of SEC_2 gave overall yields of 59% and 42%, respectively. The purified toxins were homogeneous when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Rabbits were immunized with SEB and SEC_2. Maximal titers were 80 for SEB and 40 for SEC_2. The methodology described herein can be employed to purify SEB and SEC_2 for use as reagents and as potent immunogens by processing low initial volumes of culture supernatants.

Key words: staphylococcal enterotoxins, purification, chromatography, antisera.

INTRODUCTION

Staphylococcal enterotoxins (SEs) are low molecular weight proteins (27,000 to 30,000 daltons) that can cause food poisoning when ingested. Seven serologically and biochemically different enterotoxins (SEA to SEE) have been identified and purified (2). SEC was divided into three subtypes according to minor epitopes. The proteins are relatively thermostable and thus may be present in foods in the absence of the bacterium. In addition to their ability to cause gastroenteritis, these enterotoxins can bind directly to MHC (Major Histocompatibility Complex) class II molecules and function as superantigens that induce MHC-unrestricted T cell stimulation (11). Besides the increasing interest in their mode of action, emphasis must be given to the methods of identification and quantification of SEs, which require highly purified standard enterotoxins and specific antibodies. Immunization schedules have been studied for the production of antiserum against these enterotoxins, as reported by Casman and Bennett (4) and Shinagawa *et al.* (16).

A number of procedures involving the combined use of cation-exchange resins and gel filtration have been proposed for enterotoxin purification. The methods are generally time consuming and with recovery values varying around 35% for SEA (6), 50-60% for SEB (15) and 40% for SEC_2 (1). Recently, Brehm *et al.*(3) described a single step chromatography for large scale purification of SEA, SEB, and SEC_2 using a dye ligand as matrix. Lopes *et al.* (9) employed this procedure to purify SEA, but a second purification step had to be introduced in the case of SEB and SEC_2 to optimize the recovery values. The purpose of this study was to purify SEB and SEC_2 using dye

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ligand chromatography and to raise specific antisera against SEA, SEB and SEC_2.

MATERIALS AND METHODS

Production of SEs

Microorganism - Staphylococcus aureus strains S-6 (high SEB producer and low SEA producer) and FRI-361 (SEC_2) and standard SEs were provided by Prof. Dr. Merlin S. Bergdoll, University of Wisconsin, U.S.A. Cultures were stored on nutrient agar (Difco Laboratories, Detroit, MI) at 4°C.

Culture Medium. The culture medium used for the production of SEB and SEC_2 was Brain Heart Infusion (BHI, Difco).

Production of SEs. The sac culture method of Donnelly *et al.* (7) was used with modifications (10). Bacterial cells were removed by centrifugation at 12,000 x g for 30 minutes and the supernatant fluid sterilized by filtration with a Seitz filter. Culture filtrates were combined and concentrated overnight at 4°C with polyethylene glycol 15-20,000 (PEG) (Sigma Chemical Co., St. Louis, MO). The sacs containing concentrated enterotoxin were washed several times with distilled water and 0.02 M sodium phosphate buffer at pH 5.6.

Purification Procedure

SEA

The SEA was purified as described by Lopes *et al.*(9).

SEB and SEC_2

Step I. In order to purify SEB and SEC_2, a first step purification was performed according to Chang and Bergdoll (5). A volume of 100 to 150 ml of concentrated culture fluid was adjusted to pH 5.6 with 6 N HCl . Approximately 20 ml of Amberlite CG-50 resin (Sigma Chemical Co., St. Louis, MO) in 0.02 M NaHPO4 buffer pH 5.6 were added and the final volume made to about 700 ml with distilled water. The mixture was stirred for 1h at room temperature. After 30 minutes, the liquid was separated from the resin and discarded. The resin was packed into a chromatographic tube (2,2 x 10 cm) and washed with 200 ml of distilled water. The toxin was eluted with 0.5 M NaHPO4 (pH 6.2) in 0.5 M NaCl at flow rate of 2 ml/4 min (one fraction). Fractions containing the toxin were combined and concentrated with PEG for 24 h, in order to reduce the final volume to 10-15% of the original volume.

Step II. Dye ligand chromatography. One hundred ml of Red A gel (Amicon Corp.) were washed three times with equilibration buffer (5 mM $KHPO_4$, pH 6.8 for SEB purification and 10 mM $KHPO_4$, pH 6.5 for SEC purification). The gel was treated with 100 ml of 8 M urea containing 0.5 M NaOH, transferred to a sintered-glass funnel assembled for suction and washed with several volumes of equilibration buffer. This procedure was repeated one more time. The gel was placed in a chromatographic tube (2,2 x 37 cm) and the concentrated culture fluid containing SE was applied. The column was washed with two to three volumes of equilibration buffer and the SE eluted with a stepwise increase in the molarity of the $KHPO_4$ buffer (60 - 120 - 300 mM, pH 6.8 to SEB and 60 - 150 - 300 mM, pH 6.5 to SEC) at a flow rate of 5 ml/4 min. Fractions (5 ml) were collected and their protein content measured by absortion at 280 nm. The SE content was determined by radial immunodiffusion (12) and the fractions containing SE were pooled. The chromatography was performed at 4°C.

SDS-PAGE. The purity of the enterotoxin was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide gels according to the method of Laemmli (8).

Production of antisera

Staphylococcal enterotoxins. Purified staphylococcal enterotoxins A, B, and C were used for production of the antisera.

Rabbits. Female white rabbits (2 to 2,5 Kg) were purchased from a local producer.

Preparation of inocula. Increasing doses of staphylococcal enterotoxins were mixed to a hard emulsion with Complete Freund's adjuvant (Difco). One milliliter of the emulsion was injected subcutaneously into the shoulder area of the rabbit.

Immunization procedures. The immunization schedule was that recommended by Robbins and Bergdoll (13). The rabbits were injected with increasing doses of enterotoxin. After each injection, the animals were observed during the two following days to check for changes such as diarrhea or apathy. Small amounts of enterotoxin were used in the initial injections until the rabbit undiluted serum gave a precipitin line in a double gel diffusion plate with 2 µg/ml of enterotoxin. The threshold injection was followed by 2 or 3 boosters containing 2 to 5 times the amount of antigen of the preceding injection; immunization was completed within 8 days (TABLE 1). Test bleedings were taken weekly from the marginal ear vein until the serum antibody titer approached 20, after which bleedings of 30 ml were obtained from the ear artery at regular intervals.

Determination of antibody titers. Antibody titers were determined by the optimum-sensitivity-plate (OSP) method of Robbins *et al.* (14).

RESULTS AND DISCUSSION

Purification of enterotoxins

SEB and SEC_2 were purified from sac culture supernatants by two chromatographic steps. An initial cation exchange chromatography on Amberlite CG-50 resin was followed by fractionation on Red A gel. The amount of SEB in the 100 ml of initial culture supernatant was approximately 270 µg/ml and the overall yield was 59%. SEB was eluted at the end of the 120 mM $KHPO_4$ buffer (pH 6.8). SEC_2 was purified from 150 ml of supernatant containing approximately 93 µg/ml of the enterotoxin. The overall process yield was 42% and SEC was eluted in a large peak with 150 mM $KHPO_4$, pH 6.5 buffer (TABLE 2). Pooled SEB and SEC_2 fractions were shown to be homogeneous by SDS-PAGE (FIG. 1).The purified enterotoxins were stored and used for immunization of rabbits.

In a previous work, the one-step chromatography on Red A gel was used to purify SEA and 50% of the amount applied in the column was recovered (9). However, it was not possible to obtain good results when purifying SEB and SEC_2 with this procedure, as reported by Brehm *et al.* (3). The sac culture method (10) can raise the total amount of soluble materials in supernatant fluids and an increase in growth and enterotoxin production was observed for *S.aureus*. However, higher concentrations of other extracelullar products must also occur and they remain mixed with enterotoxins in the culture supernatants. Strain S-6 is a high SEB producer that also secretes low amounts of SEA and this probably interferes with the purification of SEB.

In previous experiments using a one-step chromatographic method there was low recovery of enterotoxins B and C_2. The toxins were eluted in small peaks containing many impurities, as revealed by electrophoresis. Therefore, a fast and easy way

TABLE 1. Immunization schedules for staphylococcal enterotoxins.

Toxin type	Days										
	0	8	21	24	28	35	42	45	50	63	70
	1	2	5		10	20*	50	100	300		
SEA											
SEB	10			20	30*					60	300
SEC	10			20	30*					60	300

* Threshold injections.

TABLE 2. Purification of SEB and SEC_2 from sac culture supernatant fluids.

Entero-toxin	Strain	Toxin in initial culture (mg)	Toxin after Step I (mg)	Equilibration $KHPO_4$ buffer	Toxin after Step II (mg)	Overall process yield (%)
SEB	S-6	27	22	5 mM(6.8)	16	59
SEC_2	FRI-361	14	14	10 mM(6.5)	5.9	42

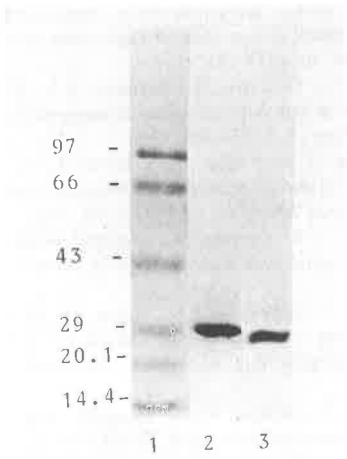

FIGURE 1. SDS-PAGE of SEB and SEC_2. Lane 1: molecular mass markers (in Kilodaltons): phosphorylase *b* (97), albumin (66), ovalbumin (43), carbonic anhydrase (29), trypsin inhibitor (20.1), and α-lactalbumin (14.4). Lane 2: 20 µl of SEB eluted from a Red A column with 120 mM $KHPO_4$ buffer, pH 6.8. Lane 3: 20 µl of SEC_2 eluted from a Red A column with 150 mM $KHPO_4$ buffer, pH 6.5.

to perform pre-purification step was introduced to remove part of the contaminants prior to Red A chromatography.

Production of antisera

Prior to immunization, serum samples from each rabbit were tested by immunodiffusion against SEA, SEB, SEC, SED, SEE and supernatant fluid of *S.aureus* FRI-823 (non-enterotoxigenic strain) cultures. No precipitin lines were observed in the assays, demonstrating the absence of antibodies against *S.aureus* extracellular products, including enterotoxins, in pre-immunization sera.

The toxicity of the enterotoxin limited the initial amount of antigen used for stimulation. When physiological changes were noted, injection of the next booster was postponed by 1 or 2 days. During immunization with SEA one rabbit died following the initial stimulation with 1 µg. No rabbits died after initial doses of 10 µg of SEB or SEC_2.

Approximately 7 days after injection of the threshold doses (20 µg for SEA and 30 µg for SEB and SEC_2) sera were obtained from each group of immunized animals. Responders with titers in the range of 10 to 20 were boostered with 50, 100 and 300 µg of SEA over an 8-day period, or 60 and 300 µg of SEB or SEC_2 over a 7-day period (TABLE 1). One week after the last dose, 30 ml of blood per rabbit were drawn. Maximal titers were 80 for SEB and 40 for SEA and SEC_2 (TABLE 3). The sera were lyophilized and stored at -20°C. The OSP method (14) was satisfactory for SE detection.

SE production and purification as described herein permits that, starting from small volumes of culture supernatants, adequate amounts of highly purified enterotoxins are obtained for use as reagents and in the production of specific antibodies. The methodology is particularly useful in small laboratories that may not have the equipment for handling large volumes of culture supernatants. An adaptation of this technique to SED purification is being currently developed.

TABLE 3. Antibody titers to SEA, SEB, and SEC_2.

Toxin	Peak titers	Average
SEA	40	27
SEB	80	60
SEC_2	40	36

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RESUMO

Purificação de enterotoxinas estafilocócicas B e C_2 por cromatografia de afinidade com corante - produção de anti-soros

As enterotoxinas estafilocócicas B e C_2 foram purificadas a partir do sobrenadante de culturas, através de cromatografia de troca catiônica em resina Amberlite CG-50 e cromatografia de afinidade com o corante Red A. Cem mililitros de sobrenadante de culturas contendo 270 µg de SEB/ml e 150 ml de sobrenadante contendo 93 mg de SEC_2/µl proporcionaram uma recuperação final de

59% e 42%, respectivamente. As toxinas purificadas apresentaram-se homogêneas quando analisadas por eletroforese em gel de poliacrilamida dodecil-sulfato de sódio. A imunização de coelhos com as enterotoxinas purificadas forneceram títulos máximos de 80 para a enterotoxina B e 40 para a enterotoxina C_2. A metodologia descrita neste trabalho mostrou-se adequada para a purificação de enterotoxinas B e C_2, visando a obtenção de reagentes e produção de anticorpos específicos, utilizando pequenos volumes iniciais de sobrenadante de culturas.

Palavras-chave: enterotoxinas estafilocócicas, purificação, cromatografia, anti-soros.

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A SIMPLIFIED METHOD FOR THE NON-RADIOACTIVE DETECTION OF HYBRIDIZED NUCLEIC ACIDS

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SHORT COMMUNICATION

ABSTRACT

We studied four different hybridization conditions for a non-radioactive genetic probe because of the common difficulty in obtaining clear results with this type of probe. A simple and reliable protocol for efficient detection of DNA using a chemiluminescence method is described for routine application in microbiological practice.

Key words: pBR-322; biotin; chemiluminescence; hybridization; non-radioactive.

One of the most common techniques currently used for DNA analysis is hybridization in solid phase (9). This approach exploits the ability of nitrocellulose or nylon filters to preserve the denatured double stranded DNA while reassociation with a labeled probe takes place (9). The labeling can be either radioactive (8) or non-radioactive (6) for detection of homologous DNA sequences. The utilization of non-radioactive probes has advantages, including safety with respect to health risks and elimination of short half-life reagents loaded with some radioactive isotopes (10). However, non-radioactive labels present some difficulties, frequently producing high background levels. The detection process of the Photogene Nucleic Acid Detection System from BRL involves three basic steps: (a) a biotin-labeled probe is hybridized to the immobilized nucleic acid; (b) streptavidin-alkaline phosphatase conjugate (SA-AP) is bound to the biotin groups; (c) the membrane is incubated with a substrate for alkaline phosphatase that luminesces when dephosphorilated (2). We describe here a simple and reliable method for the efficient detection of DNA using as a model the biotin- labeled pBR-322 probe.

Bionick Labeling System and Photogene Nucleic Acid Detection System were purchased from Gibco BRL Research Products and Formamide and Dextran Sulfate from Sigma. Unincorporated nucleotides were separated from the biotin-labeled probe using a 1.0 ml column of Sephadex G-50 resin. Plasmid pBR322 was labeled following the protocol from GIBCO-BRL. At the end of the reaction we used column chromatography made in a 1.0 ml syringe plugged with siliconized glass wool and filled with TES buffer (10mM Tris - HCl pH 7.5, 1 mM EDTA, 0.1 % SDS). The labeled DNA was equilibrated in the same buffer, applied to the column and recovered in an eppendorf tube by centrifugation at 3,000 rpm for 3 min. Photogene Nylon Membranes were rinsed in 20 mM Tris, pH 7.0, and dried (3). One hundred nanograms of pBR322 in 5.0µl were denatured by boiling for 3 min, cooled 1 to 3 min on ice and manually dotted onto the nylon membranes. Four identical membranes were exposed to U.V. light for 5 min (4) and kept at room temperature until hybridization was carried out.

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The four membranes were placed in plastic hybridization bags and pre-hybridized for 2 hours in 250µl of pre-hybridization solution per cm^2 of membrane surface area. The amount of probe for hybridization was always calculated as follows: area of membrane in cm^2 x 50 ng probe/ml x 0.1 ml/ cm^2 membrane = ng probe. In our case we used 125 ng of probe. Four protocols were employed, namely those described by Bronstein *et. al.* (2), by Flores et. al. (4), by Bellinzoni *et. al.* (1) and by Olive and Sethi (7). The latter protocol was as follows:

The filter was pre-hybridized at 55°C for 15 min. in 5 x SSC, 1 % SDS and 0.5 % bovine serum albumin. This was followed by two washes with 1% SDS in 1 x SSC at 50°C and two washes with 1% Triton X-100 in 1 x SSC at 50°C (10 min. per wash). Finally, the filter was washed twice in 1x SSC at room temperature (10 min. per wash).

The subsequent steps of blocking, application of the detection system and autoradiography of the reactions were done according to Photogene instructions.

Stability of the biotin labeled pBR 322 probe was tested by boiling for 3 min, chilling on ice and dotting of 100 ng of this probe (5 µl) onto the Photogene Nylon Membrane. Freshly labeled and one-month-old probes were used to test for stability.

FIGURE 1 shows that there was no significant difference between the detection signals emitted by the alkaline phosphatase conjugate of freshly labeled and one-month-old labeled probes. This reinforces the convenience of cold probes as compared with radiolabeled probes, which have short half-lives and, in addition, are more expensive and pose problems for their disposal. A comparison of the four hybridization protocols is presented in FIGURE 2. The protocol of Olive and Sethi (7) was initially described for alkaline phosphatase-conjugated probes using a colorimetric protocol to detect the hybridization signals. This protocol is the simplest and most reliable. It does not require formamide, dextran sulphate or sheared denatured salmon sperm DNA. The presence of bovine serum albumin in the pre-hybridization and hybridization solutions provides optimal conditions for probe specificity. During the second wash of the filter, the nonionic detergent Triton X-100 was used instead of the anionic detergent SDS. In general, nonionic and amphoteric detergents are less

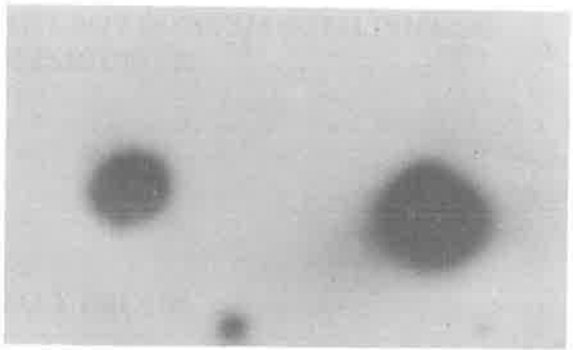

FIGURE 1. Dot Blot analysis of pBR-322 DNA. Plasmid DNA pBR-322 was biotin-labeled and dotted onto Photogene Nylon Membrane under two conditions: freshly labeled (A) and one-month-old labeled (B).

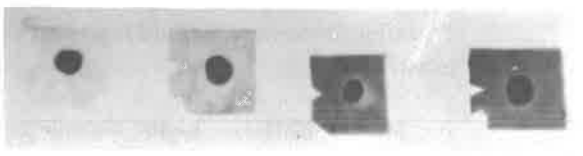

FIGURE 2. Dot Blot analysis of pBR-322 under different hybridization conditions. Plasmid DNA pBR-322 was dotted onto Photogene Nylon Membrane and hybridized with biotin-labeled pBR-322 following protocols 01 (A), 02 (B), 03 (C) and 04 (D).

denaturing to protein antigens than ionic detergents (5). Triton X-100 may better preserve the interaction of the streptavidin-alkaline phosphatase conjugate with the biotin probe. Furthermore, in association with BSA, it prevents the non-specific binding that results in high filter backgrounds. When we used the 4 protocols to be compared and developed the assays using the colorimetric approach (NBT and BCIP) we did not notice any difference among them (data not shown). The protocol described in this work can be easily applied to routine clinical practice.

RESUMO

Descrição de um método simplificado de detecção não-radioativa de ácidos nucleicos por hibridização molecular

No presente trabalho estudaram-se 4 protocolos diferentes de detecção não-radioativa de ácidos nucleicos utilizando sonda genética biotinilada.

A razão deste trabalho são as inúmeras dificuldades encontradas na padronização dos ensaios de hibridização utilizando o método quimioluminiscência principalmente devido ao grande "background" presente nos filtros. Devido às grandes vantagens que a utilização de sondas não-radioativas nos oferece, utilizando DNA plasmídico pBR-322, descrevemos uma metodologia simples, reprodutível e de baixo custo de hibridização molecular utilizando a técnica de quimioluminiscência.

Palavras-chave: pBR-322, biotina, quimioluminiscência, hibridização, não-radioativo.

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STAPHYLOCOCCUS AUREUS: RESISTANCE PATTERNS TO ANTIMICROBIALS AND PENICILLINASE AMONG STRAINS ISOLATED FROM APPARENTLY HEALTHY LACTATING COWS

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João Luciano Andrioli

ABSTRACT

Ninety-two strains of *Staphylococcus aureus* isolated from the nasal fossa and udder skin of apparently healthy lactating cows were analyzed for resistance to antibiotics and production of penicillinase.

The results showed a greater frequency of resistance patterns to penicillin and ampicillin.

All strains were sensitive to oxacyllin and gentamicin.

The most frequent Barber and Burston model was SSSS (60.90%), followed by RSSS (18.50%).

With respect to the production of penicillinase, although the Lucas method indicated a larger number of positive samples, we suggest the use of the Haight and Finland method due to a greater consistency of data obtained with it.

Key words: *Staphylococcus aureus*, antibiogram, penicillinase.

INTRODUCTION

Among the microorganisms presenting high resistance to antimicrobials, *Staphylococcus aureus* stands out due to the high frequency of isolates resistant to several groups of antibiotics (penicillin in particular) and the increasing number of cases of multiple resistance (5, 14, 16).

In parallel with these features, *Staphylococcus aureus* often determines morbid states in man and animals that range from simple infections in the skin and oropharyngeal tract to more serious disorders, often causing potentially lethal septicemias.

Staphylococcal mastitis, one of the most important infections in animals, causes great economic losses and represents an important public health problem because of the contamination of milk and its subproducts (2,3,4).

It is important to emphasize that not only sick animals but also carriers play an important role in the epidemiology of staphylococcal disease.

The association of *S. aureus* with high patterns of resistance contributed to the decision to undertake this study so as to eventually establish regional patterns and rational therapeutic approaches and also set up prophylactic measures against the microorganism (1).

Another aim of this study was to determine the frequency of penicillinase-producing strains in relation to strains exhibiting patterns of resistance to penicillin.

MATERIALS AND METHODS

Ninety-two *S. aureus* strains isolated from the nasal fossa and udder skin of apparently healthy

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lactating cows of the Ribeirão Preto and Jaboticabal region were used. The samples were collected in a farm during the summer of October 1993 and the first isolation was carried out at the same location.

The sensitivity test to antimicrobials agents was performed according to Bauer and Kirby (4) as modified by Rocha *et al.* (15).

The antibiotics tested were chosen according to their use in medical and veterinary practice and their inclusion in rations administered to lactating cows. They were: penicillin, novobiocin, ampicillin, cloramphenicol, oxacillin, gentamicin, amicacin, tetracycline, kanamycin, streptomycin, phosphomycin and sisomycin.

After the antibiogram, the strains were grouped into "models" according to Barber and Burston (3).

For the penicillinase test the following methods were employed: Haight and Finland method (8), biological method (using *Sarcina lutea* ATCC-9341, sensitive to 0.03Uml of crystalline penicillin G, as indicator strain) and the Lucas method (12), an acidometric assay, using culture medium to which crystalline penicillin G (Fontoura Wyeth) was added at a final concentration of 5000 UI/ml.

RESULTS AND DISCUSSION

TABLE 1 shows the behavior of 92 strains of *S. aureus* in relation to twelve antibiotics by the Bauer and Kirby method (6).

TABLE 1. Antibiograms of 92 strains of *S. aureus* isolated from the nasal fossa and udder skin of apparently healthy lactating cows from the Ribeirão Preto and Jaboticabal region.

Antibiotic	Sensitive		Intermediate		Resistant		
	n	%	n	%	n	%	Total*
Penicillin	68	73.90	0	0	24	26.10	26.10
Streptomycin	79	85.90	05	5.40	08	8.70	14.10
Cloramphenicol	87	94.60	0	0	05	5.40	5.40
Tetracycline	85	92.40	0	0	07	7.60	7.60
Ampicillin	69	75.00	0	0	23	25.00	25.00
Novobiocin	85	92.40	02	2.20	05	5.40	7.60
Phosphomycin	88	95.70	0	0	04	4.30	4.30
Sisomycin	86	93.50	05	5.40	01	1.10	6.50
Bacitracin	87	94.60	01	1.10	04	4.30	5.40
Kanamycin	88	95.70	0	0	04	4.30	4.30
Oxacyllin	92	100	0	0	0	0	0
Gentamicin	92	100	0	0	0	0	0

* Sum of the resistant and intermediate categories

When analyzing the results obtained and data from the literature, it can be concluded that the problem of resistance to penicillin and ampicillin is almost universal, detectable in isolates from milk samples, carrier animals or sick animals (9, 10, 11, 13).

The proposed antibiogram model (3) applied to the 92 strains permitted us to obtain 7 models, described in TABLE 2.

TABLE 2. Distribution of the 92 strains of *S. aureus* according to the Barber and Burston antibiogram*

Model	N. of strains	Percentage
SSSS	56	60.90
RSSS	17	18.50
RSSR	05	5.40
RRSS	04	4.30
SRSS	05	5.40
SSRS	03	3.30
SSSR	02	2.20
Total (7)	92	100.00

* Penicillin, Streptomycin, Cloramphenicol and Tetracycline

It should be emphasized that knowledge of the resistance levels to antibiotics is important not only for clinicians but also for the community who deals and lives with these strains daily, because through such knowledge they will be able to establish measures aiming at the rational application of antimicrobials.

Of the 92 strains of *S. aureus* studied, 24 were resistant to penicillin. The results of the penicillinase production test are shown in TABLE 3.

TABLE 3. Behavior of 92 strains of *S. aureus* concerning the production of penicillinase, as demonstrated by the methods of Haight and Finland and of Lucas.

Method		Haightand Finland				Lucas Method			
		Producer		Non Producer		Producer		Non Producer	
Category	Anti-biogram	n	%	n	%	n	%	n	%
Resistant	24	18	75	06	25	21	87.50	03	12.50
Sensitive	68	0	0	68	100	0	0	68	100

None of the strains sensitive to penicillin produced penicillinase.

The acidometric method (11) was more sensitive than the biological method (8). However, it is necessary to take into account the fact that the acidometric method is nonspecific, i.e., the color change of the acid indicator may be provoked by any acid present in the inoculum. The acid can actually be penicilloic acid produced by hydrolysis of the B-lactam ring of penicillin under the action of penicillinase present in the inoculum, as well as of one of the products of bacterial metabolism.

In contrast, the biological method (8) is specific because it is based on the action of the enzyme synthesized by the microorganism, in this case during its development on the test plates. Therefore, this method depends solely on the ability of the microorganism to synthesize penicillinase.

The above arguments point to the biological method as main choice; though being more laborious, it produces more consistent data. The acidometric method, on the other hand, is suggested for screening. However, using the statistical method of "two proportions", no significant differences were found between the two.

RESUMO

Staphylococcus aureus: Padrões de resistência a agentes antimicrobianos e penicilinase de cepas isoladas de bovinos aparentemente sadios em lactação

Noventa e duas cepas de *Staphylococcus aureus*, isoladas de fossas nasais e pele do úbere de bovinos aparentemente sadios em lactação, foram analisadas para resistência a antibióticos e produção de penicilinase.

Os resultados mostraram elevada frequência de padrões de resistência relativos à penicilina e ampicilina. Todas as cepas foram sensíveis à oxacilina e gentamicina.

O modelo de Barber e Burston mais frequente foi o SSSS (60,90%), seguido do RSSS (18,50%).

O método biológico de Haight e Finland e o método acidométrico de Lucas foram utilizados para detecção da produção da enzima penicilinase. A análise estatística pelo teste de duas proporções mostrou que não houve diferença entre os dois métodos.

Palavras-chave: *Staphylococcus aureus*, antibiograma, penicillinase.

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EVALUATION OF AN INDIRECT ELISA METHOD FOR THE DETECTION OF CHICKEN ANTIBODIES AGAINST INFECTIOUS BRONCHITIS VIRUS

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Maria do Carmo M. Galletti[3]
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ABSTRACT

An indirect ELISA method was developed and applied to detect antibodies against infectious bronchitis virus (IBV). Three hundred serum samples collected from vaccinated and non-vaccinated chickens under field conditions were tested. The ELISA results were compared with antibody titers determined by a serum neutralization assay (SN). Good coefficients of correlation ($r^2 = 0.750$) and of agreement (80%) were recorded and the ELISA reproducibility was high. The cutoff point between those techniques corresponded to ELISA level 2. It is concluded that our indirect ELISA method can advantageously replace the conventional SN test, allowing a good follow-up of IBV post-vaccination antibodies due to its convenience and applicability compared to the other assay

Key words: Infectious Bronchitis Virus (IBV), ELISA, post-vaccine serological monitoring.

INTRODUCTION

Programs for the control of Infectious Bronchitis in chickens are based on the vaccination and monitoring of post-vaccination protection, which is usually done by the serum neutralization (SN) assay (1, 9, 10), the haemagglutination inhibition (HI) test (2, 9, 12, 15, 18) or the more widely adopted commercial kits of indirect ELISA (9, 16, 21, 24, 27, 28, 32).

Kits of indirect ELISA have been routinely used to measure anti-IBV antibodies in fowls. They have permitted a rapid classification of serum samples into ELISA levels (EL), thus facilitating an accurate visualization of the degree of humoral immune responses in a large number of vaccinated birds (16, 21, 24, 27, 28).

In spite of several publications reporting the advantages of the indirect ELISA kits to quantify chicken antibodies against IBV, few studies have established the correlation coefficients, and their statistical significance, regarding SN or HI tests (9, 12, 15, 17, 18, 21, 27, 29).

This work describes the development of the essential immunoreagents and standardization of an indirect ELISA test for quantification of chicken anti-IBV antibodies and, furthermore, presents a statistical evaluation of this technique compared with the SN assay based on 300 tested serum samples.

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MATERIALS AND METHODS

Virus

The strain selected for this study was serotype M41 of chicken infectious bronchitis virus (IBV), obtained from IBV -infected allantoid fluid (AF) of specific pathogen-free (SPF) eggs on the ninth to eleventh day of embryonation, with an infecting titer of $10^{9.8}$ EID 50/μL.

Antigen purification

The virus was purified by a modification of the methods of Cavanagh *et al.*(7) and Case *et al.*(5)

Briefly, the antigen was prepared from a known amount of AF clarified by centrifugation at 2000 g for 20 minutes/ 4°C and then centrifuged at 59,000g for 150 minutes in an angular rotor. The resulting sediment was resuspended in 3 milliliters of 0.01 M TRIS buffer, 0.001 M EDTA and 0.15 M NaCl, pH 7.4, layered on a continuous 20-55% sucrose gradient (w/v) and again centrifuged for 10 hours at 80,000g. The material resulting from this ultracentrifugation was removed by means of slow injection of a 60% sucrose solution into the bottom of the tube, at a flow rate of 50 drops/min with the aid of a peristaltic pump, and fractionated into 1.5 ml aliquots using a fraction collector. The IBV presented in fractions with high absorbances at 280 nm (protein concentration) and 254 nm (RNA concentration) were re-pelleted at 90,000 g for 4 hours to remove sucrose. The sediment was resuspended in the same buffer to a final volume of 4.0 ml and used to determine protein concentration (14). The presence of intact viral particles was determined by transmission electron microscopy (4). Infectivity was determined at each stage of the purification process by inoculation of embryonated SPF eggs and the titers were calculated according to the method of Reed and Muench (1938).

Avian sera

A total of 300 serum samples were submitted to SN and ELISA tests.Two hundred and thirty six samples were collected from vaccinated broiler chickens or vaccinated egg-laying hens and 64 negative sera were obtained from non-IBV vaccinated chickens. The positive reference serum consisted of a mixture of 10 sera from chickens immunized with a commercial vaccine and selected on the basis of SN titers. The donors of these sera were chickens first vaccinated at 7 days of age, then re-vaccinated on days 49 and 84, and finally boostered with an oily vaccine on the 126th day of age. Sera with high SN antibody titers (a total of 5 samples) were mixed and used as positive reference serum. The negative reference serum consisted of a mixture of sera from non-vaccinated SPF chickens.

Anti-chicken IgG peroxidase Conjugate

Chicken IgG purified by gel filtration in sephadex G200 (3) was used to immunize two rabbits. Purified rabbit IgG anti-chicken IgG obtained by DEAE-cellulose chromatography was conjugated to horse radish peroxidase (Sigma type VI) (26,31). Rabbit anti-chicken IgG peroxidase conjugate was titrated against purified chicken IgG by a direct ELISA method and the working dilution of the conjugate was determined (26,30).

Enzyme substrate

A mixture of 0,006% H_2O_2 and 0,4 mg/ml orthophenylenodiamine, (SIGMA) in 0.1M $NaHPO_4$ buffer plus 0.1M citric acid, pH 4.9 to5.2 was prepared immediately before use.

Serum neutralization (SN) tests

The titers of serum neutralizing antibodies were determined by ß method using rigid flat-bottomed microplates containing 10^6 renal cells from SPF chicken embryos per ml (10). The neutralizing titer of a serum was calculated according to the Spearmann and Karber method (10). A SN titer ≤24 was considered negative and a SN titer ≥26 was considered positive.

Optimization of ELISA reagents

The optimum dilutions of antigen and of positive and negative reference sera were determined by checkerboard titration. Several amounts of purified antigen (12.85μg, 6.42μg, 3.21μg, and 1.60 μg) were adsorbed to the surface

of rigid microplates and placed to react with different serum dilutions (1:100, 1:200, 1:400, and 1:800). The presence of specific anti-IBV chicken IgG were detected after the addition of the optimum dilution of the conjugate (1:1000) and the mixture substrate-chromogen (30).

Indirect ELISA

The microplate wells (Nunc Immunoplate MaxiSorp F96) were adsorbed with an optimum concentration (3.21 µg) of purified virus protein diluted in sodium carbonate-bicarbonate buffer, pH 9.6, 0.1M, for 18 hours at 4°C and at 37°C for 1 hour (100µl/well). Unattached virus was then removed by washing the wells five times with a solution of PBS 0.01M PO_4^- ,0.15M NaCl, pH 7.4 containing 0.05% tween 80 (PBST). The wells were then blocked by the addition of 200 µl of PBS containing a final concentration of 10% (w/v) defatted powdered milk (PBSDPM) and plates were incubated for 45 min at 37°C. After washing five times as described above, each two wells received 100µl of a previous determined ideal dilution of test sera (1:400) in PBSDPM (0.5M NaCl). Incubation was carried out at 37°C for 1 hour. After washing as before, each well received 100µl of the ideal dilution of the conjugate in PBSDPM and the plates were again incubated at 37°C for 1 hour. After another series of washings, 50µl of substrate were added to each well and the plates were incubated for 15 minutes in the dark at room temperature. The enzymatic reaction was stopped by addition of 50µl of 2M HCl. Spectrophotometric readings were taken at 492 nm in an ELISA reader. For each test serum sample, the mean optical density (OD_{MTS}) was expressed in relation to the positive reference serum mean optical density (OD_{MPRS}) and the negative reference serum mean optical density (OD_{MNRS}) as a sample quotient / positive (S/P) value, according to the formula: $S/P = OD_{MTS} - OD_{MNRS} / OD_{MPRS} - OD_{NRS}$.

Determination of ELISA levels and the cutoff point

Samples quotient/positive (S/P) values were grouped into ELISA levels (EL) according to Martin *et al.*(20), which ranged from zero to nine (0-9). The highest upper and lower limits were determined by the mean S/P values for the positive and negative reference sera diluted 1:200 to 1:175,000, plus two standard deviations. Starting from this limit, intervals between other levels were defined by a 35% increase (20). The cutoff point was determined by graphic analysis of copositivity and conegativity of indirect ELISA and SN obtained by intercept of the copositivity and conegativity curves projected on ordinate axis.

Reproducibility of the Indirect ELISA

The reproducibility of the indirect ELISA test was evaluated against chicken sera with SN titers of 2^8 and 2^9, using a total of 15 random replicates. The coefficient of variation of ELISA values obtained in the various replicates was calculated by analysis of variance (11).

Comparison between indirect ELISA and the SN assay

The anti-IBV antibody titers detected by indirect ELISA and SN were statistically compared by linear regression analysis. The significance of the correlation coefficients was established using the student test (11) and the percentage of agreement was then calculated (20).

RESULTS

Sucrose gradient fractions showing peak absorbancy at 254nm and at 280nm were collected, the density was measured and infectivity was determined. Peak infectivity ($10^{7,8}$IED50/ml) was between fraction densities of 1,1435 and 1,1484g/ml. After re-pelleting, these fractions were analyzed by electron microscopy and revealed a well conserved virus structure, including the integrity of the envelope spikes (FIGURE 1).

Results from the checkerboard titration of different dilutions of IBV-positive and negative chicken serum against several amounts of purified viral antigen showed a maximum discrimination between these sera at dilutions of 1:400 for chicken serum and of 1:1000 for the conjugate, at an antigen concentration of 3.21µg/well. These conditions were established as optimal to analyze the 300 chicken test serum samples by the indirect ELISA method.

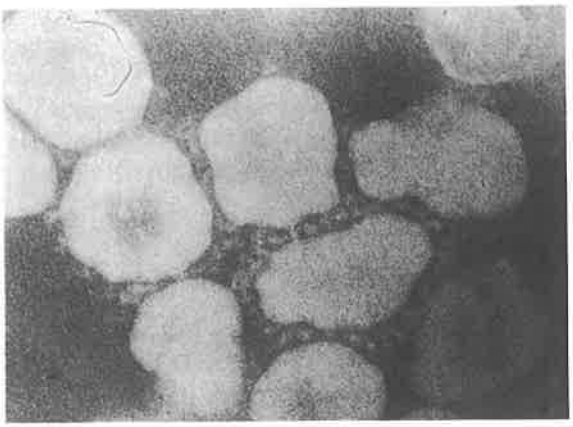

FIGURE 1. Electron micrograph of IBV strain M41 from purified virus samples identified in sucrose gradient fractions (297,000x).

Although several ELISA procedures for avian systems have been used, most of the protocols described are rather unsatisfactory, particularly with regard to the high background absorbance in the controls. In view of this, different conditions of incubation, blocking substances and buffers were tested (results not shown). The most suitable protocol adopted for the indirect ELISA is described in details in Material and Methods. The inclusion of a blocking step with skimmed milk diluted in PBS 0.01M $PO4^{=}$, 0.015M NaCl, pH 7.4, was particularly important to obtain accurate and specific results.

The correlation coefficients for antibody titers (S/P values for the indirect ELISA and log2 titers for SN) recorded by linear regression analysis was statistically and highly significant (r^2 =0,750 and p< 0,0005; FIGURE 2).

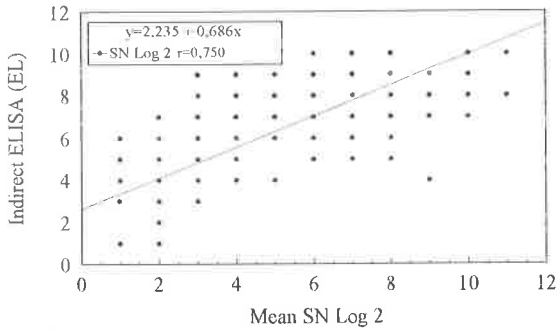

FIGURE 2. Graphic representation of the correlation and linear regression of antibodies titres obtained by indirect ELISA, expressed aso ELISA levels (EL), and SN.

TABLE 1. Checkerboard titration of differents dilutions of positive and negative serum using a constant dilution of purified antigen (3.21µg/well).

Purified antigen (3.21µg / well)		
Serum dilutions	Positive serum	Negative serum
1:100	1.193*	0.248
1:200	1.172	0.207
1:400	1.125	0.171
1:800	1.051	0.179

* Absorbance(492 nm).

When the reproducibility of the indirect ELISA method was evaluated with 15 replicates, the variance observed for the two samples of avian sera used was 0.044.

The cutoff point between the indirect ELISA and the SN was 2 (EL) and the agreement between the two tests was 80%.

DISCUSSION

The preliminary monitoring of our viral antigen preparation revealed a high infectivity titer and a well conserved virus structure, particularly regarding the presence of spikes in fractions whose sucrose densities are similar to those reported previously (5). The presence of some viral antigen components, like spikes in IBV, are fundamental for the development of certain serological reactions such as HA, HI and SN (7,23). Therefore, the final results of a comparison between any of these techniques with ELISA could be affected by such antigen structure or even by the kind of antigen preparation used in each assay.

During optimization of the indirect ELISA for detection of chicken anti-IBV antibodies, the checkerboard titration of one reagent against the other showed a wide range of combinations of serum dilutions and antigen concentrations for the highest test discrimination between positive and negative sera; these results are quite similar to those reported by Case *et al.* (5).

The additional blocking step with skimmed milk was fundamental to reduce the background in the control wells, particularly when sera were stored for a long time at -20°C, and also to increase the ratio between positive and negative sera. Similarly,

the addition of a high molarity of sodium chloride to the phosphate buffer used to dilute serum samples was an important modification (5).

Comparisons of the performance of the indirect ELISA and the SN or HI tests have been made by several authors (1, 9, 12, 17, 18, 24). However, most of these studies determined the overall agreement of the time-course of a post-infection or post-vaccination humoral anti-IBV response detected by the techniques but did not establish the correlation coefficients and their statistical significance. One exception where high correlation coefficients followed by similar high coefficients of variation (25 to 100%) were reported in a comparison of two commercial ELISA Kits with HI (29).

The correlation between our indirect ELISA and the SN assay based on 300 test sera, which included samples from vaccinated and non vaccinated chickens of different ages, was high (r^2=0.75). However, there is one significant difference between our statistical approach and that reported previously by Thayer *et al.* (29). It is very important to emphasize that our comparative study of the ELISA method and the SN assay is based on individual titers from each test analyzed in a pair-wise fashion and using least-square linear regression analysis, whereas the previous work compares indirect ELISA titer group means with specific HI titers. In fact, when our data were submitted to the same statistical analysis described by Thayer *et al.* (29), high coefficients of correlation ($r \leq 0.96$) and of variation (25 to 100%) were found.

In spite of the relatively higher correlation coefficients recorded between the indirect ELISA and SN or HI, it must be remembered that some discrepancy is expected between the performance of these techniques because of intrinsic properties of each serological test. This is particularly visualized when sera with lower antibody titers are titrated by HI, ELISA and SN (9, 15).

To conclude, our indirect ELISA method proved to be able to replace the SN assay and can be a useful alternative to the indirect ELISA commercial kits available to monitor anti-IBV humoral immune responses in chickens.

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RESUMO

Avaliação do método indireto de ELISA para detecção e monitoramento da resposta humoral anti-VBI em galinhas

Um método enzimático indireto de ELISA foi desenvolvido e aplicado para o monitoramento de anticorpos pós-vacinais contra o Vírus da Bronquite Infecciosa das Galinhas (VBIG), na análise de 300 amostras de soros examinadas. Os títulos avaliados no teste indireto de ELISA eram relacionados com aqueles obtidos na Soroneutralização (SN). O ponto de corte entre as duas técnicas correspondem ao nível 2 de ELISA. Foram encontrados coeficientes de correlação (r^2=0,750) e concordância (80%) significantes. Concluiu-se que o teste indireto de ELISA poderia substituir de forma mais eficiente o de SN no monitoramento pós-vacinal contra o VBIG.

Palavras-chave: vírus da bronquite infecciosa das galinhas; ELISA, monitoramento sorológico pós-vacinal.

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References citing "personal communication" or "unpublished data" are discouraged, although it is recognized that sometimes they must be used. In these cases, they should be cited in the text and not in the list of references. References consisting of papers that are "accepted for publication" or "in press" are acceptable. However, references of papers that are "submitted" or "in preparation" are not acceptable.

Tables
Tables should not be included in the text. Each Table must be typed in a separate page and numbered sequentially with an Arabic number. The title of a Table should be placed in the top of it and should be brief but fully descriptive of the information in the Table. Headings and subheadings should be concise with columns and rows of data carefully centered below them.

Figures
Arabic numbers should be used for numbering the Figures. Data in Tables should not be repeated in Figures. The legend of the Figures should be placed in the bottom of them.

Photographs and line drawings
Only those photographs which are strictly necessary for the understanding of the paper should be submitted. Photoprints must be of sufficient quality to ensure good reproduction. They should be numbered on the back and identified with the nominated author's name. Legends of line drawings and photographs should not exceed the printing area. All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

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