ISSN 0001-3714

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Volume 29 Número 2 Abr. - Jun. 1998

AUTHOR INDEX

REVISTA DE MICROBIOLOGIA

Volume 29 Number 2 April - June 1998

Revista de Microbiologia (1998) 29:85-92
ISSN 0001-3714

METAL BIOREMEDIATION BY MICROORGANISMS

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Submitted: February 02, 1998; Returned to authors for corrections: April 17, 1998; Approved: April 30, 1998

MINI-REVIEW

ABSTRACT

In general, metal ions are accumulated by microorganisms for maintenance of vital biological functions. However, not always microorganisms accumulate metals due to metabolic necessity. The process of metal accumulation by microorganisms can be divided into two steps. The first one involves metal adsorption around the cell envelope and is commonly a metabolism-independent process (biosorption). The second one is exclusively dependent on cell metabolism and involves active translocation of metals by microorganisms into the cell (bioaccumulation). The recent concerns on the metal uptake capability by microorganisms have prompted the development of new technologies for metal removal or recovery from waste waters.

Keywords: bioaccumulation, biosorption, metal removal

INTRODUCTION

During the past 300 years the global human population has grown from an estimated 500 million to more than 5.5 billion. This event was followed by an increase in pollution levels and subsequent disruption of many ecosystems. Water is one of the natural resources most affected by environmental pollution. Rivers and seas commonly receive wastes containing high concentrations of heavy metals, pesticides and other substances such as nitrogen, phosphorus and organic compounds. Heavy metals in particular are among the most threatening pollutants. Heavy metals can be easily transported as solutes or particulates, and often reach high concentrations in areas close to their disposal or due to biological amplification.

To reduce the most severe problems caused by the presence of elevated levels of metals in the water supply, efforts have been made to establish constantly decreasing concentration standards for these elements in industrial effluents (14). In general, treatment of effluents contaminated with metals involves physicochemical processes of flocculation and/or precipitation, electrolysis, crystallization and adsorption (8). However, in general, these processes can be expensive and/or lead to production of new contaminants. Thus, the need for more economic and practical technologies for the removal of metals from effluents has resulted in the search for new technologies that may be useful in reducing the levels of metals or even in recovering them from industrial effluents. Biological methods based on the use of microorganisms have proved to be quite promising

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and today offer an alternative method for the removal and/or recovery of metals (6, 7, 9, 61).

Microorganisms commonly isolated from industrial process solutions and polluted environments with high metal concentrations exhibit considerable tolerance to these elements (21, 23). This tolerance may be due to abiotic factors such as pH, temperature and nutrients in the environment or growth media, or to the physiological and genetic adaptations of the microorganism (1, 50). Frequently, resistance of microorganisms to metals is directly related to the ability to accumulate metals. The process of metal accumulation by microorganisms may or may not depend on metabolism (65). Living microbial cells usually exhibit two distinct phases of metal accumulation. The first involves metal adsorption around the cell envelope and is commonly a metabolism-independent process (biosorption) (65, 66). The second is exclusively dependent on cell metabolism and involves active translocation of metals by microorganisms into the cell (bioaccumulation) (Fig. 1) (6).

METAL UPTAKE BY A METABOLISM-DEPENDENT PROCESS-BIOACCUMULATION

Bioaccumulation is a much slower process of metal uptake than biosorption and may be inhibited by the absence of nutrients such as glucose, nitrogen and phosphate, and by the action of metabolic inhibitors, low temperatures and other environmental factors (20, 21, 47, 48, 52). The culture conditions used for the cultivation of microorganisms may also affect the ability of the latter to accumulate metals. Some fungi in the presence of high concentration of metals can invoke qualitative and quantitative changes in cell wall and cytoplasmic membrane composition, increasing their ability to accumulate metals. The fungi *Cunninghamella blakesleeana,*

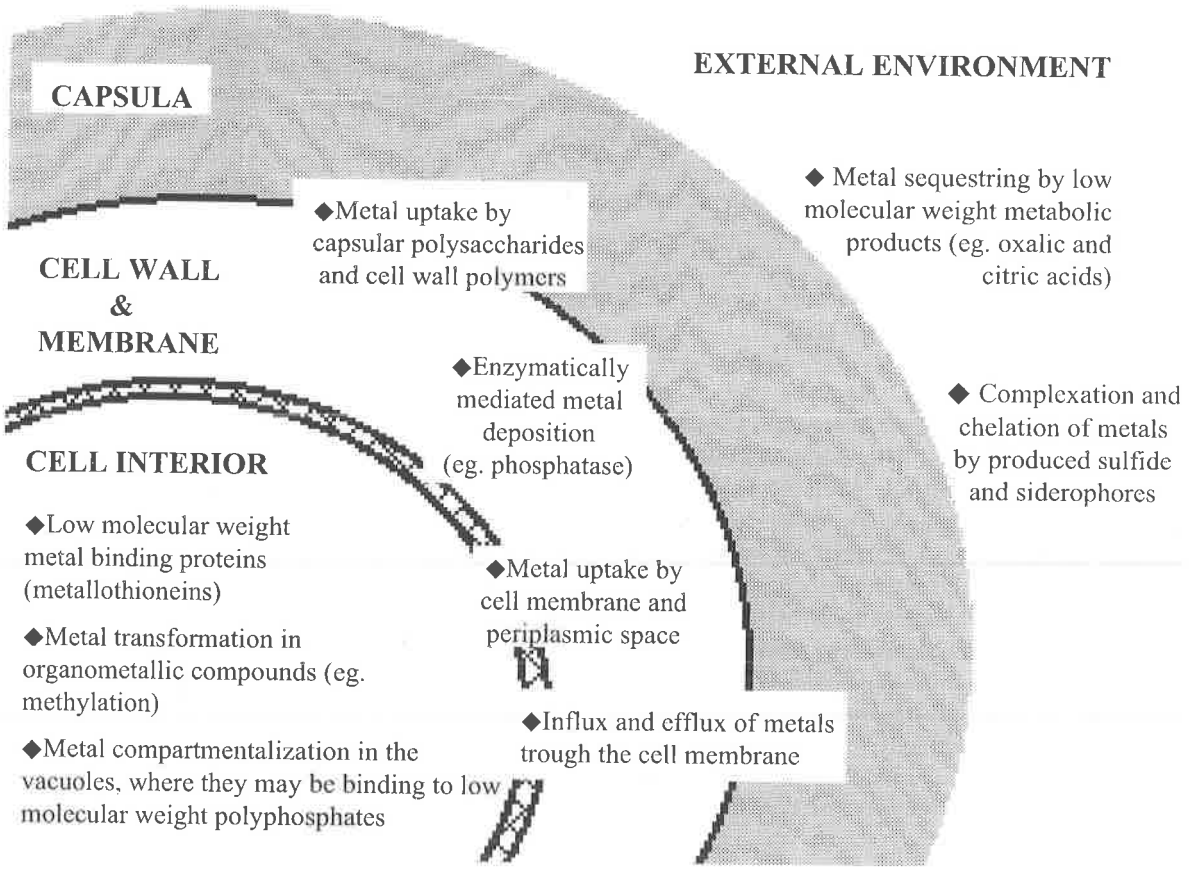

Figure 1. Simplified scheme of metal-cell interactions that contribute to the accumulation and detoxification of metals by microorganisms (24, 53, 54).

Neurospora crassa and *Trichoderma virile*, when grown in the presence of toxic copper concentrations exhibit chemical alterations of the cell wall, such as an increase in the proportion of chitosan. These changes confer more resistance on these fungi and often increase their ability to accumulate metals (13, 59, 64). Some essential nutrients may also increase or even confer the ability of the microorganism to accumulate metals. Macaskie and Dean (44) demonstrated that the bacterium *Citrobacter* sp. can efficiently accumulate cadmium and that the accumulation process is mediated by an enzyme bound to the cell wall identified as a phosphatase. This enzyme was induced during growth of the bacteria in a medium containing glycerol - 2 - phosphate as the only source of phosphorus. The accumulation of metal ions by microorganisms is of fundamental importance for their ability to grow in environments contaminated with heavy metals, as long as this tolerance is due to the ability of the microorganism to prevent the entry of potentially toxic metals or to compartmentalize or detoxify them (Fig. 1).

Microbial metabolism can be affected by metals in all of its aspects such as respiration, protein synthesis and membrane transport, leading to reduction of growth rates, increase of lag phase and changes in morphology and physiology of microorganisms (23, 36). However, some microorganisms may acquire physiological adaptation or have genes that confer high resistance to toxic metals. Genes related to metal resistance can reside in a chromosome or be located in plasmids. One of the best known chromosome gene-related mechanism is responsible for the induction of low-molecular weight metal-binding proteins (metallothioneins) (12, 38). These inducible metal-binding proteins are found in animals, plants, fungi, yeasts and bacteria. (38). In terms of plasmid-mediated resistance, the best known plasmids are those responsible for resistance to arsenate, cadmium, copper and mercury (13, 56). Arsenate resistance is achieved via an ATP-driven transport system that ejects arsenate from inside the cell. Similarly, cadmium and copper resistance is also based on an efflux system, even though in strains of *Pseudomonas syringae* copper resistance is due to chelation by copper-binding proteins. Organomercurial compounds are enzymatically converted to volatile elemental mercury (36). Such genes can be useful for construction of transgenic organisms for removal of toxic metals from contaminated sites. Some constructed transgenic plants, that express a bacterial *mer* A gene, are able to sequester mercury from the soil by converting Hg (II) to Hg (0) (18).

Not all metal ions are harmful to microorganisms. On the contrary, metal ions are essential for microbial growth and the maintenance of vital biological functions. For example, iron is an important cofactor of metalloenzymes and of cytochromes, and zinc is an essential catalytic and structural component of various enzymes. The best known mechanisms for the transport of metal ions into microbial cells include metal ion channel pumps, complex permeation, carrier mediation, lipid peroxidation, and endocytosis (6, 36). However, certain metals that have no known biological function can also be accumulated by these same metal transport systems. This is believed to occur due to the similarity of charge and ionic radius of some toxic and essential metals (metal mimicry) (e.g.: $Cd^{2+} = Ca^{2+}$, $Ag^{+} = Cu^{+}$ and $Ti^{+} = K^{+}$) or due to the presence of non specific transport systems (36).

METAL UPTAKE BY A METABOLISM-INDEPENDENT PROCESS - BIOSORPTION

In general, microorganisms mostly accumulate metals on the cell wall and in the periplasmic space. The cell wall protects the microorganisms against injury, controls the flux of substances between cytoplasm and the outer cell environment, and soluble substances in liquid medium are commonly adsorbed to this structure (23, 70). "Passive" metal ion adsorption by the cell wall or cell envelope is commonly denoted as biosorption and does not depend on cellular metabolic activity (4, 17, 33, 68). Some constituents of the bacterial cell wall structure have metal binding ability and in particular the peptidoglycan make the main sites for metal deposition (46). It is believed that the carboxyl groups present in peptidoglycan are the most electronegative sites and may represent the main site for metal binding on the cell wall of *Bacillus subtilis* and *Escherichia coli* (3, 5). Biosorption phenomena are closely related to the cell surface and vary significantly among groups, genera and species of microorganisms (Tables 1 and 2). The predominance of negatively charged free groups in several biopolymers that form the cell wall confers an anionic character on the cell surface, resulting in the passive attraction of various metal cations (19, 54, 57). Thus, the binding of metal ions to the cell surface

Table 1. Relative metal binding affinities for various microorganisms

Biomass	Main known biopolymers in cell walls for metal binding	Relative metal-binding affinity
Activated sludge	Extracellular polymers produced by floc-forming bacteria, polysaccharides, humic acids and proteins	Cr > Cd> Ag > Pb > Zn > Cu >Ni, Co, Mn, Mo (58)
Bacillus megaterium	Peptidoglycan / teichoic acid	La > Cd > Sr > Ca > Mg> K > Na (45)
Bacillus subtilis	Peptidoglycan / teichoic acid	Zn > Mg > Sr > Na > Ca> Mn > Ni (17)
Pseudomonas sp.	Peptidoglycan	Cu > Ni > Co (55)
Aspergillus niger	Chitin-β-glucan	La ≥ Ag > Cu > Cd > Mn > Zn > Co (49, 66)
Mucor rouxii	Chitin-chitosan	La ≥ Ag > Cu > Cd (49)
Penicillium sp.	Chitin-chitosan	Fe > Cu, Zn, Ni > Cd, Pb > UO_2 (26)
Penicillium chrysogenum	Chitin-chitosan	Pb > Cd > Cu > Zn > As (51)
Rhizopus arrhizus	Chitin-chitosan	UO_2 > Pb > Cd > Zn > Cu (60)
Chlorella vulgaris	Not yet completely established (polysaccharides, uronic acids and proteins)	Al, Ag ≥ Cu > Cd ≥ Ni ≥ Pb > Zn, Co ≥ Cr (2)

Table 2. Metal accumulation by microorganisms most studied for biosorption of metals.

Microorganisms	Metal	Metal uptake mg (g biomass)	References
Aspergillus niger	Ag	22.3	40, 41, 49, 62
	Au	176	
	Cd	3.74	
	Cu	1.7	
Penicillium chrysogenum	Cd	11-56	23, 51
	Cu	122	
	Pb	122	
	Zn	6.5	
Rhizopus arrhizus	Ag	54	41, 60
	Au	164	
	Cd	30	
	Cu	16	
	Hg	54	
	Mn	12	
	Pb	91	
	Zn	20	
Saccharomyces cerevisiae	Cu	1.9	25, 35, 69
	Cd	71	
	Th	119	
Bacillus subtilis	Au	70-79	10, 28, 68
	Cu	146	
	Fe	201	
	Mn	44	
	Pb	189	
Chlorella vulgaris	Ag	55	11, 16, 28, 31
	Au	80	
	Hg	40	
	Pb	165	

is assumed to be due to electrostatic interactions leading, in some cases, to complex formation between metal cations and different functional binding groups (OH^-, HPO_4^{-2}, $R\text{-}COO^-$, =C=O, $R\text{-}S^-$, $-SH^-$, NH_2^-) found in carbohydrates, lipids, proteins and other biopolymers of the microbial cell envelope. The charge distribution and geometry of these binding sites may vary with the composition of the cell envelope of each microorganism, resulting in markedly different metal-binding affinities (Table 1).

Since free metal cation concentrations can be drastically reduced in the natural and extreme environments by complexing agents, such as CN^-, SCN^-, PO_4^{3-}, HS^-, NH_3, $S_2O_3^{2-}$ and SO_3^{2-} (15), tests using metal complexes have been done to evaluate the advantages and disadvantages of microorganisms for metal removal (30, 32). Despite the fact that metal complexes are rarely adsorbed by biosorbents (9), some metal complexes can be removed by biosorption, with the pH of the medium being one of the major factors responsible for the accumulation of these complexes. The pH of the solution affects the chemistry and the availability of the metals, the activity of functional groups on the surface of the microorganisms, as well as the competition between metal ions for binding sites. Greene *et al.* (32), observed that the adsorption of the anionic complex $[Au(CN)_2^-]$ was extremely dependent on pH, with maximum adsorption occurring close to pH 3.0. Kuyucack and Volesky (41) also observed that the process of gold biosorption in the form of anionic complexes was largely dependent on pH, suggesting that the binding sites on the cell wall had affinity for anions in acid pH, and the amine-groups were probably the major ligands responsible for metal binding.

Biosorption is also influenced by other external factors such as metal speciation, the presence of co-ions, and with the possible occurrence of different types of mechanisms such as complexation, coordination, chelation, ion exchange, adsorption and microprecipitation (67). These metal - microbe interaction mechanisms are extremely dependent on the extent of affinity between metal species or their ionic forms and the active sites present in a specific molecular structure. One or more of these mechanisms may result in the immobilization of metal ions on the biosorbent (42).

MICROBIAL METAL REMOVAL AND RECOVERY FROM WASTE WATER

Living or dead cells can be used for the removal of metals from industrial solutions (9, 24, 43). However, the use of living cells involves not only the problem of metal toxicity to the microorganism, but also the action of other toxic pollutants present in the effluent. If these problems are overcome by the use of resistant microorganisms, the use of living cells can be quite efficient (29). In addition to accumulating metals, living microorganisms can have the additional ability of removing other pollutants such as hydrocarbon compounds, pesticides, cyanides and nitrates. Living cells may have all of the types of interaction with metals that occur with dead cells as well as other interactions that require active metabolism (Fig. 1) (24). Although the binding sites of the living biomass may become saturated, it is not necessary to exchange it for a non-charged one and the system is self-supplying due to microbial growth. Several studies have been published recently proposing new methodologies based on the use of living microorganisms for the removal of metals from industrial effluents (27, 43). Among them, three processes are widely known and successfully used by industries: activated sludge, continually growing biofilm and artificial wetlands (24). In the process of activated sludge the effluent is treated in tanks of mechanical aeration or air defusing. The resulting biological mass is recirculated or separated from the effluent to discard. In the biological filtration (microbial biofilm) the effluent is injected through a fluidized or fixed bed reactors where the microorganisms quickly form the biofilm. Stones, ceramic and plastic are usually used as supports for the biofilm. The artificial wetlands are stream meanders formed by naturally-occurring higher plants, algae and aerobic and anaerobic microbes. These organisms act synergistically in the removal of metals and other pollutants from industrial effluents.

Conversely, over the last decade, the use of dead microbial cells (biosorbents) has proven to be an excellent alternative or an additional technology for the removal or even recovery of metals from industrial effluents, representing a method of both economic and environmental interest (22). The use of biosorbents in metal removal presents four basic advantages: 1) low cost of the process needed to obtain biological material for biosorbent production (e.g. biomass of *Aspergillus niger* usually available cheaply as a waste product of citric acid biosyntheses), 2) the process is not ruled by physiological restrictions, 3) the metals are rapidly sequestered and easily recovered without the destruction of the biosorbent, and 4) when immobilized the biosorbents behave like an ion exchange resin and the removal of metals involves a conventional solid-liquid contact and separation process. Widely varying concentrations of the different metals are biosorbed by the microorganisms, indicating that certain species might be better suited to particular metal pollutants (Table 2). For example, *Penicillium chrysogenum* and *Bacillus subtilis* accumulate copper to a greater concentration than do the others microorganisms.

Nevertheless, microbial biomass (whether living or dead cells) has a disadvantageous characteristic for industrial application. Microbial cells have a small particle size, low mechanical strength and low density for waste water treatment. So both live and dead cells must be immobilized in supports for easier biomass/effluent separation. The immobilization by supporting agents can be achieved by entrapment (e.g. alginate, carrageenan or polyacrylamide), adsorption (e.g. sand beads, celite and wood chips) or covalent binding (e.g. hydroxyethyl acrylate) (9, 34). These techniques allow for easy separation of cells and effluent, high biomass loading, decrease in the obstruction and biomass re-use.

In conclusion, the development of an industrial-scale process for metal removal from industrial effluents based on the use of living or dead microbial cells depends on various factors such as the microbial ability to accumulate metals, the selectivity, concentration and types of metals to be removed, the easy recovery of metal and economy of the operation. Thus, it is imperative to have a good knowledge of the microorganism to be used and of the advantages and disadvantages of the use of living or dead cells. Despite the excellent prospects for industrial

application, few studies have been devoted to the evaluation of the efficiency of biosorbents (dead cells) under real treatment conditions (37, 39, 63) and thus so far there are no signs that this technology may be applied successfully to the removal and/or recovery of metals from industrial effluents. Industrial effluents are not simple and homogeneous solutions, but usually contain various metal ions and other inorganic and organic compounds that interfere drastically with the process of metal biosorption by microorganisms. In the future biosorbents have the potential to be very useful for the treatment of metal-containing effluents. However, within a biotechnological context, due to the enormous discrepancies existing between synthetic solutions formulated in the laboratory and effluent solutions originating from industrial activities, further care is needed to evaluate the efficiency of biosorbents under real treatment conditions in order to develop an effective technology for metal removal and recovery from waste waters.

RESUMO

Bioremediação microbiana de metais

Em geral, íons metálicos são acumulados por microrganismos para manutenção de funções biológicas vitais, porém, nem sempre os metais são acumulados devido a necessidades e exigências metabólicas. O processo de acumulação de metais pode ser dividido em duas etapas: a primeira envolve adsorção do metal ao redor do envelope celular e é um processo independente metabolismo (biossorção); a segunda é exclusivamente dependente do metabolismo celular e envolve a translocação ativa de metais através do envoltório celular para o interior da célula (bioacumulação). As recentes descobertas, a respeito da capacidade dos microrganismos em acumular metais, vêm incitando o desenvolvimento de novas tecnologias para a remoção ou recuperação de metais de soluções e efluentes industriais.

Palavras-chave: bioacumulação, biossorção, remoção de metais.

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Revista de Microbiologia (1998) 29:93-98
ISSN 0001-3714

PURIFICATION AND CHARACTERIZATION OF ALKALI-TOLERANT XYLANASES FROM *ASPERGILLUS TAMARII*

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Submitted: March 14, 1997; Returned to authors for corrections: March 23, 1998; Approved: April 16, 1998

ABSTRACT

A strain of *Aspergillus tamarii* isolated from soil produced two different cellulase-free xylanases when developed on corn cob powder as the only carbon source. The two enzymes, designated xylanase I and II, were purified to apparent homogeneity by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose and Sephadex G-100. The molecular weigths estimated by SDS electrophoresis and Sephadex G-100 filtration were 12,500 and 28,000 Da, respectively to xylanase I and II. The enzymes I and II had a carbohydrate content estimated as 39 and 12.7%, respectively. The apparent Km values, using birchwood xylan as substrate, were 5.8 and 8.4 mg/ml and Vmax. values were 6.94×10^{-2} and 1.7×10^{3} $\mu mol.ml^{-1}.mg^{-1}$, respectively to enzymes I and II. The hydrolysis patterns demonstrated that the xylanases were endoenzymes. Both enzymes yielded mainly xylobiose and higher xylooligosaccharides from xylan. The *Aspergillus tamarii* xylanases were stable at large range of alkaline pH and they were able to hydrolyse xylan at pH 8.0.

Key words: Alkali-tolerant xylanase, *Aspergillus tamarii*, cellulase-free xylanase, hemycellulose, endoxylanase

INTRODUCTION

Xylan, the major component of hemicelluloses, consists of a linear chain of β 1,4 linked xylose residues carrying different substituints such as L-arabinose, 4-O-methyl-D-glucuronic acid and O-acetyl groups (24). The hydrolysis of these complex molecules requires the concerted action of a number of different types of enzymes, among which xylanases (1,4 β-D-xylan xylanohydrolase, E.C. 3.2.1.8) and β xylosidases (1,4 β-D-xylan xylohydrolase, E.C. 3.2.1.37) are the most well-characterized. Other enzymes such as phenolic acid esterases, acetyl esterases, α-glucuronidases and α-L-arabinofuranosidases are involved in the cleavage of different xylan substituints to total hydrolysis of xylan (3).

The process of xylan degradation is of biotechnological interest, and the xylanases are being utilized increasingly in the pulp, paper, food feed and textile industries (18). Besides, the study of physical properties of xylanases from several microorganisms can contribute to understanding the degradation of heterogeneous polymers such as the native xylans. A larger number of microorganisms that produce xylanases have been found in extremely diverse natural habitats and a great number of different *Aspergillus* species have been reported to produce cellulase-free xylanases (2, 4, 5, 22). Cellulase-free xylanases active at alkaline pH may be particularly useful for the treatment of alkaline pulps. Activity and stability at alkaline pH have been reported for xylanases from several bacteria, but only a few fungi are able to produce xylanases active at alkaline pH (6,

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10, 17, 19, 20). Recently we isolated from soil a strain of *Aspergillus tamarii* able to produce high cellulase-free xylanase activity when developed on corn cob powder as the major substrate (11, 12). The present paper describes the production and purification of two xylanases active at alkaline conditions.

MATERIALS AND METHODS

Microorganism: *Aspergillus tamarii* was isolated from soil during a screening program for xylanase-producing microorganisms (11). The organism was maintained on potato dextrose agar slants (21) at 4°C and subcultured in intervals from 10 to 30 days. The cultures were incubated on potato dextrose agar slants at 30°C for 5 days for spore production. The conidia suspensions were prepared by adding 10 ml of sterilized water to slant cultures and the surface gently rubbed with a sterilized wire loop.

Enzyme Induction: For production of the extracellular xylanolytic complex, *A. tamarii* was grown in 500 ml Erlenmeyer flasks containing 100 ml of Vogel minimal media (16) and 4% (w/v) corn cob powder (40 mesh) as the carbon source. One ml of conidia suspension (10^4 spores) was used as the inoculum. The cultures were incubated at 30°C on a rotary shaker at 120 rpm and after 5 days the mycelia were removed from the culture media by filtration.

Enzyme assays: Xylanase was assayed using birchwood xylan (Sigma) as substrate. A 1.0 ml reaction mixture contained 0.5 ml of appropriately diluted enzyme solution and 0.5 ml of a 1% (w/v) suspension of xylan in 0.05 M citrate buffer, pH 5.4. The mixture was incubated at 50°C for 10 min., and the reducing sugars produced assayed by the dinitrosalicylic acid method (15) using D-xylose as the standard. A unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugars per min.

Analytical methods. The protein concentration was measured according to Lowry *et al* (14) using bovine serum albumin as the standard. The carbohydrate content in the purified enzymes was measured by the phenol-H_2SO_4 method (8), using D-mannose as the standard.

Purification of the enzymes: All operations were carried out at 4°C. Proteins in the culture filtrate (300 ml) were precipitated by using ammonium sulfate. The 0 to 30% saturation precipitate was removed by centrifugation (30 min, 11,000 g) and discarded. The supernatant solution was precipitated again with 30 to 75% $(NH_4)_2SO_4$ saturation. After centrifugation the pellet was ressuspended in water and dialyzed in a bag membrane (Mr cut off: 10,000) against at least two changes of an excess of distilled water. Undissolved material was removed by centrifugation and the supernatant was applied to a DEAE-cellulose column equilibrated with 0.01 M potassium phosphate buffer, pH 6.8, and eluted with a linear gradient (0-0.3M) of sodium chloride dissolved in the same buffer. Two peaks with xylanase activity, designed as xylanase I and II, were obtained. The pooled active fractions I and II were dialyzed against water, concentrated by freeze-drying and applied to a Sephadex G-100 columm (2.5 x 51 cm) equilibrated with 0.01 M citrate buffer, pH 5.4. After dialysis against water, the purified xylanases were concentrated as previously described and stored at -20°C.

Molecular weight determination: SDS/PAGE (13) were performed using 10% acrylamide and the gels were stained for proteins using Coomasie Blue. The following Mr standards (Sigma) were used: bovine serum albumin, 66,000 Da; ovoalbumin, 43,000 Da; glyceraldeyde 3-phosphate dehidrogenase, 36,000 Da; carbonic anhydrase 29,000 Da; trypsinogen, 24,000 Da; trypsin inhibitor, 20,100 Da and α-lactoalbumin 14,200 Da. The molecular weight of the purified enzymes were also estimated by Sephadex G-100 gel filtration (1).

Optimum pH and temperature: The purified enzymes were incubated with 1% (w/v) birchwood xylan in 0.05 M sodium acetate buffer (pH 4 - 5.5), 0.05 M citrate buffer (pH 3 - 7), 0.05 M phosphate buffer (pH 7 - 8) or 0.05 M glycine buffer (pH 8 - 10) under standard conditions. For determination of the temperature optimum, the standard assay was performed at temperatures ranging from 30 to 70°C.

Temperature and pH stability: The purified enzymes were dissolved in 0.05 M citrate buffers with pH values ranging from 5 to 9 and incubated for two days at 40°C. Thermal stability was investigated by incubating the enzyme at 50 and 60°C after exposure times ranging from 0 to 150 min. Immediately afterwards the enzymes were immersed in a ice bath and then the activities were tested under standard conditions.

Hydrolysis of xylan at alkaline conditions and determination of xylan degradation products: The hydrolysis was carried out in stoppered tubes with 1 U of each xylanase and 25 mg of birchwood xylan or oat spelt xylan contained in 0.05 M glycine buffer, pH 8.0, in a reaction volume of 0.5 ml at 40°C. The aliquots were removed at different time intervals, and

the reducing sugars were assayed by the dinitrosalicylic method using xylose as standard. Percentage hydrolysis was calculated according to Dey *et al.* (7). The end products released were analysed by paper chromatography. Samples (15 µl) were spotted onto Whatman n° 1 paper and descending chromatography was performed at room temperature using benzene: n-butanol:piridine:water solvent system 1:5:3:3 and stained with a silver nitrate reagent. (23).

RESULTS

A summary of the purification scheme for the enzymes is given in Table 1. Two chromatographic steps were required to obtain electrophoreticaly-homogeneous enzymes. 13 ml of the concentrated culture was loaded onto DEAE-cellulose column. The xylanase activity was eluted in two peaks of activity, designated as xylanase I and II. The fractions with activity were pooled, concentrated by lyophilization and rechromatographed in a Sephadex G-100 column. After this treatment xylanases I and II were obtained as homogeneous preparations.

The molecular weights of xylanases I and II estimated by SDS electrophoresis and Sephadex G-100 chromatography were 12,500 Da and 28,000 Da, respectively. The enzymes I and II contains 39 and 12.7% (w/v) carbohydrate as estimated by the phenol-sulfuric method. The apparent Km and Vmax. values, determined from the Lineweaver-Burk plot, using birchwood xylan as substrate were respectively 5.8 mg/ml and 6.94 x 10^2 µmol.ml^{-1}.mg^{-1} for xylanase I and respectively 8.4 mg/ml and 1.7 x 10^3 µmol.ml^{-1}.mg^{-1} for xylanase II.

Xylanase I was most active at 60°C, whereas xylanase II had an optimum temperature at 55°C (Fig. 1). Thermal inactivation was determined at 50 and 60°C (Fig. 2). Both enzymes were stable at 50°C.

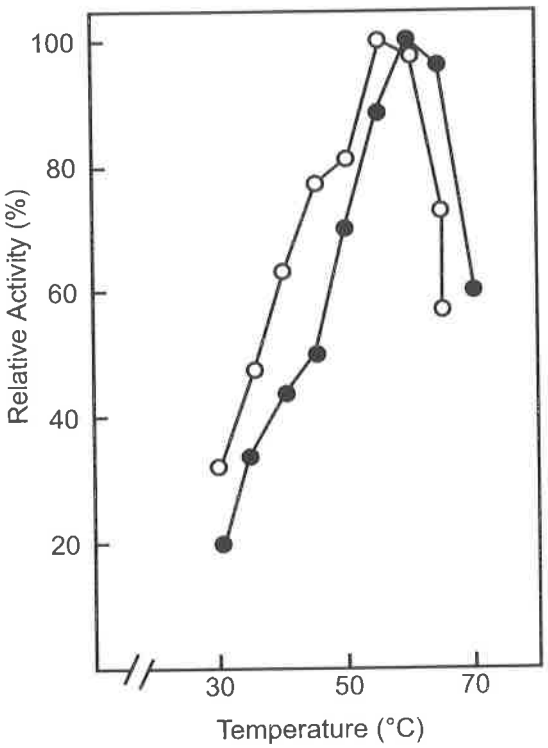

Figure 1. Effect of temperature on xylanase activity. (●) xylanase I; (○) xylanase II

The half lives at 60°C of xylanases I and II were 100 and 18 min, respectively. There was no loss of activity after 8 days at 40°C for both enzymes. Xylanases I and II at 50°C showed optima pH at 6.0 and 5.5, respectively, but had about 90 and 60% of maximum activity at pH 8 for xylanase I and II, respectively (Fig. 3). Both enzymes were stable (100% of activity) between pH 5 and 9 for 48 h at 40°C (Data not shown).

The enzymes were incubated at alkaline conditions with birchwood or oat spelt xylan as substrate and the reaction mixtures were periodically withdrawn and the reducing sugars produced were

Table 1. Purification of xylanases from *Aspergillus tamarii*.

Steps	Vol. (ml)	Xylanase (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Purification (fold)	% recovery
1. Culture supernatant	300	305.2	3.79	80.5	1.00	100
2. 30-75% (NH4)2SO4 precipitation	13	4999.3	28.40	176.0	2.18	71
3. DEAE-cellulose						
Xylanase I	57	337.3	1.61	209.5	2.60	21
Xylanase II	250	161.0	0.35	460.0	5.71	44
4. Sephadex G-100						
Xylanase I	55	133.1	0.42	316.9	3.94	8
Xylanase II	123	138.4	0.22	629.1	7.81	19

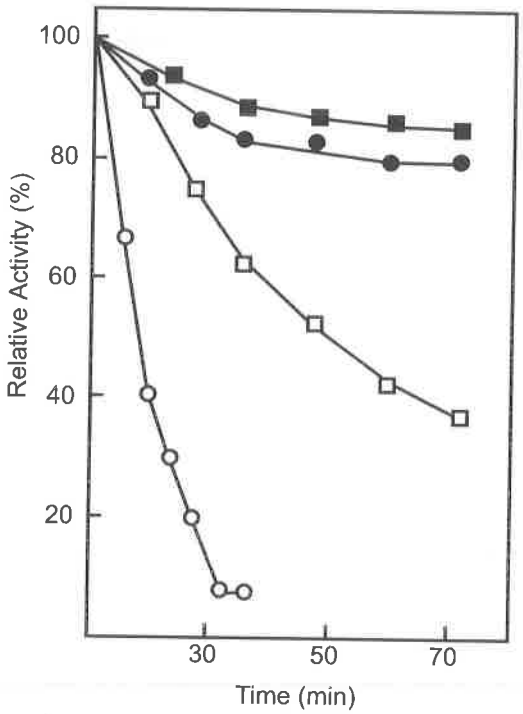

Figure 2. Thermal stability of xylanases. The enzymes were incubated at 50 and 60°C in 0.05 M citrate buffer, pH 5.4. At different time intervals the aliquots were withdrawn, and residual activities were measured under standard conditions. Xylanase I: (●) at 50°C; (○) at 60°C; Xylanase II: (■) at 50°C; (□) at 60°C.

determined. After 24 h, 70% of oat spelt xylan was hydrolysed by xylanase II and 50% by xylanase I (Fig. 4A). When birchwood xylan was the substrate, the maximum degree of hydrolysis after 24 h was 40 and 50% respectively by xylanase I and II (Fig. 4B). The end products released were analysed by paper chromatography. Xylanase I and II produced xylobiose as the main product along with xylotriose, xylotetraose and xylooligosacharides. Xylose was not detected as hydrolysis product (Data not shown).

DISCUSSION

The two xylanases detected in the culture filtrate of *A. tamarii* differed in their molecular weights, Km values and degree of carbohydrate content. A comparison of Km values suggests that xylanase I has higher affinity for birchwood xylan than xylanase II. Both enzymes released xylooligosaccharides of intermediate size from xylan indicating that they are endoxylanases. The occurrence of multiple xylanases in a microorganism immediately raises questions concerning the function and origin of each enzyme. In the nature, xylan is a integral component of lignocellulose material and it is in close association with cellulose, lignin and other hemicelluloses. It

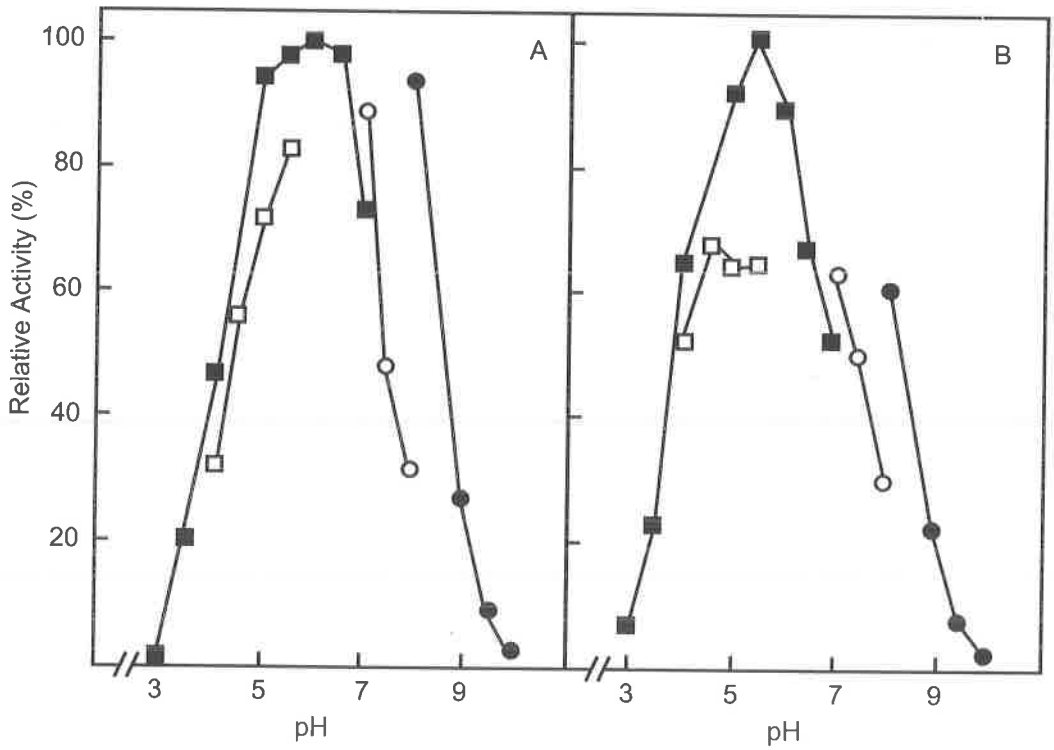

Figure 3. Effect of pH on activity of xylanases. A: xylanase I; B: xylanase II. (■) citrate buffer; (□) acetate buffer; (○) phosphate buffer; (●) glycine buffer.

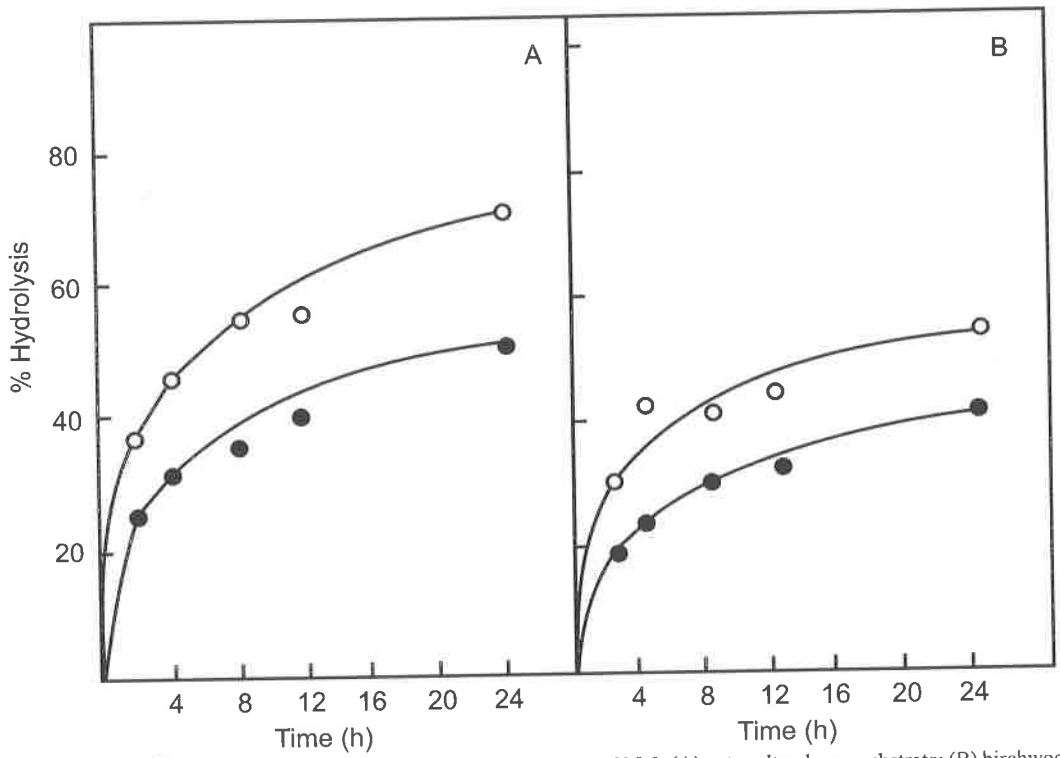

Figure 4. Time course of hydrolysis of xylan (25 mg) by 1 U of xylanases at pH 8.0. (A) oat spelt xylan as substrate; (B) birchwood xylan as substrate. (○) xylanase II; (●) xylanase I.

appears that xylosidic linkages in lignocellulose material are not all equivalent and equally acessible to xylanolytic enzymes (25). The production of a system of enzymes, each enzyme with specialized function, may be a strategy that a microorganism use to achieve superior xylan hydrolysis.

The optimum temperature (55-60°C) and pH (5.5-6.0) of the two xylanases did not show any striking difference and are similar to that for xylanases from other *Aspergillus* strains (5, 9). However, both xylanases from *A. tamarii* were found to be stable at pH 5 to 9 and showed about 90% (xylanase I) and 60% (xylanase II) of maximum activity at pH 8. It is unusual fungi to produce xylanases able to hydrolyse xylan at alkaline conditions. For this reason, *A. tamarii* is a potential source of xylanases for the selective hydrolysis of xylan residues in pulp applications, in which alkaline pulping and bleaching conditions are often used.

ACKNOWLEDGEMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal Superior (CAPES) and Universidade Estadual de Maringá. The authors are grateful to M.A.F. Costa for her technical assistance.

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Revista de Microbiologia (1998) 29:99-103
ISSN 0001-3714

PRODUCTION OF AMYLASES BY *ASPERGILLUS FUMIGATUS*

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Submitted: May 15, 1997; Returned to authors for corrections: July 14, 1997; Approved: March 16, 1998

ABSTRACT

The effect of carbon and nitrogen sources, substrate concentration, pH and temperature on growth and production of glucoamylase and α-amylase by *Aspergillus fumigatus* was examined. Low constitutive levels of both enzymes were obtained with growth on glucose, xylose and lactose, while significantly higher levels were produced from maltose, raffinose, glycogen, starch and starchy sources (wheat bran, oat bran, manioc and canola meal). Stationary cultivation led to significantly higher yields than those obtained using shaking culture. $NaNO_3$ and NH_4NO_3 seemed to be good nitrogen sources for amylase synthesis, while $(NH_4)_2SO_4$ and NH_4Cl were poor substrates for this purpose. Initial pH between 4 to 10 and temperature between 30-45°C could be utilized to produce amylolytic activity. The highest levels of glucoamylase and α-amylase were obtained with stationary cultures grown on 3% (w/v) oat bran as carbon source and 0.2% (w/v) sodium nitrate as nitrogen source at pH 6.0 and 30°C.

Key words: glucoamylase, α-amylase, *Aspergillus fumigatus,* enzyme induction.

INTRODUCTION

Amylases are widely distributed in the nature and play a very significant role in starch-processing industries. Several types of enzymes are involved in the degradation of starch, mainly α-amylases, β-amylases and glucoamylases (6, 11, 18). Many microorganisms, including bacteria, yeast and filamentous fungi are able to produce amylolytic enzymes. Amongst the fungal amylases, those of *Aspergillus niger* and *Aspergillus oryzae* have received most attention because of their high productivity. These species are well-known commercial sources of α-amylases and glucoamylases (16). Several other *Aspergillus* species produce amylases but some species, such as *A. flavus* and *A. fumigatus,* produce toxic substances when cultivated under determined conditions. In this case, the use of these amylases has only a restricted commercial use. Hizuriki et al. (1) obtained a raw-starch-digesting amylase from a strain of *Aspergillus fumigatus* deposited as *Aspergillus* sp K-27. Two enzymes, a glucoamylase and an α-amylase, were purified and partially characterized (2). Recently, the α-amylase, was more completely characterized (14). Another strain of *Aspergillus fumigatus,* isolated from soil, produces both liquefying (α-amylase, 1,4-α-glucan glucanohydrolase, E.C. 3.2.1.1) and saccharifying (glucoamylase, 1,4 α-glucan glucohydrolase, E.C. 3.2.1.3) activities induced by soluble starch and lignocellulosic materials (8). Some characteristics of this strain, such as the ease of cultivation and the high production of extracellular amylases in an inexpensive medium, make this soil isolate a potential source of amylases. The purpose of this study was to investigate the effect of some

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environmental and nutritional factors on the production of α-amylase and glucoamylase by *Aspergillus fumigatus*.

MATERIALS AND METHODS

Microorganism: *Aspergillus fumigatus* was isolated from soil during a screening program for amylase-producing microorganisms (8). It was routinely maintained on potato dextrose agar at 30°C. Spore suspensions were prepared by adding 10 ml of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop.

Growth medium and amylase production. The cultures were obtained in 250 ml Erlenmeyer's flasks containing 50 ml of the following medium: 1 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4.7H_2O$ and 1 g of yeast extract dissolved in 1 l of deionized water. The media were supplemented with different carbon and nitrogen sources. Inoculated flasks were incubated at 30°C on a rotary shaker at 120 rpm or under stationary condition. Flasks were harvested at periodic intervals, the contents filtered through tared Whatman # 1 filter paper and the mycelia dried to constant weight at 60°C. The biomass production was not determined when the cultures were supplemented with insoluble carbon sources. Culture filtrates were assayed for α-amylase and glucoamylase activities, protein and reducing sugars contents. Results were expressed as the mean of at least three different experiments.

Enzyme assays: The starch-digesting activity of the culture filtrate enzyme was assayed in the following two different ways: **i). glucoamylase assay**: Glucose liberation from starch was measured by the peroxidase-glucose oxidase assay (4) utilizing 0.5% (w/v) starch in 0.05 M phosphate buffer pH 6.0 at 40°C. One unit of glucoamylase catalyses the liberation of 1 μmol of glucose per min under the specific conditions; **ii).** α-amylase assay: the dextrinizing activity of the culture filtrate was determined by the iodometric method (9) as described in Wilson and Ingledew (19). One unity of α-amylase was defined as the amount of enzyme that hydrolyses 0.1 mg of starch in 10 min at 40°C when 4.0 mg of starch is present. In both cases, the enzyme samples were diluted appropriately to maintain linearity over the assay interval. The results for both enzymes are presented as specific activity (U/mg extracellular protein).

Protein estimation: Extracellular protein was estimated as described by Lowry *et al.* (10) using bovine serum albumin as the standard.

Reducing sugar estimation: Reducing sugars were estimated using dinitrosalicylic acid reagent (12) with glucose as the standard.

RESULTS AND DISCUSSION

The ability of *A. fumigatus* to grow and to produce amylases on different carbon source were studied. The organism was grown in liquid culture with 1% carbon source (w/v) at 30°C quiescently and on a rotary shaker for 6 days. The results of amylase and biomass production produced are shown in Table 1. Significant growth was detected on all carbon source.

Table 1. Effect of carbon source on the growth and amylase production by *Aspergillus fumigatus*.

Carbon source	Stationary culture			Shaking culture		
(1% w/v)	Biomass (mg)	Glucoamylase (U/mg)	α-amylase (U/mg)	Biomass (mg)	Glucoamylase (U/mg)	α-amylase (U/mg)
none	28	0.03	68	45	0.02	40
glucose	355	1.50	132	590	0.80	110
xylose	270	1.90	175	550	1.14	130
lactose	240	2.10	260	490	1.82	143
raffinose	250	8.10	510	430	3.60	350
maltose	264	15.56	670	440	2.87	330
glycogen	310	33.64	2140	420	5.16	670
starch	290	35.71	2390	390	4.10	680
wheat bran	Nd	20.80	1650	Nd	4.80	650
oat bran	Nd	23.70	1890	Nd	4.10	690
manioc	Nd	17.50	1400	Nd	3.30	510
canola meal	Nd	6.60	850	Nd	2.50	310

The cultures were obtained in 250 ml Erlenmeyer flasks containing 50 ml mineral medium supplemented with 0.2% $NaNO_3$ (w/v) as nitrogen source and different carbon sources at 1% (w/v) quiescently or on a rotary shaker at 120 rpm at 30°C for 6 days. Nd=not determined.

Low constitutive levels of glucoamylase and α-amylase were observed after growth on glucose, lactose, and xylose. High amylase activities were observed when the organism was grown on maltose, raffinose, glycogen, starch or starchy sources (wheat bran, oat bran, manioc and canola meal). These data suggest the inductive nature of production of glucoamylase and α-amylase by saccharides with α 1,4 linkages. The inductive nature of production of amylase has been suggested in other *A. fumigatus* isolated from soil. However, the levels of glucoamylase (35 U/mg) and α-amylase (2400 U/mg) obtained in starch cultures in this work were superior that obtained with that *A. fumigatus* strain (1, 2). Although more biomass was obtained on shaking cultures, the strain of *A. fumigatus* utilized for the present study produced more than fourfold of both glucoamylase and α-amylase activities when grown under stationary condition than with shaking. Similar results were obtained with other *Aspergillus* strains (17). In both stationary and shaking cultures, incubation over 6 days did not increase significantly the percentage of amylases produced (data not shown).

The effect of substrate concentration on the production of amylases was investigated (Fig. 1). For lower substrate concentrations (up to 1%, w/v), the cultures utilizing soluble starch as substrate produced more glucoamylase than those grown on wheat bran, oat bran or manioc. Nevertheless, for an initial substrate concentration greater than 1%, higher amylolytic activities were obtained with starchy sources. The amylase production was characterized by a steady decline at higher starch concentrations. In these cultures, high levels of reducing sugars were present (data not shown). The presence of accumulated hydrolysis products resulted in catabolite repression of enzyme production at higher starch concentration.

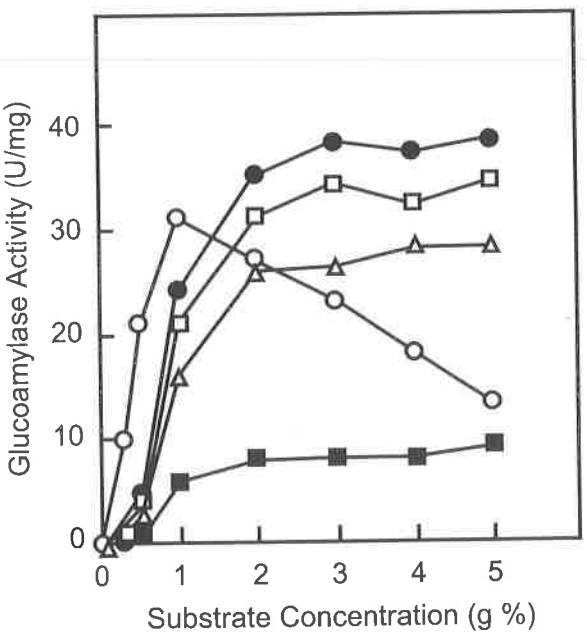

Figure 1. Effect of substrate concentration on production of glucoamylase by *Aspergillus fumigatus*. (○) Starch; (●) Oat bran; (□) Wheat bran; (△) Manioc; (■) Canola meal. The cultures were obtained quiescently at 30°C for 6 days in mineral medium supplemented with 0.2% (w/v) NaNO3 as nitrogen source.

The effect of different inorganic and organic nitrogen sources on the production of amylases is shown in Table 2. $NaNO_3$ and NH_4NO_3 seemed to be the best sources for amylase synthesis by this fungi while $(NH_4)_2SO_4$ and NH_4Cl were poor substrates for this purpose. Many studies have related the importance of nitrogen source in the production of amylases Generally, NH_4^+ salts and different NO_3^- have been described as good nitrogen sources for amylase synthesis (3, 5, 7, 13, 15). Although the fungus had produced more biomass, none significant enhancement in the enzyme production was observed when an organic nitrogen source was employed, instead of inorganic ones.

The effect of the initial pH of the medium on the growth and amylase production was studied within a range of 3 to 12 (Table 3). At initial pH levels between 3 to 6, the growth resulted in an increase in

Table 2. Effect of nitrogen source on growth and amylase production by *A. fumigatus*.

Nitrogen source (0.2%, w/v)	biomass (mg)	glucoamylase (U/mg)	α-amylase (U/mg)
none (control)	140	2.0	210
$NaNO_3$	290	35.0	2300
KNO_3	280	26.8	1700
NH_4NO_3	140	31.	2200
$(NH4)_2SO_4$	280	18.2	460
NH_4Cl	268	6.0	380
ammonium acetate	531	3.3	550
yeast extract	250	16.1	1500
glutamic acid	260	16.9	1080
serine	510	15.3	1300
glycine	300	15.1	1460

The cultures were obtained in Erlenmeyer flasks with 50 ml mineral medium supplemented with 1% (w/v) starch as carbon source and 0.2% (w/v) different nitrogen sources quiescently at 30°C for 6 days.

Table 3. Effect of initial pH on the amylase production by *A. fumigatus*

Initial pH	Final pH	Glucoamylase (U/mg)	α-amylase (U/mg)
3	4.8	6.1	690
4	5.7	28.1	1690
5	6.1	32.7	2300
6	6.8	33.1	2430
7	6.8	34.7	2200
8	7.1	31.7	2170
9	7.7	33.3	2240
10	9.2	31.0	1930
11	9.6	13.4	860
12	10.1	4.2	360

The cultures were obtained in mineral medium supplemented with 0.2% (w/v) NaNO3 as nitrogen source and 2% (w/v) oat bran as carbon source quiescently at 30°C for 6 days.

Table 4. Effect of temperature on amylase production by *A. fumigatus*

Temperature (°C)	Glucoamylase (U/mg)	α-amylase (U/mg)
25	28.5	1590
28	29.7	1680
30	34.3	2280
32	33.6	2420
35	32.9	2370
40	35.1	2470
45	31.3	2320
48	14.1	1100
50	3.1	290

The cultures were obtained in mineral medium supplemented with 0.2% (w/v) NaNO3 as nitrogen source and 2% (w/v) oat bran as carbon source quiescently at different temperature for 6 days.

the pH of the filtrates. When the initial pH was superior to 6, the growth resulted in decrease of pH. Initial pH between 4 and 10 was found to be more favourable for production of high yields of amylases. Growth and enzyme production were inhibited when the initial pH of the medium was above 10 or below 4.

The effect of temperature on the growth and enzyme production is shown in the Table 4. The strain of *A. fumigatus* utilized in this study was able to produce high levels of amylases under elevate temperature (45°C). The fungi did not grow at temperatures above 48°C. These data show that the *A. fumigatus* strain is a heat-resistant mold.

The data presented in this work suggest that the strain of *A. fumigatus* is able to present high glucoamylase and α-amylase activities under different conditions.

ACKNOWLEDGEMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Universidade Estadual de Maringá. The authors are grateful to M.A.F. Costa for her technical assistance.

RESUMO

Produção de amilases por *Aspergillus fumigatus*

O efeito de diferentes fontes de carbono e nitrogênio, concentração do substrato, pH e temperatura de cultivo no crescimento e produção de glucoamilase e α-amilase por *Aspergillus fumigatus* foi estudado. Níveis constitutivos baixos de ambas as enzimas foram obtidos utilizando-se glucose, xilose e lactose como substrato, enquanto altos níveis de atividade enzimática foram produzidos em cultivos com maltose, rafinose, glicogênio, amido e materiais amiláceos tais como, farelo de trigo, farelo de aveia, mandioca e farinha de canola. A produção de amilases em culturas estacionárias foi significativamente maior do que em culturas sob agitação. Nitrato de sódio e nitrato de amônia foram boas fontes de nitrogênio para síntese das amilases, enquanto que sulfato de amônio e cloreto de amônio não foram adequados para este propósito. Altas atividades enzimáticas foram produzidas em culturas com pH inicial na faixa de 4 a 10 e temperaturas entre 30 e 45°C. Os maiores niveis de glucoamilase e α-amilase foram obtidos em crescimento estacionário a pH 6,0 e 30°C, utilizando-se farelo de aveia a 3% (p/v) como fonte de carbono e nitrato de sódio 0,2% (p/v) como fonte de nitrogênio.

Palavras-chave: glucoamilase, α-amilase, *Aspergillus fumigatus*, indução enzimática.

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Revista de Microbiologia (1998) 29:104-108
ISSN 0001-3714

PHYSIOLOGICAL CHARACTERIZATION OF YEASTS ISOLATED FROM ARTISANAL FERMENTATION IN AN *AGUARDENTE* DISTILLERY

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Submitted: October 29, 1997; Returned to authors for corrections: March 12, 1998; Approved: April 02, 1998

ABSTRACT

The physiological characteristics of 210 yeast isolates from an *aguardente* distillery of the State of Minas Gerais were studied. Among these isolates, 186 were able to grow at 35°C, and 23 at 42°C. All isolates were able to grow in medium containing 25% of glucose, except for an isolate of *Candida karawaiewii*. The majority of the isolates grew in medium with 5% ethanol, a concentration similar to that of the fermented must. Killer toxin production was detected in isolates of *Candida sake, Kluyveromyces lactis* var. *drosophilarum*, *Saccharomyces kluyveri*-like and *Saccharomyces servazzii*-like. Forty-two isolates, which showed measurable growth in medium containing 10% (V/V) ethanol and were also sugar-tolerant, thermotolerant, and neutral or killer toxin producers, were tested for invertase production. Ten isolates of *S. cerevisiae* and one of *C. sake* showed the highest invertase activity.

Key words: Yeast physiology, spontaneous fermentation, *aguardente* production.

INTRODUCTION

Brazilian sugar-cane *aguardente* is made by spontaneous fermentation using the microbiota that develops spontaneously in a starter. The starter is prepared with sugarcane juice, crushed corn, lemons, rice, and other substrates, and allowed to ferment naturally for 5-20 days. Typical conditions during starter formation are the high concentration of fermentable sugars (between 16 and 25° BRIX) and amounts of ethanol increasing to 5 to 7 g/l in the end of the process (8). The yeast flora arises from equipment, vats and the sugarcane. After this, the starter is added to the vats and sugarcane juice is added. The fermentation cycles normally last 18-48 hours, after which 4/5 of the fermented must is distilled and fresh sugarcane juice is added to the remainder to begin a new fermentation cycle. The fermentations are carried out at temperatures around 28°C. In the state of Minas Gerais, more than 8,000 distilleries produce about 130 million's liters/year, from May to December, and the production is completely artisanal.

The short fermentation cycles, the daily addition of sugarcane juice, and the low amounts of ethanol in the must are the usual characteristics of artisanal *aguardente* production. The yeast communities present in such fermentations are in constant succession, and the species present in the fresh sugarcane juice are constantly introduced in the microenvironment of the fermentation vat. *Saccharomyces cerevisiae* is the prevalent species, but apiculate yeasts (mainly *Kloeckera japonica*), *Candida, Kluyveromyces* and *Pichia* species are

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frequently isolated (8). Other important characteristic of spontaneous fermentations is the presence of killer yeasts (13). It has been suggested that the killer phenomenon is a mechanism of antagonism among yeasts during spontaneous fermentations and that killer strains may dominate the end fermentations in wineries (1, 4). Morais et al. (8) have shown that the majority of the strains isolated from an *aguardente* distillery were neutral to toxins produced by killer yeasts.

The physiological abilities of the yeast strains isolated from fermented must have a special relevance in understanding the mechanisms involved in must colonization, and determining the optimum conditions to maintain healthy fermentations. In this work, osmotolerance, ability to grow at high temperatures and high alcohol concentrations, patterns of killer toxin resistance and sensitivity among prevalent yeasts and invertase activity from strains isolated of an *aguardente* distillery in the State of Minas Gerais were studied.

MATERIALS AND METHODS

Two hundred and ten yeasts were isolated from one *aguardente* distillery in State of Minas Gerais, as described in Morais et al. (8). The strains were characterized according to standard methods (12), and identified by the keys of Kreger-van Rij (5), Barnett et al. (2) and Vaughan-Martini and Martini (14).

The yeast isolates from the fermentation vats were grown on modified Sabouraud agar (glucose 2%, peptone 1%, yeast extract 0.5%, and agar 2%) at room temperatures for 24 hours, and 0.1 ml of a suspension containing 1 x 10^7 cells was inoculated in the following media: Sabouraud broth with 15, 20 and 25% of glucose, to test osmotolerance; Sabouraud broth with 5, 8 and 10 g/l of ethanol (ethanol was added after sterilization and the tubes were covered with a parafilm to avoid ethanol evaporation); and in Sabouraud broth incubated in water bath at 32, 35, 37, 40 and 42°C. Osmotolerance and ethanol resistance was determined at room temperature (25 ± 3°C). Yeast growth was evaluated by visual turvation of the liquid medium and the viability by the methylene blue method (6).

The killer toxin production was screened according to Young (15) and Starmer et al. (11), using *Candida glabrata* NCYC 388 and *Saccharomyces cerevisiae* NCYC 1006 as sensitive reference strains. Killer strains obtained were tested for killer activity against all the isolates obtained from the fermentation. YM agar supplemented with 0.003% methylene blue and buffered to pH 4.2 with sodium acetate, previously seeded with the sensitive strains, was inoculated with the killer strains (10 per plate) and incubated at 22°C for four days. Strains were identified as killer if they produced a halo of dark blue dead cells or a zone of inhibition surrounded by a halo of dead cells.

Strains that were able to grow at 37°C, in medium with 25% of glucose and 10% of ethanol, and that produced killer toxins or were neutral to toxins produced by killer strains were tested to invertase production. The invertase activity was measured by the dinitrosalicilic acid (DNS) colorimetric test, as described in Ekinsanmi and Odunfa (3). Each yeast was grown on Sabouraud agar for 48 hours, the cells were diluted in sterile water, washed by centrifugation, and 0.1 g wet weight of each was resuspended in 10 ml of acetate buffer, pH 5.0. One ml of each cell suspension was added to 2 ml of 4% sucrose solution in the same buffer and incubated for 5 min. at 30°C. One unit of invertase activity was defined as the amount of enzyme which liberated one µmol/min of reducing sugars under these conditions. All the experiments were made in triplicate.

RESULTS AND DISCUSSION

The microenvironment of the *aguardente* fermentation was selective for osmotolerant yeasts. All strains grew in medium with 25% of glucose, except for a strain of *Candida karawaiewii*. The majority of the yeast isolates were able to grow at 35°C, but only 10.9% grew at 42°C (Table 1). Some strains, including three isolates of *K. japonica*, grew in temperatures higher than those described for this species. This characteristic was also observed with apiculate yeasts isolated from *Drosophila* in Atlantic Rain Forests in Rio de Janeiro (7). In general, *S. cerevisiae* isolates were the most resistant to the high alcohol concentration: 75% were able to grow in the presence of 10% of alcohol. The yeast viability in the above experiments was around 90%. These results showed that the yeasts isolated from *aguardente* fermentation were adapted to grow at alcohol concentrations typical of those found at the end of the fermentative process (5 to 7 g/l).

Killer strains were isolated throughout the fermentation process as well as in the starter ferment. They included *C. sake, K. lactis* var. *drosophilarum*, *S. kluyveri*-like and *S. servazzii*-like (Table 2). None

Table 1. High temperature growth and ethanol tolerance levels of yeasts isolated from an *aguardente* distillery.

Species	Number of isolates	Temperatures (°C)				Alcohol concentration (%)		
		35	37	40	42	5	8	10
Candida citrea	6	6	4	-	-	6	5	2
Candida maltosa	1	1	1	1	1	1	-	-
Candida sake	27	27	24	8	-	27	22	3
Candida sorbosa-like*	9	9	7	-	-	9	8	4
Candida sorboxylosa	5	4	4	-	-	4	4	3
Candida stellata	3	2	1	-	-	3	3	1
Candida karawaiewii	2	1	1	-	-	2	2	1
Geotrichum sp.	5	4	4	-	-	4	4	-
Hanseniaspora occidentalis	1	1	1	-	-	1	1	-
Hanseniaspora uvarum	2	1	-	-	-	2	2	1
Kloeckera japonica	30	15	9	3	1	22	17	7
Kloeckera javanica	4	4	2	-	-	2	2	1
Kluyveromyces marxianus	2	2	2	1	-	2	1	1
Kluyveromyces lactis var. *drosophilarum*	12	12	8	4	3	12	11	1
Kluyveromyces thermotolerans	1	1	1	1	1	1	1	-
Pichia etchelsii	2	2	2	1	1	2	2	-
Pichia guillermondii	2	2	2	2	2	2	2	-
Pichia kluyveri	2	2	2	2	-	2	2	-
Pichia membranaefaciens	6	2	2	-	-	6	4	2
Saccharomyces cerevisiae	76	76	57	35	11	76	76	57
Saccharomyces kluyveri-like	3	3	3	3	2	3	3	1
Saccharomyces servazzi-like	8	8	5	3	1	8	8	4
Torulaspora delbrueckii	1	1	1	1	-	1	1	1
TOTAL	210	186	143	64	23	198	181	90

* Probable nes species similar to the species indicated.

of the killer yeasts species dominated the fermentative process for *aguardente* production. The four killer isolates of *C. sake* obtained from the starter killed 64 isolates of 23 species (Table 2), including two isolates of *S. cerevisiae* and all species considered transients or contaminants (8). Killer isolates of *C. sake* from the fermentation vats had a narrower spectrum of activity than those of the starter. *Kluyveromyces lactis* var. *drosophilarum*, isolated from the *aguardente* fermentation, had two different killer phenotypes. Four isolates killed 13 species, including *S. cerevisiae* and *C. sake*, whereas another isolate killed solely *P. kluyveri* var. *kluyveri*, *C. stellata*, *K. japonica*, *P. membranaefaciens* and *K. marxianus*. Radler et al. (10) isolated four different killer phenotypes of *Hanseniaspora uvarum* and

Table 2. Frequency of isolation and detection of yeast producing killer toxins and sensitive isolates from the starter and fermentation vats.

Species	Killer isolates*	Origin	Spectrum of Activity		
			Species n=23	Isolates n=210	*S. cerevisae* n=76**
Candida sake	4 (6)	Starter	23	64	16
	5 (21)	Fermentation vat	13	47	12
Kluuveromyces lactis var.drosophilarum A	4 (12)	Fermentation vat	13	49	15
Kluuveromyces lactis var. drosophilarum B	1 (12)	Fermentation vat	6	10	-
Pichia kluyveri	1 (2)	Starter	4	11	1
Saccharomyces kluyveri - like	2 (3)	Starter	14	54	11
Saccharomyces servazzi - like	1 (8)	Starter	6	9	-

* Number in parentesis represent the total number of isolates tested for killer activity.
** Number of *Saccharomyces cerevisiae* isolates are included in the total number of isolates, and represent the number of isolates of this species that are sensitive to killer toxins.

Table 3. Invertase activity of selected *aguardente* yeasts

Species	Number of isolates tested	Invertase activity*				
		<10	11-50	51-100	101-200	>200
Candida sake	2	1	-	-	-	1
Candida stellata	1	-	1	-	-	-
Saccharomyces cerevisiae	35	10	3	12	10	-
Saccharomyces servazzii-like	3	-	2	1	-	-
Torulaspora delbrueckii	2	-	-	2	-	-
TOTAL	43	11	6	15	10	1

* µmol reducing sugar/mg/cell/min. at 30°C

seven killer phenotypes for *P. kluyveri*. So far at least three killer phenotypes have been observed in *S. cerevisiae* (9, 15).

Ekusanmi and Odunfa (3) have shown that high invertase activity is required for yeast growth in molasses, where the principal carbohydrate is sucrose. From 42 *aguardente* yeast isolates tested for invertase activity, 10 isolates of *S. cerevisiae* produced more than 100 µmol/min reducing sugar (Table 3); in one strain of *Candida sake*, the rate was 400 µmol/min. Most of these *S. cerevisiae* isolates would be suitable as a starter for *aguardente* production. From this, we concluded that the yeasts found in Brazilian *aguardente* fermentations were well adapted to the environmental conditions prevailing in the fermentation vats, and that some of the strains isolated could be used advantageously as starter cultures for the production of sugarcane *aguardente*.

ACKNOWLEDGMENTS

We acknowledge financial support from the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG- processo nº 2659), the Pró-Reitoria de Pesquisas-UFMG, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thank the Vale Verde Distillery for allowing us to use its plant facilities, and Dr. Marc-André Lachance's suggestions to improve the manuscript.

RESUMO

Caracterização fisiológica das leveduras isoladas na fermentação artesanal em uma destilaria de aguardente

Foram estudadas as características fisiológicas de 210 linhagens de leveduras isoladas de um alambique de aguardente artesanal do Estado de Minas Gerais. Das linhagens isoladas, 186 cresceram a 35°C e 23 cresceram a 42°C. Todos os isolados, exceto uma linhagem de *Candida karawaiewii*, cresceram em meio contendo 25% de glicose. A maioria dos isolados cresceram em concentração de 5% (v/v) de etanol, concentração similar àquela encontrada no mosto fermentado. As linhagens de *Candida sake*, *Kluyveromyces lactis* var *drosophilarum*, *Saccharomyces kluyveri*-similar e *Saccharomyces servazzii*-similar produziram micocinas. Quarenta e duas linhagens que foram capazes de crescer em meio contendo 10% de etanol, e mostraram-se termotolerantes, osmotolerantes, neutras ou produtoras de micocinas, foram testadas quanto a capacidade de produzir invertase. Dez isolados de *S. cerevisiae* e uma linhagem de *C. sake* apresentaram maior atividade invertásica.

Palavras-chave: Fisiologia de leveduras, fermentação espontânea, produção de aguardente

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Revista de Microbiologia (1998) 29:109-112
ISSN 0001-3714

PATHOGENICITY OF ISOLATES OF *METARHIZIUM ANISOPLIAE* (METSCH.) SOROKIN TOWARDS THE CATTLE TICK *BOOPHILUS MICROPLUS* (CAN.) (ACARI: IXODIDAE) UNDER LABORATORY CONDITIONS

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Submitted: March 17, 1997; Returned to authors for corrections: October 27, 1997; Approved: March 16, 1998

ABSTRACT

The pathogenic activity of *Metarhizium anisopliae* on *Boophilus microplus* females was assessed using five fungal isolates. Groups of 8 engorged ticks collected from cattle on free pasture were inoculated by immersion for 5 seconds in a spore suspension containing 2.5×10^8 conidia/ml. The groups were transferred to moist chambers in which they were maintained at 27 ± 1°C for about 15 days, their mortality being determined every 2, or occasionally 3, days. The isolate that produced the best results in this assay was used to inoculate female ticks with spore suspensions containing 0, 7.5×10^5, 7.5×10^6, 7.5×10^7 and 7.5×10^8 conidia/ml, under the same conditions as described above. The pathogenic action of *M. anisopliae* on engorged females was clearly demonstrated. Isolates E9 and AM were more effective, causing high tick mortality as well as reduced oviposition. The concentration of 7.5×10^8 conidia/ml was the most effective, with the fungus sporulating on 91.1% of the ticks. Mean percent oviposition was highest in the control treatment and lowest in the treatment with 7.5×10^8 conidia/ml.

Key words: *Metarhizium anisopliae*, *Boophilus microplus*, entomopathogenic fungi, cattle tick, biological control.

INTRODUCTION

The tick *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae) is geographically distributed at a latitude between the 32nd parallel North and the 35th parallel South, involving areas of South and Central America, Africa, and Australia (13). It is an ectoparasite that feeds on blood from warm-blooded animals. In addition to parasitizing other species including humans, it preferentially parasitizes cattle, especially dairy breeds (*Bos taurus*). The exact damage caused by this parasite is difficult to calculate, with direct and indirect losses including mortality of breeds of European origin, elevated morbidity with reduced milk and meat yields, losses in leather production, and costs of antiparasitic agents (5). It is also the major transmitter of bovine parasitic sadness (13).

The control of these ticks is of great importance for cattle raising in tropical and subtropical regions. The methods utilized thus far are limited to the application of synthetic chemical products in combination with management measures. However, the indiscriminate use of drugs against ticks has led to serious problems such as environmental pollution and the appearance of resistance to the active ingredients of these products (1,3,5,8).

Research on the use of microorganisms for the biological control of cattle ticks is still incipient (7). Some studies (7) have reported the presence of

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bacteria in pathogenic association with this host. Brum and coworkers have investigated different aspects of the incidence of bacterial infection of *Boophilus microplus*, especially *Cedecea lapagei* (7). As regards the use of fungi, some authors experimentally infected *Ixodes ricinus* females with *Beauveria bassiana*, *Botrytes cinerea* and *Penicillium insectivorum* (5). Later studies have investigated some aspects of infection of *B. microplus* (2,4,5,6,12), *Rhipicephalus appendiculatus* and *Amblyomma variegatum* (11) with the fungi *Metarhizium anisopliae* and *Beauveria bassiana*, with promising results.

The objective of the present study was to assess the *in vitro* pathogenicity of different *Metarhizium anisopliae* isolates for engorged *B. microplus* females, to select the most pathogenic isolate and to determine the spore concentration that produced the highest tick mortality.

MATERIALS AND METHODS

Isolates evaluation

Five *Metarhizium anisopliae var. anisopliae* isolates from various regions of Brazil were cultured in BDAY medium (potato, dextrose, agar and yeast extract). The following isolates were used: E9 (isolated from *Deois flavopicta*, in the State of Espírito Santo), AM (non-identified insect, State of Amazonas), AL (*Mahanarva posticata*, State of Alagoas), MT (*Deois* sp., State of Mato Grosso), and SP (*Deois flavopicta*, State of São Paulo). The cultures were kept in an incubator at 27 ± 1°C until satisfactory fungal sporulation was obtained. Inocula were prepared in 0.1% aqueous Tween 80 solution with a spore concentration of 2.5 x 10^8 conidia/ml, estimated in a Neubauer chamber, for all isolates. Groups of 8 ticks collected from cattle on free pasture were immersed for 5 seconds in 10 ml of the spore suspension according to the technique of Drummond *et al.* (9) and Green and Connole (10).

Each group was kept in a moist chamber which had an average RH of 88%, ranging from 82 to 97%.The chambers were incubated for 14 days at 27 ± 1°C, and mortality caused by the fungus as well as absence of oviposition were checked at 2, or occasionally 3, day intervals. The female ticks were considered dead if they didn't move after being touched several times using a stylet.

Effect of spore concentration

Isolate E9, which presented the best result in the previous assay, was selected to test the potential of different spore concentrations. A conidial suspension of 7.5 x 10^8 conidia/ml was prepared and concentrations of 7.5 x 10^7, 7.5 x 10^6 and 7.5 x 10^5 conidia/ml were obtained by serial dilution. The ticks were inoculated and assessed for mortality and oviposition as described for the previous assay. They were maintained for 16 days at 27 ± 1°C and 87% RH, ranging from 80 to 97% RH.

Statistical analysis

The experiments were arranjed as a completely randomized design (CRD) composed of 5 replications per treatment and 8 (1st assay) or 9 (2nd assay) individuals in each replication. Means were compared by the Tukey test at 5% level of significance.

RESULTS AND DISCUSSION

The pathogenic action of the fungus was demonstrated by the statistically significant difference observed between the control and the treatments with the isolates used (Table 1). All

Table 1. Mean mortality rates and mean ovipositing values for *Boophilus microplus* ticks up to the 14 h day after inoculation with isolates of *Metarhizium anisopliae*.

Treatment	Dead ticks		Females that oviposited	
	Mean number[2,3]	Mean percentage	Mean number[2,3]	Mean percentage
Control	1.000 D	0.0	2.9657 C	97.5
AL	2.1655 C	47.5	2.3972 B	60.0
SP	2.3357 BC	57.5	2.2315 B	50.0
MT	2.7919 AB	85.0	2.4068 B	60.0
AM	2.9657 A	97.0	1.6957 A	25.0
E9	3.0000 A	100.0	1.6293 A	22.5

M. anisopliae isolates

[2] Means transformed to $\sqrt{x + 1}$ values

[3] Means followed by the same letter in the column are not significantly different by the Tukey test ($P < 0.05$)

treatments were efficient in causing tick mortality, with emphasis on isolates E9, AM and MT which respectively induced 100, 97 and 85% mortality. In addition, it should be pointed out that on the 6th day after fungal application isolate E9 had already caused 50% tick mortality.

Another important feature was the decrease in ovipositing ability (Table 1), which means a smaller number of descendants acting in subsequent infestations. Within this context, E9 and AM were equally outstanding, reducing oviposition by more than 70%. These results clearly show that oviposition decreases with increasing pathogenic action of the fungus.

The statistical analysis of the effect of different spore concentrations applied to female ticks (Table 2) showed that all spore suspensions had pathogenic action. When the effect of these spore concentrations on ovipositing ability is considered (Table 2), a reduction of more than 80% in oviposition is observed, by the only treatment (7.5×10^8 conidia/ml) that differed significantly from the control.

Similar results were described by other investigators. *B. microplus* ticks sprayed with 2.74×10^7 and 2.74×10^8 conidia/ml of the E9 strain of *M. anisopliae* respectively presented 60 and 97.4% mortality on the 12th day after fungus application (12). The larval stage also proved to be susceptible to the action of fungi, with mortality rates caused by *M. anisopliae* ranging from 29.3 to 97.3% (4) and mortality rates caused by *Beauveria bassiana* ranging from 18.8 to 88% (6). In both cases the highest conidial concentrations (10^8 and 10^9 conidia/ml) caused the highest mortality rates.

Other aspects of the biological cycle of the tick were also affected by the action of the fungi. Among them, reduced ovipositing ability (5, 12) and reduced ovipositing period (5). The action of *M. anisopliae* and *B. bassiana* in preventing or reducing larval hatching has been presented by several studies (2, 5, 6), although Mendes *et al.* (12) reported that the application of *M. anisopliae* did not interfere with larval hatching rate.

The present assays permitted us to conclude that *M. anisopliae* has a pathogenic action against *B. microplus*. The isolates used differed in pathogenicity and the highest spore concentrations were more effective.

The susceptibility of *B. microplus* to the action of microorganisms is little known but studies available indicate a promising future with more than one alternative for the control of this tick. Under laboratory conditions, *M. anisopliae* showed excellent potential for use in the microbial control of this arthropod, although it is necessary to determine the pathogenicity of the fungus against ticks under field conditions.

ACKNOWLEDGMENT

The authors wish to thank Probion - Indústria de Medicamentos Veterinários for financial support.

RESUMO

Patogenicidade de isolados de *Metarhizium anisopliae* (Metsch.) Sorokin para o carrapato de bovinos *Boophilus microplus* (Can.) (Acari: Ixodidae) sob condições de laboratório

A atividade patogênica de *Metarhizium anisopliae* para fêmeas de *Boophilus microplus* foi avaliada utilizando-se 5 isolados do fungo. Suspensões de esporos contendo $2,5 \times 10^8$ conídios/ml foram usadas para inocular, através de imersão por 5 segundos, grupos de 8 teleóginas ingurgitadas, coletadas de bovinos em regime de pasto. Os grupos foram transferidos para o interior de

Table 2. Mean mortality and mean oviposition values for *Boophilus microplus* ticks up to the 16th day after inoculation with different *Metarhizium anisopliae* concentrations.

Treatment	Dead female		Females that oviposited	
	Mean number[2,3]	Mean percentage	Mean number[2,3]	Mean percentage
Control	1.000 B	0.0	2.8284 B	77.7
$7,5 \times 10^5$	2.7048 A	72.2	2.3862 AB	52.8
$7,5 \times 10^6$	2.8685 A	80.5	2.2894 AB	47.2
$7,5 \times 10^7$	2.9520 A	86.1	2.1771 AB	41.6
$7,5 \times 10^8$	3.0382 A	91.1	1.5454 A	19.4

M. anisopliae isolates

[2] Means transformed to $\sqrt{x} + 1$ values

[3] Means followed by the same letter in the column are not significantly different by the Tukey test ($P < 0.05$)

câmaras úmidas, mantidas a 27 ± 1°C por cerca de 15 dias, avaliando-se a mortalidade a cada 2 ou ocasionalmente 3 dias. Com o isolado que produziu melhor resultado neste ensaio, submeteu-se, em idênticas condições, fêmeas do carrapato a suspensões de esporos que continham 0, 7,5 x 10^5, 7,5 x 10^6, 7,5 x 10^7 e 7,5 x 10^8 con./ml. Evidenciou-se claramente a ação patogênica de *M. anisopliae* sobre fêmeas ingurgitadas. E9 e AM, os isolados mais eficazes, provocaram alta mortalidade de fêmeas e reduziram a oviposição. A concentração de 7,5 x 10^8 con./ml mostrou-se mais efetiva tendo o fungo esporulado em 91,1% das carrapatas; a porcentagem média de oviposição foi máxima no tratamento testemunha e mínima no tratamento 7,5 x 10^8 con./ml.

Palavras-chave: *Metarhizium anisopliae*, *Boophilus microplus*, fungo entomopatogênico, carrapato de bovino, controle biológico.

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Revista de Microbiologia (1998) 29:113-117
ISSN 0001-3714

POSSIBLE INVOLVEMENT OF AN OUTER MEMBRANE PROTEIN IN THE PATHOGENICITY OF A CHICKEN SEPTICEMIC *ESCHERICHIA COLI* ISOLATE

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Submitted: July 01, 1997; Returned to authors for corrections: September 19, 1997; Approved: May 4, 1998

ABSTRACT

A mutant variant of a septicemic *Escherichia coli* strain (L3) isolated from an outbreak in chickens was constructed by the insertion of TnphoA transposon. Seven mutant derivatives were analyzed regarding the pathogenicity. Two of them (XP2, XP4) were less pathogenic in the one-day-old chick pathogenicity assay. The expression of several outer membrane proteins of mutant XP2 strain was suppressed, and strain XP4 had a 47.8 ^{kDa} protein that was not expressed. None of these proteins was correlated to the iron-acquisition system. Mutant XP2 could have suppression of a regulatory protein responsible for the expression of other proteins not related to pathogenicity but important for the rapid bacterial growth, while mutant XP4 did not express a 47.8 ^{kDa} protein. We propose that the 47.8 ^{kDa} protein could be associated to the pathogenicity process of *Escherichia coli* strains responsible for septicemia in poultry.

Key words: *Escherichia coli*, fowl, pathogenicity, membrane protein

INTRODUCTION

Avian *Escherichia coli* strains responsible for septicaemia in poultry cause high economic losses to the poultry industry and have been extensively studied in regard to their pathogenicity traits. Colibacillosis probably begins as an opportunistic infection after a respiratory disease caused by a virus (1). This invasion leads to organ lesions such as airsacculitis, pericarditis, perihepatitis, septicaemia and finally death (11). There have been several investigations to determine microbial characteristics associated with the pathogenesis of this group of *E. coli*. Among the known traits of these strains, expression of fimbriae (5,6,7), aerobactin iron-acquisition system (8, 15), serum resistance (7,9,31) and outer membrane proteins (OMPs) (10,18,22) seem to be among the most important ones for pathogenicity. In this work, expression of OMPs, colicin production and serum resistance by a septicaemic virulent strain (L3) and two less virulent derivatives obtained by transposon mutagenesis (TnphoA) were studied and their association with pathogenicity for one-day-old chicks was analysed.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth media

Escherichia coli L3 was isolated from the liver of a chicken with colisepticemia in Campinas, São Paulo state, Brazil. *E.coli* strain MS101 (lac$^-$, Streptomycin resistant) is serum sensitive and non-pathogenic for poultry. *E.coli* strains XP2 and XP4 (this work) are transconjugants isolated after conjugation between L3 and SM10 lambda pir strain (transposon TnphoA) and had their pathogenicity

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decreased by several logs. Culture media used in this work were Luria-Bertani broth (LB) and agar (LA) (17), and BHI (brain heart infusion), from Biobrás. All strains were stored at -70°C in LB broth with 15% glycerol to avoid plasmid losses. Plasmids used as reference for molecular weight estimation were p307 (12) and pRP4 (4). *E.coli* V517 (16) was also used as source for reference plasmids. Strain SM10λpir (plasmid pRT733-TnphoA) was a gift from Dr. James B. Kaper from the Center for Vaccine Development, Maryland, USA. All the antimicrobial drugs used were from Sigma.

Pathogenicity test

The one-day-old male chickens pathogenicity assay was performed as described previously (10). The LD_{50} was calculated by the method of Reed and Muench (20).

Colicin expression assay

Determination of the colicin production was performed as described previously (10).

Plasmid DNA extraction and agarose gel electrophoresis

Plasmid extraction and agarose gel electrophoresis were performed as described by Sambrook et al. (21).

Transposon mutagenesis

Mutagenesis with the transposon TnphoA, using plasmid pRT733, was accomplished as described by Taylor et al. (26).

Hemagglutination assay

D-mannose-sensitive hemagglutinating fimbriae (type 1) (MSH) and D-mannose-resistant hemagglutinating fimbriae (MRH) were assayed with guinea pig erythrocytes treated, either with or without D-mannose, using a slide agglutination technique, as described by Fantinatti et al. (10).

Serum resistance determination.

The resistance of different strains to the bactericidal activity of serum was determined by a quantitative method as described previously (10).

Outer membrane protein (OMP) extraction and SDS-PAGE Electrophoresis.

The bacteria were grown in LB broth, with and without 200 μM of alpha-alpha-dipyridil (Sigma Chemical Co.), and incubated overnight. The cells were collected by centrifugation (8000g for 5 min at 4°C) and the OMPs were prepared as described previously (22). The concentration of protein was determined, before resuspension in the last buffer, as described by Bradford (3). Proteins were separated by SDS-PAGE as described by Laemmli (14) with a 3% stacking and a 12.5% separating gel. An aliquot of 2.0ug of each sample was applied to each well. Protein bands were visualized by silver staining as described by Blum et al. (2). Molecular weights were estimated by comparison with the relative molecular mobilities of standard protein markers (Sigma).

Growth curve analysis

Bacteria were grown overnight in LB broth at 37°C at 150 rpm as a pre-inoculum and afterwards diluted 1:100 into 100 ml of the same medium and again incubated at 37°C (150 rpm). Aliquots of 100 ul were removed every 1.5 h, diluted in sterilized 0.85% NaCl solution and plated onto LA medium. These plates were incubated at 37°C overnight and the number of colony forming units (CFU) determined.

Lipopolysaccharide analysis

The lipopolysaccharide preparations were made as described by Helander (13). For analysis, 5 to 10 ul of each reaction was loaded in 15% SDS-PAGE gels and, after running, the gel was silver stained, as described by Tsay and Frasch (28).

RESULTS

Seven blue-kanamycin resistant colonies were isolated after conjugation of strain SM10 lambda pir (pRT733) with strain L3. With the one-day-old chick pathogenicity assay, we verified that two of them (XP2 and XP4) were less pathogenic. The LD_{50} of L3, XP2 and XP4 strains are shown in Table 1. As can be noted, the LD_{50} of mutants XP2 and XP4 were four and six logs higher than the wild type strain, respectively. Some other characteristics as production of colicins, expression of fimbriae and plasmid profiles were also analyzed. The wild type and both mutant strains produced colicins E1 and E3, expressed type 1 (F1) fimbriae and harbored at least seven plasmid DNA bands of 95, 69, 37, 20, 10, 6 and 5 MD (Table 1). The LPS profile was the same for all three strains (results not shown).

Determination of serum resistance in L3, XP2, and XP4 strains showed that XP2 and XP4 had a lower serum resistance (results not shown). The growth curves of L3 and XP4 strains were similar

Table 1. Phenotipic characteristics of L3 *E.coli* and mutant strains XP2, XP4.

STRAIN	LD_{50}(CFU [1])	COLICIN PRODUCTION	EXPRESSION OF FIMBRIAE	PLASMID PROFILE (MD^2)
L3	$<3.7x10^2$	E1 and E2	Type 1 (F1)	95,69,37,20,10,6, and 5.
XP2	$1.8x10^4$	E1 and E2	Type 1 (F1)	95,69,37,20,10,6, and 5.
XP4	$3.5x10^8$	E1 and E2	Type 1 (F1)	95,69,37,20,10,6, and 5.

1. CFU: Colony forming units.
2. MD: Mega Daltons.

while XP2 strain had a lower number of CFU per hour (Fig. 1).

The SDS-PAGE of OMPs from strains XP2 and XP4 grown in the presence of iron (Fig. 2, Lane D, F) showed that protein bands of 127.7, 76.6, 41, 38.5, 35.7, 35.2, 27.3 and 14.7 kDa were missing in strain XP2 and one 47.8 kDa protein band was lacking in strain XP4, when compared with strain L3 (Fig. 2, Lane B).

SDS-PAGE of OMPs from strains L3, XP2, and XP4 grown in the absence of iron (Fig. 2, Lanes C, E, G) when compared with the same strains grown in the presence of iron (Fig. 2, Lanes B, D, F) showed that five protein bands of 122.7, 55.7, 35.7, 27.3 and 19.4 kDa were induced by the absence of iron in strain L3 (Fig. 2, Lane C) and the same bands were expressed by strains XP2 and XP4.

DISCUSSION

Several works trying to understand the pathogenicity mechanisms of *Escherichia coli* strains responsible for septicemia in poultry were done in the last two decades. One of the first characteristics to be studied was colicin V production (23), but today this is not considered a virulence factor (24,19). Plasmids carrying colicin V gene also carry other genes which are responsible for the pathogenic traits observed in these strains (30,25,29). These virulence factor genes would code for the expression of iron-acquisition systems, which are inducible by the low levels of body-circulating iron (30), and would enable the strains to overcome the iron-limiting conditions existing in the host-body fluids. Expression of *tra*T and *iss* outer membrane proteins could modify sites on the bacterial cell surface, making them resistant to the bactericidal effect of the serum (27) or could express toxins (10), or outer membrane proteins (10,18,22). However the resulting effects on pathogenicity are not well understood.

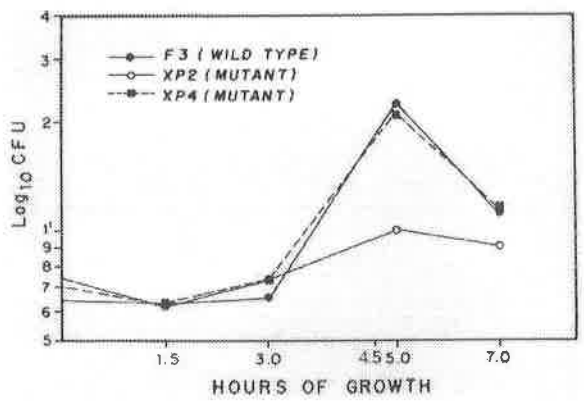

Figure 1. Wild-type (L3) and mutants (XP2, XP4) *E. coli* growth curves.

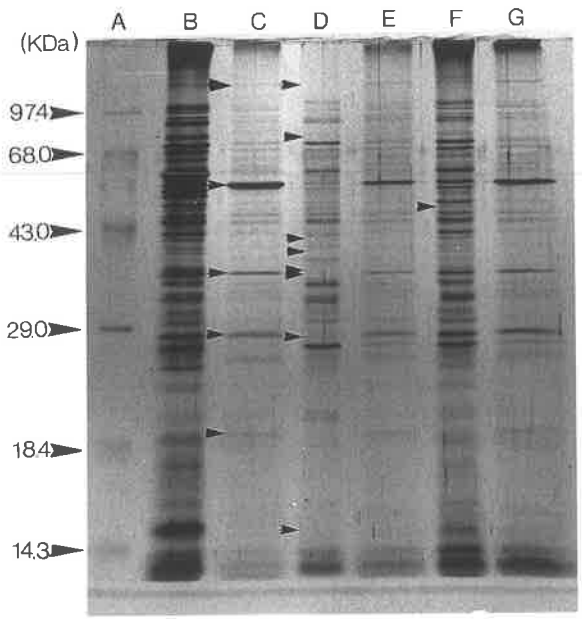

Figure 2 - 12.5% SDS-PAGE gel of outer membrane proteins of colicepticemic strain L3 and mutants XP2 and XP4 grown in the presence and absence of iron. Lanes: A (molecular weight markers of 97.4kDa, 68kDa, 43kDa, 29kDa, 18.4kDa, 14.3kDa); B and C (strain L3); D and E (strain XP2); F and G (strain XP4). Lanes B, D and F: presence of iron. Lanes C, E and G: absence of iron. The arrowheads point the OMPs expressed in the absence of iron (lane C), and OMPs not expressed in the presence of iron (lanes D and F).

In this study an avian-septicemic *E.coli* strain (L3) was conjugated with a strain containing a suicidal plasmid (pRT733), harboring TnphoA, to obtain outer membrane protein mutants less pathogenic than the wild type.

Two mutants obtained by TnphoA mutagenesis (XP2 and XP4) were less virulent than the wild type strain in one-day-old-male chickens, and had the expression of eight OMPs (XP2) and one OMP (XP4) suppressed in the presence of iron, and also had a decrease in serum resistance. This serum susceptibility was not due to the suppression of any of the proteins related to iron acquisition systems since, in the absence of iron (presence of alpha-alpha dypiridil), all the mutants had the same OMP profile as L3. When the growth curves of these mutants were analyzed, we found that strain XP2 had slower growth. Based on these results we suggest that, in strain XP2, TnphoA was inserted in a regulatory protein which is essential for the expression of several OMPs that are not related to the pathogenic process but are essential for rapid growth.

Since, in mutant XP4 the LD_{50} was four logs higher than in mutant XP2 and six logs higher than in wild type strain (L3), and had only one missing OMP(47.8 kDa), we believe that this protein could be directly involved in the pathogenicity process.

Since the agarose gel electrophoresis data of plasmid DNA showed that the bands harbored by L3, XP2 and XP4 strains did not have any variation in weight, we propose that TnphoA insertions in XP2 and XP4 mutants were in the chromosome, although we can not exclude the possibility of insertions in one of the high molecular weight plasmids in our experimental condition, since a 7kb DNA (TnphoA sequence) insertion in these plasmids could not be detected. However, transfer of all plasmids from strain L3 to a non-virulent, serum sensitive *E.coli,* did not render isolates with an increased pathogenicity, although some of them exhibit resistance to the bactericidal action of the serum (unpublished results), suggesting that the presence of these plasmids *per se* is not enough to change a non virulent strain into a virulent one.

ACKNOWLEDGMENTS

This work was supported by grant nº 94/4457-2 from FAPESP and grant nº 520358/94(SU) from CNPq. The authors are indebted to Dr. M.A.H. Dolder and Dr. V. Sperandio for reviewing the manuscript.

RESUMO

Possível envolvimento de uma proteína de membrana externa na patogenicidade de uma de *Escherichia coli* isolada de frangos septicêmicos

Uma linhagem de *Escherichia coli* septicêmica (L3), isolada de um surto epidêmico em aves (frangos), foi mutagenizada com o transposon TnphoA. Sete linhagens mutantes foram isoladas e analisadas quanto à patogenicidade. Dois mutantes (XP2 e XP4) foram menos patogênicos que a linhagem selvagem em testes com pintos de um dia de idade. A expressão de várias proteínas de membrana externa (OMPs) foi suprimida no mutante XP2 enquanto que o mutante XP4 não expressou uma OMP de 47,8kDa. Nenhuma destas OMPs foi correlacionada ao sistema de aquisição de ferro. O mutante XP2 pode conter mutação em uma proteína regulatória responsável pela expressão de outras proteínas não relacionadas à patogenicidade mas importantes para o rápido crescimento bacteriano, enquanto que o mutante XP4 apresenta deficiência na expressão de uma OMP de 47,8kDa. Estes resultados sugerem que esta última proteína poderia estar relacionada ao processo de patogenicidade em linhagens de *Escherichia coli* septicêmicas para aves.

Palavras-chave: *Escherichia coli*, aves (frangos), patogenicidade, proteínas de membrana

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Revista de Microbiologia (1998) 29:118-121
ISSN 0001-3714

A PRELIMINARY NOTE ON YEAST COMMUNITIES OF BROMELIAD-TANK WATERS OF RIO DE JANEIRO, BRAZIL

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Submitted: March 17, 1997; Returned to authors for corrections: October 27, 1997; Approved: February 02, 1998

SHORT COMMUNICATION

ABSTRACT

Bromeliad tank waters were studied as an example of yeast diversity in tropical ecosystems. Many isolates found in these microhabitats were phenotypically similar to *Saccharomyces cerevisiae* and including a probable new species. Other frequent isolates were *Candida intermedia, Debaryomyces hansenii* (+*Candida famata*), and *Cryptococcus albidus*. The prevalent species of yeast communities in bromeliad tanks included mostly species found previously in tropical forests.

Key words: yeast diversity, bromeliad-tank waters, tropical ecosystems

Brazil has a large diversity of ecosystems most of which have received little study as a sources of germplasm. The characterization of yeast communities associated with different habitats could be a good indicator of microbial diversity in the tropics (5). Studies of yeasts associated with tropical habitats have indicated the presence of many different biotypes and putative new species with possible application in biotechnology (1, 7, 12). High specificity between some yeast communities and their microhabitats can be influenced both by habitat characteristics and vectors (11, 12, 14). The bromeliads are typical neotropical plants distributed in diverse ecosystems (10). The accumulated water in the tanks of some species should be relatively stable microhabitats with complex trophic structures supporting many species of animals and plants (9). Microorganisms present in these microhabitats play an essential role in nutrient cycling and food webs. Since they are visited by many animals that could serve as yeast vectors and most yeasts survive well in fresh water, the ascomycetous yeasts in these microhabitats could reflect the biodiversity of their ecosystems. Our objective was to study the potential use of water and associated decomposing material (phytotelmata) of bromeliad tanks as a model for studies of yeast community diversisty in tropical ecosystems.

Water samples were collected assepticaly from the tanks of five different species of tank bromeliads (*Quesnelia arvensis, Nidularium procedurum, Neoregelia cruenta, Aechmea nudicaulis* and *Vriesia procera*) at 4 different sites between October 23, 1995 and September 27, 1996: mangrove ecosystems at Coroa Grande, RJ (1) and Bracuí, RJ, Atlantic Rain Forest at the IBAMA Poço das Antas Biological Reserve, Silva Jardim, RJ, and coastal sand dune ecosystem at Maricá, RJ (11). Aliquotes of 0.1 ml of serial dilutions were spread (in triplicate) on YM-Agar medium (malt extract 0.3%, yeast extract 0.3%,

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peptone 0.5%, glucose 1%) with sodium propionate 0.1%, cloramphenicol 0.04% and agar 2%, pH adjusted to 4.5 with HCl. After incubated at 25°C for 3 days, representative colonies were isolated and submitted to identification procedures according to standard methods (2, 3, 6). A matrix of relative frequences of the yeasts species from each bromeliad was calculated as $F = N_x/N$, where N_x is the number of bromeliads where the yeast specie 'x' was isolated and N is the total number of bromeliads sampled. Similarities between pairs of communities were calculated as the Pearson's correlation coefficient, and clustering was carried out by the unweighted pair-group arithmetic average (UPGMA) procedure (13).

We isolated 327 strains of yeasts belonging to 36 species of mostly ascomycetous affinity (Table 1). *Saccharomyces cerevisiae* – like was the prevalent yeast group with some phenotypic variantions among these isolates many of which did not form ascospores, but all close to the physiological profile of the standard description of *Saccharomyces cerevisiae*. This group occurred in all the bromeliad species sampled and the high frequency of isolation

Table 1. Relative frequency of yeast species isolated from bromediad tank waters of different tropical ecosystems

Species	Marica[a] (n=10)[b] *N. cruenta*[c]	Coroa Grande (n=19) *Q. arvensis*	A Bracui (n=18) *A. nudicaulis*	Bracui (n=18) *V. procera*	Poço das Antas (n=17) *Q. arvensis*	Poço das Antas (n=17) *N. procedurum*
Aureobasidium sp.	0,10	0,00	0,00	0,00	0,00	0,00
Bullera variabilis	0,00	0,00	0,00	0,00	0,06	0,00
Candida etchelsii	0,00	0,05	0,00	0,06	0,00	0,00
C. famata	0,00	0,00	0,00	0,00	0,00	0,24
C. glucosophila	0,00	0,00	0,00	0,00	0,06	0,00
C. intermedia	0,00	0,05	0,17	0,22	0,12	0,06
C. lipolitica	0,00	0,05	0,00	0,00	0,00	0,00
C. magnoliae	0,00	0,11	0,00	0,06	0,06	0,00
C. rugosa	0,20	0,05	0,00	0,00	0,00	0,00
C. terebra	0,00	0,05	0,00	0,00	0,00	0,00
C. tropicalis	0,00	0,21	0,17	0,00	0,06	0,06
Cryptococcus albidus	0,80	0,42	0,00	0,06	0,12	0,24
Cr. laurentii	0,10	0,00	0,00	0,00	0,00	0,00
Debaryomyces hansenii	0,20	0,21	0,00	0,00	0,29	0,00
D. vanrijiae	0,00	0,00	0,00	0,00	0,00	0,12
Kluyveromyces aestuarii	0,00	0,00	0,06	0,00	0,00	0,00
K. africanus	0,00	0,00	0,11	0,00	0,00	0,00
K. termotolerans	0,00	0,00	0,00	0,06	0,00	0,00
Kluyveromyces sp.	0,00	0,00	0,00	0,00	0,00	0,06
Kloeckera apis	0,00	0,00	0,00	0,00	0,00	0,06
Picchia membranaefaciens	0,00	0,00	0,17	0,06	0,00	0,00
P. anomala	0,00	0,00	0,00	0,00	0,06	0,00
Rhodotorula aurantiaca	0,00	0,11	0,00	0,06	0,00	0,00
Rh. bacarum	0,10	0,00	0,00	0,00	0,00	0,00
Rh. glutinis	0,10	0,00	0,06	0,00	0,00	0,00
Rh. minuta	0,00	0,00	0,00	0,00	0,06	0,00
Rh. mucilaginosa	0,10	0,16	0,00	0,00	0,12	0,06
Saccharomyces cerevisiae - like	0,40	0,26	0,28	0,44	0,47	0,24
Schwanniomyces occidentalis	0,10	0,00	0,00	0,00	0,00	0,00
Sporodiobolus	0,10	0,00	0,00	0,00	0,00	0,00
Sporopachydermia	0,10	0,00	0,00	0,00	0,00	0,00
Tremella foliaceae	0,00	0,00	0,00	0,00	0,06	0,00
Trichosporon bergelli	0,00	0,00	0,06	0,00	0,00	0,00
Williopsis saturnus	0,00	0,00	0,00	0,00	0,12	0,00
Yarrowia lipolitica	0,00	0,05	0,00	0,00	0,00	0,00
Zygosaccharomyces sp.	0,00	0,00	0,00	0,06	0,00	0,00

[a] Sampling site
[b] Total of bromeliads sampled
[c] Bromeliad specie
Relative Frequency= nº of bromeliads in which the specie 'x' was isolated

observed may indicates that these plants could be a natural habitat for *S. cerevisiae*. Hagler *et al.* (4) working in Coroa Grande mangrove with *Q. arvensis* (syn. *Q. quesneliana)* isolated a *Saccharomyces unisporus*-like yeast. At least some of the strains appear to belong to this same new specie of the *Saccharomyces 'sensu lato'* group (A. Vaughn-Martini personal communication), but having an ITS size of 850 bp which is typical of the *Saccharomyces "sensu-stricto"* group (15). *Debaryomyces hansenii* with its anamorph *Candida famata, Debaryomyces vanrijiae* and the phenotypically similar species *Candida intermedia* were the other more frequently isolated ascomycetous yeasts. The most frequently isolated species of basidiomycetes affinity were *Cryptococcus albidus* and *Rhodotorula mucilaginosa*. Although ascomycetous species are generally more tolerant to high temperatures, these species were more frequently isolated in bromeliads located in shaded habitats where the water temperature tend to be lower than in the non-shaded plants. Yeast communities associated with bromeliads exposed to intense sunlight, such as *Neoregelia cruenta* sampled in the Maricá sand dune ecosystem, were dominated by the presence of basidiomycetous species. This pattern may be due to basidiomycetous species being generally typical of the phyloplane and more resistant to solar radiation than ascomycetous species (4). There is also a difference in the trophic structure of these microhabitats in that primary production in shaded tanks comes mainly from plants debris that fall into the tanks and are degraded, whereas primary production in unshaded tanks is largely from growth of microalgae and cyanobacteria.

Cluster analysis (Fig. 1) showed that the yeast community structures from the bromeliads *A. nudicaulis* and *V. procera* located at the Bracui mangrove were very similar, but this was not observed for the yeast communities of *N. procedurum* and *Q. arvensis* located in the Atlantic Rain Forest. Since several animal species, including potential dispersers of yeasts, are able to use different species of bromeliads as refuges and feeding sites, it is possible for different bromeliad species in the same habitat to present similar yeast communities. However, the yeast communities observed for the bromeliad species located in the Atlantic Rain Forest did not show this, perhaps because the number of samples was still not sufficient to adequately represent the rare species. *Q. arvensis* was the only species that occurred in two distinct ecosystems

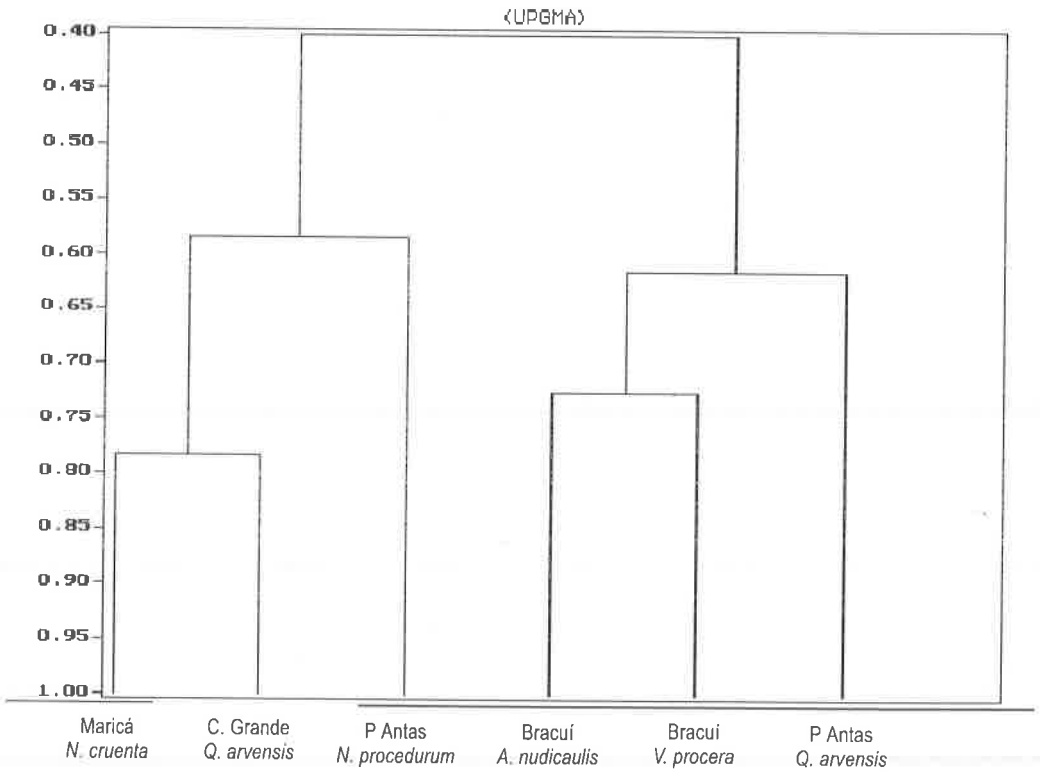

Figure 1. Similarity between yeast communities isolated from bromeliads of different tropical ecosystems

(mangrove and Atlantic Rain Forest) and showed distinct yeast communities in the two sites probably influenced by the diversity of these ecosystems. Morais *et al.* (8), studying yeasts associated with differents groups of *Drosophila* in tropical forests of Rio de Janeiro, observed a specificity between yeast species and *Drosophila* food resources and showed that the highest yeast species diversity was present in *Drosophila* groups that were found in forests with more diversified food resources (7). We did not find specific associations of yeast species, with the possible exception of the *Saccharomyces unisporus* –like new specie, contrasting with the results of Starmer *et al.* (14) and Rosa *et al.* (11, 12) studying communities of yeasts associated with cacti. The necrotic tissue of these cacti had yeast communities dominated by typical cactophilic species. The 5 prevalent yeast groups in bromeliad tanks were mostly of species that have been found in other microhabitats and are not exclusively associated with bromeliads. Because of this lack of specificity, the yeast communities found in this preliminary study of bromeliad tanks show a good potential as a model for studies of yeast diversity in neotropical ecosystems.

ACKNOWLEDGEMENTS

We thank Fabio Scarano and Tania Wendt for their cooperation in locating and identification of the bromeliads and discussions on their ecology. This work was financed by fellowships and bench fees from the Conselho Nacional de Pesquisa (CNPq) and by an European Economic Community fixed contribution contract nº C11* - CT 93-0054.

RESUMO

Comunidades de leveduras em águas de bromélias-tanque de diferentes ecossistemas tropicais do Rio de Janeiro, Brasil

Águas de bromélias-tanque foram estudadas como modelo de diversidade de leveduras em ecossistemas tropicais. O grupo de leveduras mais frequentes encontrada nestes microhabitats é formado por isolados fenotipicamente similar a *Saccharomyces cerevisiae* e provavelmente inclui novas espécies. Outras espécies isoladas frequentemente foram *Candida intermedia, Debaryomyces hansenii (+Candida famata)* e *Cryptococcus albidus*. As espécies de leveduras mais prevalentemente encontradas nestes tanques de bromélias incluiram em sua maioria espécies encontradas previamente em florestas tropicais.

Palavras-chave: Diversidade de leveduras, águas de bromélias-tanque, ecossistemas tropicais.

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Revista de Microbiologia (1998) 29:122-126
ISSN 0001-3714

SELECTION OF STRAINS OF *SACCHAROMYCES CEREVISIAE* MEYEN BY QUANTIFICATION OF TOTAL SOLUBLE CARBOHYDRATES PRESENT IN THE CELLULAR LYSATE

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Submitted: September 18, 1997; Returned to authors for corrections: December 12, 1997; Approved: April 23, 1998

ABSTRACT

In order to select strains of *Saccharomyces cerevisiae* that can act as elicitors of phytoalexin in plants, of the content of all soluble carbohydrates present in the lysate fraction of different laboratory strains and of hybrids resulting from their crossings was studied. The results gave direct evidence that it is possible to select laboratory strains which are highly variable in this characteristic. The hereditary pattern showed to be determined by quantitative factors. Hybrids with higher content of soluble carbohydrates demonstrated the possibility of non-commercial strains to be selected for stimulation of phytoalexin production in plants and use in plant resistance induction.

Key words: Elicitors, phytoalexin, plant-resistance induction, *Saccharomyces cerevisiae*

INTRODUCTION

Plants need to defend themselves against attack from fungi, viruses, invertebrates, and even other plants. Each plant cell must posses a preformed and/or inducible defense capability. Many researchers have tested the hypothesis that the plant defense reaction (by phytoalexin accumulation) is activated by an elicitor stimulus that may be activated by a great variety of microorganisms (5,8,16,21,23,24,31). Considerable knowledge has since accumulated on the biochemical and genetic basis of disease resistance and elicitor activity. Ayers et al (1) were the first to demonstrate that a pathogen wall component is implicated in plant disease resistance.

Hahn and Albersheim (14) isolated an elicitor of glyceollin from the commercial extracts of the yeast *Saccharomyces cerevisiae* Meyen which possesses several characteristics in common with the isolated elicitor from *Phytophora megasperma* Drechs. var. *sojae* A. A. Hildebrand. Among these characteristics is the fact that the yeast isolated elicitor can be separated from other macromolecules containing carbohydrates, using the same employed methodology for the isolation of the *P. megasperma* elicitor. Both molecules are β-glucans with 1-3; 3-6 bonds and require the same quantity of glucans to elicit glyceollin. This suggests that soybean, and other plant species, may detect the presence of other fungi by recognizing some of their glucans.

The surface structure of fungi, the cell wall, has called the attention not only of mycologists but also of researchers in other biological fields. The cell wall has been studied under different aspects and these studies have contributed to a better understanding of many biological phenomena related to the wall surface as well as to the host-pathogen molecular interactions. A potential pathogen has to be capable of recognizing plant characteristics which signal the plant receptivity of the parasite.

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The properties of the cell wall, as such as mechanical power, morphological attributes and biological activity are, for sure, based on their particular chemical composition. The main components of fungal walls are polysaccharides which constitute 80-90% of the dry mass of the wall (15), other substances being proteins, lipids, occasionally melanin, phosphates and inorganic salts. The generic name "glucans" is applied to a great number of D-glucose polymers which are different in type in the relative proportions of their individual glucosidic bonds (9).

In *S. cerevisiae* specifically, the cell wall is constituted by a complex containing glucans, mannans, small quantity of chitin, phosphate and proteins (19). Studies on the chemical structure of the cell wall of the yeasts are well documented in literature (2, 7,11, 12,17, 18, 19, 20,25,30).

The use of non-pathogenic fungi which carry elicitors in the cell wall as well as endo or extracelular elicitors constitutes an excellent tool in the search for replacement of agricultural defensives, such as fungicides, and also to stimulate, again and again, the use of alternative methods of control in agriculture, such as biological control and plant-resistance induction.

The objective of this work was to study the carbohydrate content in the lysate fraction of different laboratory strains and in the resulting hybrids of their crosses aiming at the selection of strains that could act as phytoalexin elicitors in plants.

MATERIALS AND METHODS

Yeast strains DC-6, AH-22, C1, 1783, FL, GRF-18, RC631, D-665.1, TD-20 and ATCC 26698 were obtained from the Instituto de Microbiologia of the University of São Paulo, Brazil. Cultures were maintained and grown on nutrient YEPD agar. Lysed fraction of the strains were obtained by cryotrituration with carborundum for 1 h, centrifuged (15 000 g) and filtered through Whatman filter paper (n° 1) . Total soluble carbohydrate contents of the yeast fractions was determined by the anthrone procedure (6). Crosses were made using the two strains which presented higher carbohydrate content. Two hours after mixing MATa and MATα cells, a sample of the conjungation mixture was spread onto dissection agar. Newly formed zygotes were separated from the bulk of cells with a Micromanipulator (E. Leitz) and incubated at 37°C for two or three days in the sporulation medium (10). The partial digestion of the ascus was made by the β-glucuronidase enzyme (Sigma-type Hp_2 from *Helix pomatia* "crude solution"). This solution was made by mixing 0.5 ml of sorbitol 1M and 0.03 ml of the enzyme (100,000 units/ml at pH 5). This mixture was then incubated for 15 minutes, at 37°C. The spores inside each ascus were separated by micromanipulation and allowed to grow into colonies for two to three days. Each colony was then one by one planted onto Petri dishes with complete medium (YEPD) taking care to identify the strains from each ascus.

Statistical analysis

Statistical analysis of the data was carried out with analysis of variance. Also a Tukey test was employed to asses differences with the strains carbohydrate content data.

RESULTS AND DISCUSSION

The results of carbohydrate contents of different strains determined by the anthrone method after cryotrituration are shown in Fig. 1. The values are practically the same for some strains but different for others.

Most strains presented a mean value of carbohydrate content within the range 13 and 27 Eq .μg of glucose/ml, not significant . The crossing of the GRF-18 MATα strain with the D 665-1A MATa strain resulted in seventy six haploid strains, obtained by micromanipulation. Strains were separated according to the ascus from which they came. Samples derived of six different asci originated from the crossing GRF-18 X D. 665-1A strains were analyzed. Chemical tests with these strains showed quantitative differences in polysaccharide content in samples from a single ascus and in samples from different asci. It was observed that strains with higher carbohydrate content than both parents were predominant. Fig. 2 shows the total soluble carbohydrate content of twenty four haploid strains. These strains which were originated from laboratory crosses ranged from 13 to 145 μg of glucose/ ml. This is a clear demonstration of the variability of carbohydrate content in each strain.

The cellular lysis of several strains of the yeast *S. cerevisiae* showed the presence of different contents of total soluble carbohydrates statistically significant. In most cases the carbohydrate content of the daughter strains was higher than the average. It is

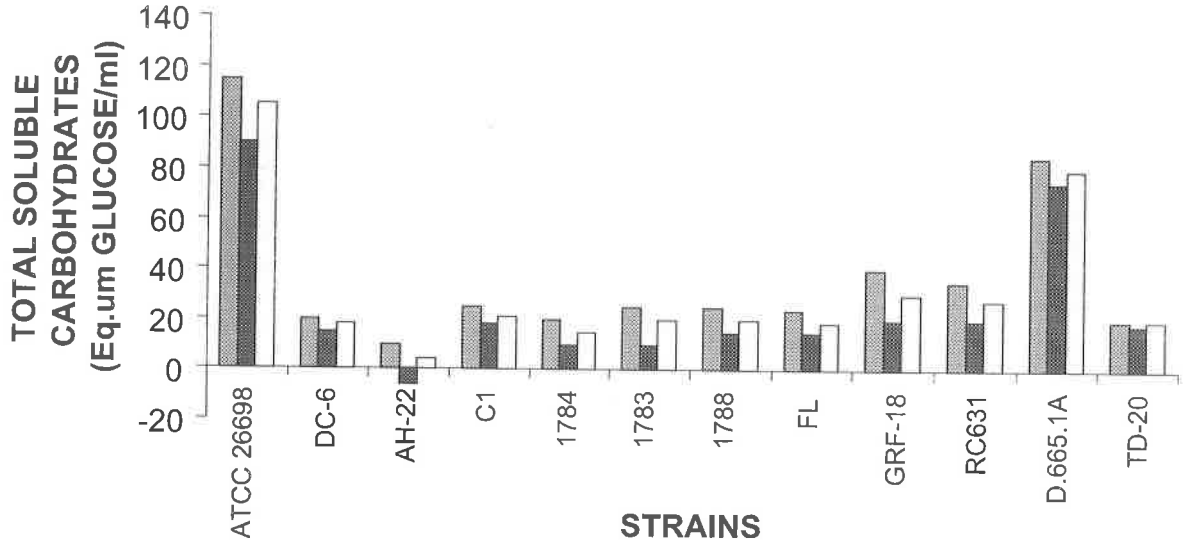

Figure 1- Total soluble carbohydrate contents of lysate of different strains of *S. cerevisiae* determined by anthrone method in Eq.μg of glucose/ml. The averages of the strains ATCC 26698, GRF-18 and D. 665 1A, are significantly different at 5% level (p 0.05) (Tukey test). Averages of three repetitions.

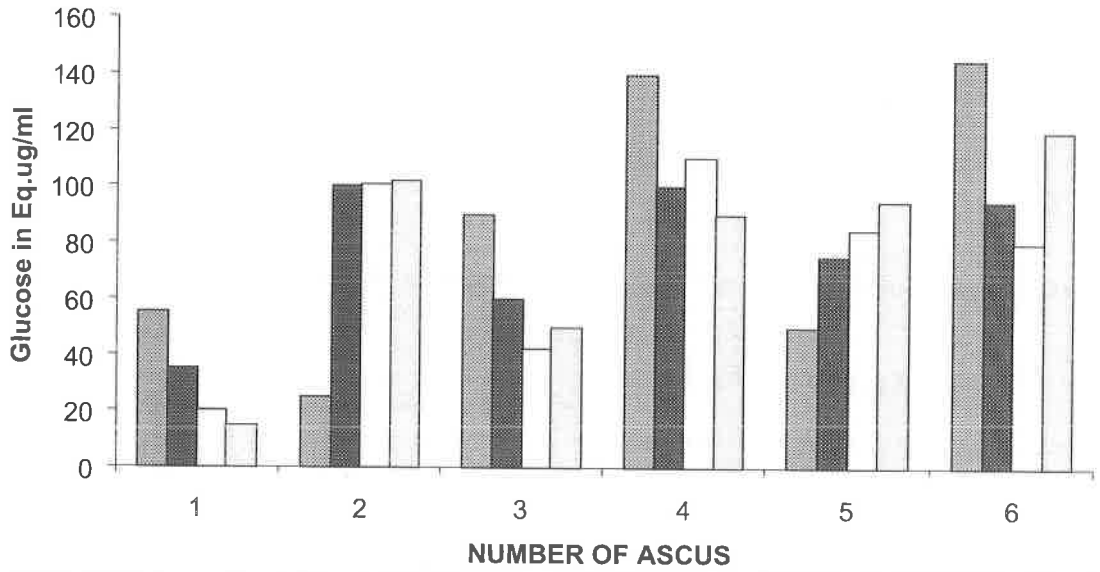

Figure 2- Total soluble carbohydrate contents of the lysate fraction of twenty four tetrads derived of asci originated from the crossing of *S. cerevisiae* GRF-18 X 665-1A strains. Total soluble carbohydrate contents determined by the anthrone method in Eq.μg of glucose/ml.

an evidence that it is possible the arising of strains with even higher carbohydrate content due to more favorable genetic combinations. The crossing of the most productive strains aimed at improving this characteristic. The present study has given direct evidence that it is possible the selection of laboratory strains which are highly variable in this characteristic. This allows the utilization of carbohydrate content as an important marker in the search for phytoalexin elicitors. The possibility of non-commercial strains of *S. cerevisiae* to induce phytoalexin production in plant tissues is confirmed by the values of glyceollin accumulation in bioessays in sojae cotyledon. The cotyledon assay for elicitor activity was used to test for their ability to stimulate glyceollin accumulation in cotyledons of soybean

(23). Researchers have been using lysed extracts of commercial strains for the lack of a program of selection and improvement of strains for this purpose. As a consequence researchers have to work with strains which have been genetically manipulated to suit the interests of industries. Laboratory strains should be manipulated to comprise a strain with the essential characteristics for a good elicitor of phytoalexin, e. g., high carbohydrate content linked in β-configuration. They should also have a chemical moiety that maximizes elicitor activity (27,28,29).

McMurrough and Rose (22) showed that the relative proportions of polysaccharides (glucan and mannan) in yeast cell walls are largely dependent on the nature of the substrate limitation imposed on the culture.

The presence of a greater proportion of glucan than mannan in the walls appeared on the whole to be associated with yeast grown in cultures containing high concentration of glucose. McMurrough's data deserved to be studied in more detail in order to establish the ideal culture conditions to increase elicitor yield. Guzzo (13) pointed out the importance of mannans, which are also linked in β-configuration, in the chemical composition of the urediniospores extracelular elicitor of coffee (*Coffea arabica*) pathogen *Hemileia vastatrix* Berk et Br.

The crossing of 665-1A X Grf-18 strains produced hybrids with higher carbohydrate contents than their parents. This result demonstrates not only that the content of total soluble carbohydrates is variable in different strains but also that there are genetic combinations which are conducive to a greater production of carbohydrates. Possibly, differences in culture growing conditions might account for discrepancies found among tetrads derived from a single ascus as the composition of the culture medium is likely to affect cell wall formation. Nonetheless, two aspects must be taken into consideration: 1) culture growing conditions were rigorously controlled so that all strains would be handled in the same way and be used in the same cellular cycle; 2) graduation of obtained values is an evidence of quantitative inheritance, therefore, several genes may be actuating. The genetic study of genes actuating in this characteristic will have to be made step by step, as the various kinds of sugar present in the lysate are identified; as specific mutants are obtained; and as dialelic crossings are made to identify epistatic combinations that may occur. There is evidence that (1-6)-β-glucan chains are made within the cytoplasm. Brown et al (4) have identified in *S. cerevisiae* several killer-toxin-resistant mutants defective in (1-6)-β-glucan synthesis. Their findings suggest that this polymer is synthesized sequentially and involves the products of several (KRE) genes, some of which are in the secretory pathway, while others are cytoplasmic or membrane proteins. Autoradiography has shown that chitin and (1-3)-β-glucan are directly deposited outside the plasma membrane (30). In conclusion, we can state that there is genetic variability present in different strains of *S. cerevisiae* in relation to the content of total soluble carbohydrates; the crossing between compatible strains may result in hybrids that present genetic combinations, such as higher content of carbohydrates; total soluble carbohydrate contents proved to be determined by quantitative genetic factors and may be used as a marker when selecting strains of *S. cerevisiae* with the purpose of plant-resistance induction.

ACKNOWLEDGEMENTS

Thanks are due to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), to Dra. Elizabeth Vicente for donating the strains of *Saccharomyces cerevisiae* and to Maria Lúcia Mendes Gomes for English assistance.

RESUMO

Seleção de linhagens de *Saccharomyces cerevisiae* Meyen pela quantificação do teor de carboidratos totais solúveis presentes no lisado celular

Com o objetivo de selecionar linhagens de *Sacharomyces cerevisiae* que possam atuar como elicitores de fitoalexinas em plantas, analisou-se o teor de carboidratos totais solúveis presente no lisado de diferentes linhagens de laboratório e de híbridos resultantes de seus cruzamentos. O presente estudo proporcionou evidência direta de que é viável a seleção de linhagens de laboratório altamente variáveis quanto a esta característica. O tipo de herança mostrou ser determinado por fatores quantitativos. Os híbridos que apresentaram maiores teores de carboidratos totais solúveis demonstraram a posibilidade de linhagens não-comerciais serem selecionadas para uso em programas de indução de resistência em plantas.

Palavras-chave: Elicitores, indução de resistência, fitoalexinas, *Saccharomyces cerevisiae.*

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Revista de Microbiologia (1998) 29:127-129
ISSN 0001-3714

EVALUATION OF A RAPID METHOD FOR THE DETECTION OF DNAse ACTIVITY IN *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI*

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Submitted: August 19; Returned to authors for corrections: October 28, 1997; Approved: March 26, 1998

SHORT COMMUNICATION

ABSTRACT

Hänninem's DNAse method for *Campylobacter* biotyping was evaluated. From the 105 strains studied, 68 (64.8%) were DNAse positive by this method, appearing 6 as false DNAse negative by Lior's reference method. Hänninem's method was more sensitive (100%) than Lior's method (91,2%) to detect DNA hydrolisis activity but both showed 100% specificity.

Key words: *Campylobacter*, DNAse, biotyping

Campylobacter jejuni and *C. coli* have been recognized as important enteropathogens for human beings in developed and developing countries (1,3). Since the epidemiology of the infection is complex, due to the wide environmental distribution of these zoonotic bacteria among domestic and wild mammals and birds, various marker systems have been devoloped for the better understanding of their epidemiological relationships. These systems could be classified into molecular and phenotypical techniques, being biotyping methods the most widely used, probably due to their easy performance (10,11). Biotyping methods could divide *Campylobacter* strains into biotypes on the basis of biochemical reactions. DNA hydrolysis is one of the discriminatory tests included in most of the biotyping procedures that have been proposed (5,7,8,11). Firstly, methylene green-DNA agar was used (5,7) and later, toluidine blue-DNA agar (TB-DNA agar) was introduced with this purpose (8). In 1989, Hänninem reported better results employing a rapid well-agar diffusion method for the detection of DNAse activity in *Campylobacter* species, that uses TB-DNA agar and the pretreatment with polymyxin B of the strains under study (4).

The aim of this work was to evaluate the TB-DNAse diffusion method proposed by Hänninem having the one proposed by Lior (7) as the reference method.

One hundred and five strains (46 *C. jejuni* and 59 *C. coli*), previously identified by conventional biochemical and phenotypic tests (12) were studied. The strains *C. jejuni* LIO36 biotype II and *C. jejuni* LIO1 biotype I, kindly provided by Dr. Manuel García from the Laboratory Centre for Disease Control, Ottawa, Canada, were used as positive and negative DNAse controls respectively. Each strain was tested for DNAse activity by means of the Lior (8) and the Hänninem (4) methods.

When DNA hydrolysis was tested by Lior's method (8), a heavy inoculum of each strain was seeded on TB-DNA agar forming a circular area of

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about 0.5 cm of diameter, and incubated at 42°C under a microaerophilic atmosphere for 48 h. A clear-colorless or pinkish zone around the inoculum was considered as a positive reaction.

TB-DNA agar, composed of deoxyribonucleic acid 0.3 g; calcium chloride (0.01M) 1 ml; sodium chloride 10 g; agar Oxoid L28 6.5 g; 0.05M Tris Buffer pH 9 1,000 ml and 3% toluidine blue "O" 2.5 ml, was prepared by mixing all ingredientes except toluidine blue "O" and heat to boiling to dissolve DNA and agar completely. Then, the mixture was cooled to 50°C and toluidine blue "O" was added, mixed well and poured (about 25 ml/plate) into petri dishes. The medium, that did not require sterilization, was stored at room temperature in the dark for no longer than one week.

When DNA hydrolysis was tested by Hänninem's method (4), each strain was suspended in 1 ml of nutrient broth at a density equivalent to 1 MacFarland standard [ca. 3.8 x 108 CFU/ml as determined by the method of Miles and Misra (9)], treated with 50 µl of polymyxin B (2 mg/ml) and incubated at 4°C for 15 min. Then, the suspensions were inoculated into wells (5 mm diameter, 12 wells per plate), previously made in the TB-DNA agar plates, and incubated aerobically, at 37°C for 24 hours. A clear-colorless or pinkish zone around the inoculated well was considered as a positive reaction and their diameter recorded. The sensitivity and specificity of both methods were determined by means of the equation proposed by Borobio (2).

Of the 105 strains studied, 68 (64.8%) were DNAse positive by the Hänninem's method, (*C. jejuni* 29 and *C. coli* 39). When they were tested by the Lior's method, 4 strains of *C. jejuni* and 2 of *C. coli* appeared as DNAse negative; these were considered as false negative results. These results are shown in Table 1. The biotyping scheme proposed by Lior, using hippurate hydrolysis, rapid H_2S production and DNA hydrolysis, identify 4 biotypes in *C. jejuni* and 2 in *C. coli*, being *C. jejuni* biotypes I and II and *C. coli* biotype II DNAse positive (7,8). With these DNA hydrolysis results and considering the hippurate and H_2S test previously done, 17 of the strains under study were identified as *C. jejuni* biotype I, 29 as *C. jejuni* biotype II, 20 as *C. coli* biotype I and 39 as *C. coli* biotype II. With these results, the method proposed by Lior appeared to be less (91,2%) sensitive than the test of Hänninem (100%). However, the specificity was identical for both methods (100%). The better performance of the latter method could be due to the pretreatment of the bacterial suspension with polymyxin that, as occurred with lincomycin in *Vibrio cholerae* (13), allowed the release of the membrane-bound DNAse. So, the test evaluate the extracellular DNAse and the cell-associated DNAse activities producing, around the inoculated well, a clear, well defined halo, whose diameter is proportional to the DNAse concentration present in the bacterial suspension (4). In this study, the diameter of the halos varied from 10 to 13 mm and, in some instances, the positive reactions were evident within the first 6 to 12 hours of incubation. This suggests that the pretreatment with polymyxin induces a rapid enzimatic liberation. All the positive reactions were clearly visible at 24 h of incubation but no new positive strains were found extending the incubation period for 48 hours. This is in accordance with the observations done by Lachica *et al* (6) using a metachromatic well-agar diffusion technique for the identification of staphylococcal nuclease.

Although the method proposed by Lior could be considered as a reference method, the Hänninem's method shows some comparative advantages such as its higher sensitivity, the use of a standardized inoculum and a shorter incubation period at 37°C, avoiding the use of a microaerophilic atmosphere. Moreover, due to the use of wells done in the agar, at least 12 strains could be tested simultaneously per 10 cm diameter Petri dish.

Considering the results obtained in this study and the above comparative advantages, the method proposed by Hänninem was adopted in our laboratory as the routine DNAse test method.

Table 1. DNAse activity in 105 strains of *Campylobacter* on TB-DNA agar using the Lior's and Hänninem's methods.

Species biotypes	DNA hydrolysis			
	TB-DNA Lior		TB-DNA Hänninem	
	+	-	+	-
C. jejuni biotype I	0	17 (100)	0	17 (100)
C. jejuni biotype II	25 (86.2)	4 (13.8)	29 (100)	0
C. coli biotype I	0	20 (100)	0	20 (100)
C. coli biotype II	37 (94.8)	2 (5.2)	39 (100)	0
Total	62 (91.2)	43 (63.2)	68 (100)	37 (100)

ACKNOWLEDGMENTS

The present work was supported by Grants S-95-45 and S-97-21 from the Dirección de Investigación y Desarrollo, Universidad Austral de Chile and Fondecyt 1930353.

RESUMO

Avaliação de um método rápido para a detecção de atividade de hidrólise de DNA em *Campylobacter jejuni* e *Campylobacter coli*

O método da DNAse de Hänninem para a biotipagem de *Campylobacter* foi avaliado. Das 105 amostras estudadas, 68 (64,8%) foram positivas por este método. Pelo método de referência de Lior, 6 foram falsos negativas. O método de Hänninem foi mais sensível (100%) que o de Lior (91,2%) para detectar atividade de hidrólise de DNA. Ambos apresentaram a mesma especificidade (100%).

Palavras-chave: *Campylobacter*, DNAse, biotipagem.

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Revista de Microbiologia (1998) 29:130-133
ISSN 0001-3714

COMPARISON OF THE E TEST WITH AGAR DILUTION FOR DETERMINING SUSCEPTIBILITY OF *STREPTOCOCCCUS PNEUMONIAE* TO PENICILLIN

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Submitted: December 12, 1997; Returned to authors for corrections: April 22, 1998; Approved: May 04, 1998

ABSTRACT

Infections due to penicillin-resistant *Streptococcus pneumoniae* are increasing worldwide. Definition of resistance of *S. pneumoniae* to penicillin is made by determination of minimal inhibitory concentration (MIC) of each isolate. The E test is a system used to determine MIC of different organisms. In this study, 56 isolates (susceptible, n=26, intermediately resistant, n=28 and high level resistance, n=2) of *S. pneumoniae* isolated from patients of Porto Alegre, RS, were submitted in parallel to the E test and to a reference method (agar dilution test). Agreement between both systems was 96.4 per cent and in 6/56 (10.7 percent) minor interpretative errors occurred. E test represents an accurate and reliable alternative for determining penicillin MICs of *S. pneumoniae*.

Key words: resistance, pneumococcal infection, treatment

INTRODUCTION

Infections due to *Streptococcus pneumoniae* cause between 3 and 5 million deaths each year (22). Treatment of these infections is being limited by the occurrence of antimicrobial resistance. Penicillin resistance is a worldwide problem (10) and deserves special attention (5,7). Resistance of *S. pneumoniae* to penicillin is a quantitative concept and, according to this concept, susceptible strains are those showing minimal inhibitory concentration (MIC) of less than 0.12 μg/ml. Strains with MICs between 0.12 and 1.0 μg/ml are considered intermediately resistant to penicillin. Finally, high level resistance to penicillin is present in strains with MICs equal to or greater than 2.0 μg/ml (15). Thus, definition of MIC by dilution methods is essential in order to define resistance of *S. pneumoniae* to penicillin.

The E test (AB Biodisk, Solna, Sweden) is a product used to determine MIC of several microorganisms. The principle of this alternative system is based on the concept of agar diffusion tests applied to a quantitative format. The product consists of a strip containing a predefined exponential gradient of a specific antibiotic. The strip is marked with a scale which permits reading of MICs in μg/ml.

Studies done by other investigators showed a good performance of the E test in determining MIC of *S. pneumoniae* to penicillin (3,6,8,9,12,13,14,16,21). The agreement between the E test and the reference method (agar or microbroth dilution) observed in these studies was about 90%.

Our objective was to study the performance of the E test in defining MICs to penicillin in isolates of *S. pneumoniae* obtained from patients in Porto Alegre, RS, Brazil.

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MATERIALS AND METHODS

A total of 56 isolates of *S. pneumoniae* was submitted in parallel to the E test and a reference method, agar dilution test for definition of MIC to penicillin. Isolates included susceptible strains (n=26), intermediately resistant strains (n=28) and strains with high level of resistance to penicillin (n=2) as defined by the reference method. All isolates were from patients from Porto Alegre, RS, with significant pneumococcal infections. *S. pneumoniae* ATCC 49619 was used for quality control.

E test (AB Biodisk, Solna, Sweden) with penicillin in a gradient covering a continuous concentration range of 0.002 to 32 µg/ml was used. Manufacturer's recommendations, with necessary modifications for testing *S. pneumoniae*, were followed. Mueller-Hinton agar was supplemented with 5% defibrinated sheep blood, incubation was done in a CO_2 enriched atmosphere at 35°C during 20-24 hours. After this period, MICs were read from the intersection of the inhibition ellipse with the test strip.

Agar dilution tests were done following the procedure described by the National Committee for Clinical Laboratory Standards (15). Some modifications were introduced in order to test a fastidious organism like *S. pneumoniae*. Mueller-Hinton plates were prepared with 5% defibrinated sheep blood, and incubations were performed in a CO_2 enriched atmosphere. Penicillin was provided by the Quality Control Laboratory, School of Pharmacy, Universidade Federal do Rio Grande do Sul.

Results of both the alternative method, E test, and the reference method, agar dilution, were obtained independently. Results were considered in agreement when E test MICs were within plus or minus one dilution of the reference method.

RESULTS

Table 1 shows all results obtained by both methods. Agreement between E test and agar dilution (MIC of the alternative method within plus or minus one dilution of the reference method) was 96.4 per cent. A more detailed view of the agreement observed between both methods can be seen in Table 2.

In terms of interpretive discrepancies, no major or very major errors were seen. We observed 6/56 (10.7 per cent) minor error of the E test in relation to the reference method. Table 3 contains interpretive discrepancies observed.

Table 1. Results of MICs to penicillin obtained by agar dilution and E test in 56 isolates of *Streptococcus pneumoniae*

Isolate number	MIC to penicillin by agar dilution (µg/ml)	MIC to penicillin by E test (µg/ml)
015	= or < 0.03	0.016
026	= or < 0.03	0.016
040	= or < 0.03	0.023
044	= or < 0.03	0.016
054	0.25	0.25
057	= or < 0.03	0.023
065	= or < 0.03	0.016
068	0.50	1.0
070	0.12	0.19
095	0.06	0.064
099 [ab]	0.25	0.094
109 [b]	1.0	2.0
110	2.0	2.0
121	= or < 0.03	0.023
123	0.06	0.064
126	= or < 0.03	0.023
128	= or < 0.03	0.023
129	0.06	0.064
140	= or < 0.03	0.032
143	= or < 0.03	0.032
151	= or < 0.03	0.032
153	0.12	0.125
162	0.25	0.19
165	0.25	0.19
171 [a]	0.50	0.125
173	0.12	0.19
179	0.06	0.094
183	0.25	0.38
184	0.25	0.19
193	1.0	1.5
195	= or < 0.03	0.047
200	0.06	0.094
205	0.12	0.19
212	= or < 0.03	0.032
220	0.06	0.064
225	= or < 0.03	0.016
227	0.12	0.19
239 [b]	0.12	0.094
250	0.12	0.125
261	0.25	0.19
263 [b]	0.12	0.094
274	0.12	0.19
276	0.06	0.094
299 [b]	0.12	0.064
303	0.06	0.094
321 [b]	1.0	2.0
326	= or < 0.03	0.032
327	0.06	0.064
335	2.0	4.0
343	0.12	0.125
348	0.12	0.19
360	0.06	0.064
370	0.12	0,125
371	0.12	0.19
428	0.12	0.19
439	0.25	0.19

[a] Isolates showing disagreement between E test and agar dilution.
[b] Isolates in which minor errors were observed

Table 2. Agreement between results of minimal inhibitory concentrations (MIC) to penicillin of the E test and of agar dilution tests in 56 isolates of *Streptococcus pneumoniae*.

CIM by agar dilution test (μg/ml)	MICs within log_2 dilution of the reference method			
	-2	+ or - 1	+2	total
< 0.12	0	26	0	26
0.12 - 1.0	2	26	0	28
> 1.0	0	2	0	2
total	2	54	0	56

Table 3. Interpretive discrepancies observed between E test and agar dilution in 56 isolates of *Streptococcus pneumoniae*. Analysis of minor errors

Number of the isolates	Minimal inhibitory concentration (μg/ml) to penicillin	
	agar dilution	E test
099	0.25 (IR)	0.094 (S)
109	1.0 (IR)	2.0 (HLR)
239	0.12 (IR)	0.094 (S)
263	0.12 (IR)	0.094 (S)
299	0.12 (IR)	0.064 (S)
321	1.0 (IR)	2.0 (HLR)

S: susceptible, IR: intermediate resistance, HLR: high level resistance.

DISCUSSION

Resistance of *S. pneumoniae* to antimicrobic agents is a problem of great importance. Surveillance of resistant strains is one of the measures to control the spread of resistance and clinical laboratories would have a crucial role in the detection of resistant strains (7). Since definition of resistance of *S. pneumoniae* to penicillin is quantitative, it would be necessary that clinical laboratories have a convenient and accurate system to test this microorganism against such drug. Some commercial systems have failed in effectively recognize resistance of *S. pneumoniae* to penicillin (9,12,21,17,20).

Although expensive, E test is showing a good performance when compared with both microbroth (8,9,12,14,16,21) and agar dilutions (3,6). Such studies show an agreement ranging from 90 to 100 per cent when results of E test and the reference method are compared in the definitions of MIC of *S. pneumoniae* to penicillin. Only minor interpretive error were observed by Tenover et al. (21) (3.6 per cent), Kiska et al. (9) (8.8 per cent) and Jorgensen et al. (8) (9.5 per cent). Other investigators, however, found 17.5 per cent (14) and 18.0 per cent (12) of minor errors of the E test in defining resistance of *S. pneumoniae* to penicillin.

Our results do not differ from the ones obtained by the investigators mentioned above. Agreement of results of the E test and agar dilution was observed in 96.4 per cent of the isolates tested and interpretive discrepancies were seen in 10.7 per cent of the isolates. These discrepancies included six isolates defined as intermediately resistant to penicillin by the reference method (Table 3). In four of these isolates the E test results were within the susceptible category (isolates number 099, 239, 263 and 299) and in the remaining two were defined as having high level resistance (isolates number 109 and 321). Except for isolate number 099, results obtained by agar dilution were near the MIC breakpoint separating susceptible and intermediately resistant strains. Such discrepancies, at least for isolates number 239, 263 and 299, are not unexpected as the E test determines MIC from a continuous scale and the reference method does it in a discontinuous doubling dilution scale. This kind of discrepancy was pointed out by other authors (6,8,12,14). Discrepancies observed in isolates number 109 and 321 (intermediately resistant by agar dilution and high level resistance by the E test) were seen in other study (21) but can not have the same explanation.

Another issue to be considered in our study is that only two isolates with high level resistance to penicillin were included. The prevalence of strains with MIC to penicillin > 1.0 μg/ml in *S. pneumoniae* isolated in Brazil is low (1,2,4,11,19), but seems to be increasing according to a recent investigation (18).

Results of our study, together with the studies mentioned above, are in favor of the E test as a practical, accurate and reliable system to determine MICs of *S. pneumoniae* to penicillin. However, the benefits of this system must be considered in relations to its cost.

ACKNOWLEDGEMENTS

The authors thank to FAPESP for support Maria Beatriz Cirne Lima and Rosângela Agne for technical assistance and to the Quality Control Laboratory of the School of Pharmacy of Universidade Federal do Rio Grande do Sul for providing the penicillin used in agar dilution tests.

RESUMO

Comparação entre E test e diluição em ágar na determinação de suscetibilidade de *Streptococcus pneumoniae* à penicilina

Resistência de *Streptococcus pneumoniae* aos antimicrobianos vem sendo detectada de maneira crescente em diversos países. A definição da resistência de *S. pneumoniae* à penicilina é feita pela determinação da concentração inibitória mínima (CIM) de cada cepa. O E test é um sistema empregado para determinação de CIM de diversos microrganismos. Em nosso estudo, 56 amostras de *S. pneumoniae*, incluindo amostras suscetíveis, intermediárias e plenamente resistentes (26, 28 e 2 amostras, respectivamente), isoladas de pacientes em Porto Alegre, RS, foram testadas em paralelo pelo E test e por um método de referência, teste de diluição em ágar. A concordância observada entre os dois métodos foi de 96,4%, sendo que erros interpretativos menores ocorreram em 10,7% das amostras. O E test representou uma alternativa confiável e de boa exatidão na determinação de CIMs de *S. pneumoniae* à penicilina.

Palavras-chave: Resistência, infecção pneumocócia, tratamento

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Revista de Microbiologia (1998) 29:134-137
ISSN 0001-3714

PRODUCTION OF EXTRACELLULAR LIPASE BY A *CANDIDA RUGOSA* STRAIN ISOLATED IN PERNAMBUCO, BRAZIL

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Submitted: August 28, 1997; Returned to authors for corrections: October 28, 1997; Approved: May 4, 1998

ABSTRACT

Extracellular lipases are produced by many species of microorganisms. In this work several *Candida* species isolated in Pernambuco were screened for extracellular lipase production in olive oil containing medium. Among the species tested, *C. rugosa* displayed higher level production of this enzyme. This strain of *C. rugosa* produced more enzyme than other strains previously reported. In shake-flask culture experiments, it was demonstrated that specific lipase activity was higher after 72 hours of cultivation in a medium containing 0.5% olive oil during the stationary phase of growth. This enzyme showed optimal activity at pH 8.0 in tris/HCl buffer at 37°C. Optimal temperature of activity was 40°C. The values of apparent K_M and V_{max} for this lipase were determined as 0.8×10^{-4} M and 0.315 $\mu M.min^{-1}$, respectively, using pNPP as substrate at pH 8.0 and at 40°C.

Key words: Enzyme, extracellular lipase, *Candida rugosa*, olive oil

INTRODUCTION

Lipases (glycerol ester hydrolase E.C. 3.1.1.3) are defined as enzymes which hydrolyse the fatty acyl ester bonds of acyl glycerols (5). This group of enzymes also catalyses the synthesis and transesterification of glyceride and phosphoglyceride ester bonds (5), and the synthesis and hydrolysis of various non-glyceride esters, like carbohydrate esters used as surfactants in food and cosmetic industry (12,13). Thus, lipases have been used in industry to modify fats and oils, improving the taste, flavour, colour, softness and/or structure of many different foods (12,13). The stereo and enantioselectivity of lipases are used to resolve racemes mixtures of drugs (12). Several other applications of lipases have been described elsewhere (13).

Many microorganisms are described as good lipase producers when grown in medium containing oil (10). Multiple forms of isoenzyme lipases produced by *C. rugosa* have been demonstrated and characterised (1,7,11) and the cloning of the *C. rugosa* lipase genes was reported (2). In this work, we report the screening of extracellular lipase-producing species of *Candida* isolated in the State of Pernambuco, Brazil, and the characterisation of the shake-flask fermentation and kinetic parameters of the best producer.

MATERIALS AND METHODS

1. Microorganisms

Candida species, listed in Table 1, were obtained from the Collection of the Department of Mycology of the Universidade Federal de Pernambuco.

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2. Screening for lipase production

Candida strains were cultivated in yeast medium containing (g/l) yeast extract 10, KH_2PO_4 1.0, $MgSO_4$ 0.1 and olive oil 2.5 ml/l for 24 hours at 30°C in a rotatory shaker (180 min^{-1}). The optical density (O.D.) of the culture was measured at 600 nm after appropriate dilution. The cells were harvested by centrifugation and the lipase activity was measured in the supernatants by the colorimetric method with p-nitrophenyl palmitate (pNPP) as substrate. One unit of lipase is defined as the amount of enzyme that causes the cleavage of 1µmol/min of pNPP, detected at 410 nm (8,11).

3. Shake-flask fermentation

The selected yeast strain was cultivated in the yeast medium containing different olive oil concentrations and the lipolytic activity was measured up to 96 hours of cultivation. Fermentation profile analysis included pH changes, lipase activity, lipase specific activity (unit of enzyme per milligram of total extracellular protein) and cell concentration (O.D. 600nm) in the medium containing 0.5% (v/v) olive oil. Protein concentration was determined according to Lowry's method (4).

4. Kinetic characterization

Optimal pH was evaluated by measuring the lipase activity in citrate buffer (pH 5.0-7.0) and tris/HCl buffer (pH 7.0-9.0). Optimal temperature was evaluated by incubating the enzymatic reaction at the range of 20°C to 60°C. Parameters of apparent K_M, and V_{max} were measured in the best conditions of pH and temperature using pNPP as substrate. The results were analysed according to Lineweaver-Burk plot.

RESULTS AND DISCUSSION

The screening for lipase production by yeast cells indicated that *C. rugosa* 968 strain produced 0.290 U/ml of lipase activity and was the best lipase producer among the isolates tested (Table 1). Moreover, *C. vini* 152 produced 0.127 U/ml and the other yeasts produced less than 0.1 U/ml. Thus, *C. rugosa* 968 was chosen for further experiments. Lipase activity of 0.149 U/ml by *C. rugosa* was detected by Rapp and Backhaus (10) only in cultures of 240 hours with 1% olive oil using pNPP as substrate. These authors cultivated yeast cells in a pre-inoculum containing glucose, which could have inhibited lipase production during the initial period of cultivation. Therefore, we demonstrated that this strain of *Candida rugosa* produced more lipase in a short period of incubation than it has been previously reported in a medium containing fatty acids as the main carbon source (10).

Shake-flask fermentations of *C. rugosa* 968 were done in the medium containing different oil concentrations. The low lipolytic activity of 0.150 U/ml in 0.25% (v/v) olive oil medium was reached after 96 hours of cultivation. Maximum lipase activity of 0.25 U/ml was detected in 1% (v/v) olive oil medium in 48 hours of cultivation, followed by a significant decrease thereafter. Lipolytic activity in 0.5% (v/v) olive oil medium reached a plateau from 24 to 48 hours of cultivation. However, maximum lipase activity of 0.44 U/ml was observed after 72 hours of cultivation in this medium (Fig. 1). Gordillo *et al.* (3) and Ohnish *et al.* (6) demonstrated that the lipase production by *C. rugosa* and *Aspergillus oryzae* decreased after reaching a maximum in the media containing higher concentration oleic acid and soybean oil, respectively. Therefore, concentration of 0.5% olive oil was chosen for further experiments. The presence of glucose inhibited lipase production in oil-containing media (Fig. 1).

The fermentation profile in the medium containing 0.5% olive oil is showed in Fig. 2. Cell concentration increased up to 24 hours of cultivation and reached stationary phase of growth. The values of pH in the supernatant culture remained stable between 6.0 to 7.0 during the period of *C. rugosa* cultivation and lipase production (Fig. 2). The lipolytic activity showed a plateau of 0.22 U/ml from 24 to 48 hours of incubation, which might correspond to the changes in cell physiology during the first stage

Table 1. Production of extracellular lipase by different species of *Candida* after 24 h of cultivation in yeast medium containing 0.25% olive oil.

Candida species	Lipase activity (U/ml)[a]
C. guilliermondii	0.012
C. kruzei	0.009
C. luzitanial	0.021
C. melibiose	0.021
C. parakruzei	0.024
C. pseudotropicalis	0.009
C. rugosa	0.290
C. valida	0.009
C. vini	0.127
C. utilis	0.024

A One unit of lipase is defined as the cleavage of 1µmol/min pNPP detected at 410 nm (9).

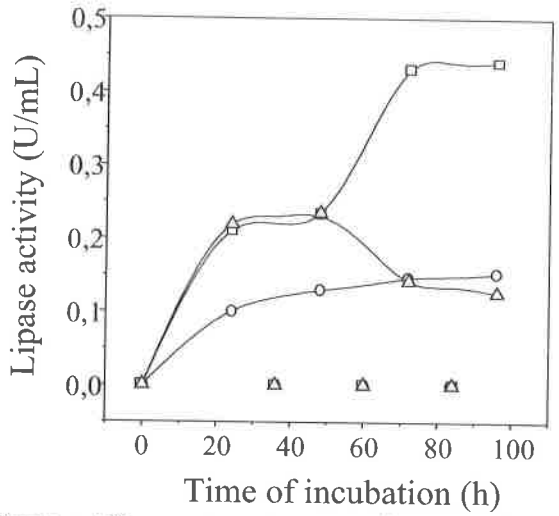

Figure 1. Effect of olive oil concentrations in the lipase production by *Candida rugosa* 968 in shake-flask cultivations at 30°C. The cells were grown in yeast medium containing 0.25% (○), 0.5% (□) or 1% (Δ) of olive oil. Symbols without lines represent cultivation in the presence of glucose. Mean values of two cultures are shown.

of the stationary phase, and increased thereafter to a peak of 0.44 U/ml after 72 hours of cultivation. Higher lipase production during the stationary phase of growth has been well documented for filamentous fungi (6,8,9). The specific lipase activity increased slowly until 48 hours of cultivation and grew faster after this time, which correspond to the second stage of the lipase production described by the lipase activity curve (Fig. 2). Therefore, lipase production by *C. rugosa* is uncoupled to the growth phase and lipase should be produced preferentially during

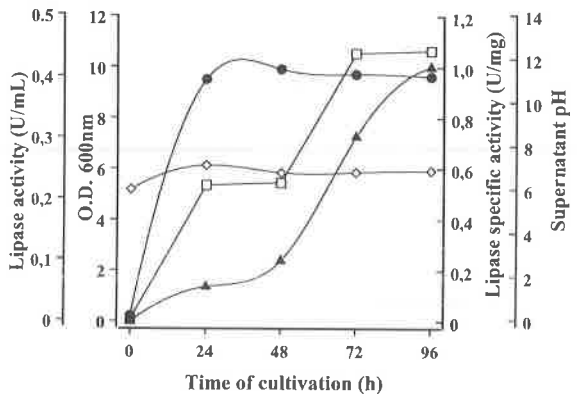

Figure 2. Fermentation profile for lipase production by *C. rugosa* 968 in yeast medium containing 0.5% olive oil. The parameters evaluated were cellular growth (●), lipase specific activity (▲), lipase activity (❑) and supernatant pH (◇). Mean values of two cultures are shown.

stationary phase of growth in relation to other proteins.

The effect of pH in lipase activity was evaluated using different pH values (Fig. 3). A peak of optimal lipase activity was observed at pH 8.0 in tris/HCl buffer, as previously demonstrated for *C. rugosa* lipase by Gordillo et al. (3). Reduced lipase activity was observed at pH below 7.0 in citrate buffer and at pH 9.0 in tris/HCl buffer. Additionally, lipase activity was evaluated by incubation in different

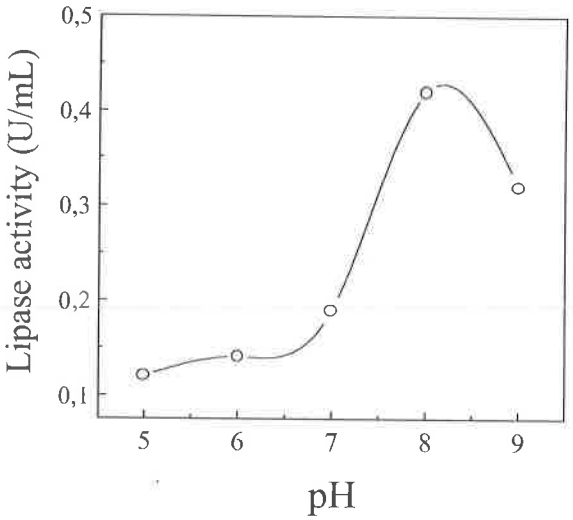

Figure 3. Effect of the pH in the activity of the lipase produced by *C. rugosa* 968. Lipase assay were done in different values of pH in citrate buffer (pH 5.0-7.0) or tris/HCl buffer (pH 7.0-9.0) at 37°C using pNPP as substrate. Mean values of two assays are shown.

temperatures. The best activity increased from 25°C up and reached a peak at 40°C (Fig. 4), with a drastic decrease in the enzyme activity above this temperature. A decrease in the enzyme activity of the *C. rugosa* lipase was also detected by Gordillo *et al.* (3), but observed only after 50°C. Kinetic parameters of *C. rugosa* 968 lipase were determined at pH and temperature conditions of maximal activity. The values calculated for apparent K_M and V_{max} were 0.8 x 10^{-4} M and 0.3146 $\mu M.min^{-1}$, respectively, using pNPP as substrate. Lipase type B, purified from commercial *C. rugosa* lipase type VII and produced by Sigma Co. (St. Luis, USA), has an apparent K_M of 0.9 x 10^{-4} M (10). Previously, an ion-exchange chromatographic fraction I from this Sigma lipase type VII showed a lipase profile, while fraction II showed a esterase profile (1). Multiple forms of lipases isoenzymes produced by *C. rugosa* have been reported (7) and the existence of five different lipase

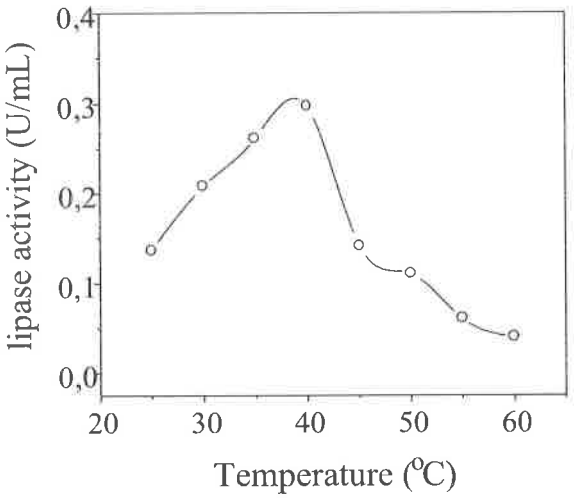

Figure 4. Effect of the temperature in the activity of the lipase produced by *C. rugosa* 968 strain. Lipase assay were done different temperatures of the emulsion at pH 8.0 in tris/HCl buffer using pNPP as substrate. Mean values of two assays are shown.

genes was demonstrated in this yeast, which should be originated through multiple duplication events of an ancestral gene (2). Until now, it is not known whether all these genes are expressed at the same time, or if each strain express typically one of them. This should explain differences in activity and productivity of lipase from different strains of *C. rugosa* in the literature.

ACKNOWLEDGEMENTS

This work was supported by grants from Fundação de Amparo à Ciência do Estado de Pernambuco (FACEPE), Financiadora de Estudos e Projetos (FINEP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Banco do Nordeste do Brasil (BNB).

RESUMO

Produção de lipase extracelular por uma linhagem de *Candida rugosa* isolada no Estado de Pernambuco, Brasil

Lipases extracelulares têm sido produzidas por vários microrganismos. Neste trabalho, algumas espécies de *Candida* isoladas no Estado de Pernambuco foram testadas para a produção de lipase extracelular em meio de cultura contendo óleo de oliva. Dentre as espécies testadas, a *C. rugosa* demostrou a maior atividade lipolítica. Além disso, a produção de lipase por esta linhagem de *C. rugosa* foi comparativamente maior do que têm sido previamente demonstrado. Cultivos em batelada demonstraram que a maior produção de lipase foi alcançada no meio contendo 0,5% (v/v) de óleo de oliva, pelo período de cultivo de 72 horas, principalmente durante a fase estacionária de crescimento do fungo. A lipase produzida apresentou atividade máxima quando testada no pH 8.0 em tampão tris/HCl a 37°C. A temperatura ótima de atividade da lipase foi de 40°C. Os valores de K_M aparente e V_{max} calculados para esta lipase foram de 0.8 x 10^{-4} M e 0.315 $\mu M.min^{-1}$, respectivamente, utilizando o pNPP como substrato a pH 8.0 e 40°C.

Palavras-chave: Enzima, lipase extracelular, *Candida rugosa*, óleo de oliva

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Revista de Microbiologia (1998) 29:138-142
ISSN 0001-3714

OCCURRENCE OF FILAMENTOUS FUNGI AND AFLATOXINS IN POULTRY FEEDSTUFFS

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Submitted: November 27, 1997; Returned to authors for corrections: February 26, 1998; Approved: March 16, 1998

ABSTRACT

Sixty samples of poultry feedstuffs obtained from one manufactory and four farms located in Manaus, Amazonas State, Brazil, were studied for filamentous fungi and aflatoxin contamination. The following parameters were analyzed: fungal contamination, aflatoxigenic potential of *Aspergillus flavus* strains isolated, occurrence of aflatoxins and influence of abiotic factors (moisture content and water activity) on the mycoflora. The fungi were isolated on Sabouraud dextrose agar with chloramphenicol. Detection of aflatoxins was carried out by thin-layer chromatography (TLC). The genus *Aspergillus* was the most frequent (71.7%), followed by *Rhizopus* (28.3%), *Absidia* (26.7%), *Penicillium* (11.7%), *Mucor* (11.7%), *Fusarium* (10%), *Cladosporium* (1.7%), and *Crysosporium* (1.7%). Among the *Aspergillus* species, *A. flavus* was the most frequently isolated (53.3%). 44% of *A. flavus* strains were toxigenic and produced only Group B aflatoxins. The fungi were recovered from samples with moisture content and water activity ranging from 11.0% to 24.6% and from 0.91 to 0.61, respectively. The number of colony forming units of *Aspergillus* pergram (CFU/g) varied from 0.5 to 21.5 x 10^3. No aflatoxins were detected in the 60 samples analysed.

Key words: Feedstuff, poultry, filamentous fungi, aflatoxins

INTRODUCTION

Aflatoxins are potent carcinogenic, mutagenic and teratogenic metabolites produced primarily by the fungal species *Aspergillus flavus* and *A. parasiticus*. Food and feeds, especially in warm climates, are susceptible to invasion by aflatoxigenic *Aspergillus* species and the subsequent production of aflatoxin during preharvesting, processing, transport or storage (5, 10, 25).

Great improvements have taken place in the production of poultry feedstuffs since the beginning of this century, when cereal based feeds were first used in poultry farming (6). Maize, the main ingredient of these feeds, has a high nutritional value and is employed at levels that vary between 60 and 70%. From 75,2 million tons of cereal grains produced in Brazil during 1993 and 1994, 42,6 million were of maize (20).

Brazil exported 416,952 tons of poultry meat in 1993, when 12 million tons of feeds were produced for birds rearing (24).

During storage, cereal grains are exposed to infections between physical, chemical and biological factors that can lead to grain deterioration. Heat treatment of grain mixtures used in the production of feedstuffs reduces the number of viable microorganisms and thus improves their preservation. However, favorable conditions of temperature and relative humidity of the air added to

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the moisture content of the finished product can bring about biodeterioration. (1, 4).

Considering the economical and nutritional importance of feedstuffs in poultry farming and the scarceness of pertinent studies, especially in Brazil, the aim of the present investigation was to evaluate the mycoflora, the aflatoxigenic potential of *Aspergillus flavus* strains isolated and the occurrence of aflatoxins in samples of poultry feedstuffs produced in Manaus, MA, Brasil.

MATERIALS AND METHODS

Samples

A total of 60 samples of feedstuffs (1 Kg each) were obtained from a factory (12 samples) and from feedboxes of 4 poultry farms (48 samples) located near the city of Manaus, Amazonia State, Brazil. This region is characterized by a hot and humid tropical climate, with rainy (winter) and dry (summer) seasons. Sampling was carried out during the months of February and March, 1995.

Moisture content and water activity of samples

Moisture content was determined immediately after sampling according to the Analytical Methods of the Instituto Adolfo Lutz (18). Water activity (Aw) was determined by automated analysis using AQUALAB CX-2 (Decagon Devices Inc).

Determination of mycoflora according to Swanson *et al.* (23)

Subsamples (10g each) of ground corn were mixed with 90 ml of sterile distilled water to obtain a 10^{-1} stock dilution and ten fold serial dilutions up to 10^{-6} were then made using the same diluent. Duplicates of 0.1 ml volume of each dilution were added to Petri dishes containing 10 to 15 ml of Sabouraud Dextrose Agar, followed by incubation at 25°C for 5 days. The Petri dished were observed daily for fungal growth. Fungal colonies were identified at the genus level; only the genera *Aspergillus* and *Fusarium* were further characterized at the species level as described by ARX (2); Raper and Fennel (21) and Nelson *et al.* (16).

Toxigenicity of aflatoxin-producing isolates

Aspergillus spp. strains were inoculated onto Coconut Agar medium and incubated for 10 days at 25°C. 10g the medium were transferred to a 200 ml becker containing 30 ml of chloroform. The mixture was macerated, filtered through filter paper and the filtered material was evaporated in a water bath. The dry extract was solubilized in chloroform and chromatographed as described by Lin and Dianese (11).

Determination of aflatoxins

Samples were evaluated by thin layer chromatography (TLC) for the presence of aflatoxins using the methodology developed by Soares and Rodriguez-Amaya (22).

RESULTS AND DISCUSSION

The mycoflora recovered from the 60 samples of poultry feedstuff, listed in decreasing order of frequency, were as follows: *Aspergillus* (71.7%), *Rhizopus* (28.3%), *Absidia* (26.7%), *Penicillium* (11.7%), *Mucor* (117.%), *Fusarium* (10%), *Cladosporium* (1.7%), *Crysosporuim* (1.7%) (Fig. 1). The predominance of *Aspergillus* spp observed in our study is in agreement with the results obtained by Neumanova *et al.* (17) and Fiorentin (7), who also reported this prevalence in poultry feedstuffs.

Within the genus *Aspergillus,* the occurrence of the species *A. flavus* (53.3%), *A. alutaceus* (5.0%), *A. niger* (5.0%), *A. sydowi* (3.3%), *A. fumigatus* (3.3%), *A. terricola* (1.7%) was observed (Fig. 2). High frequency values for *A. flavus* in animal feeds have similarly been found by other authors (7, 13, 14, 15); in our work, the predominance of this isolate was associated with moisture content values of 11 to 15% and water content values of 0.61 to 0.79. Accordingly, Kozakiewicz and Smith (8) observed that the minimum water activity necessary for growth of *A. flavus* and aflatoxin production were 0.71 and 0.82, respectively.

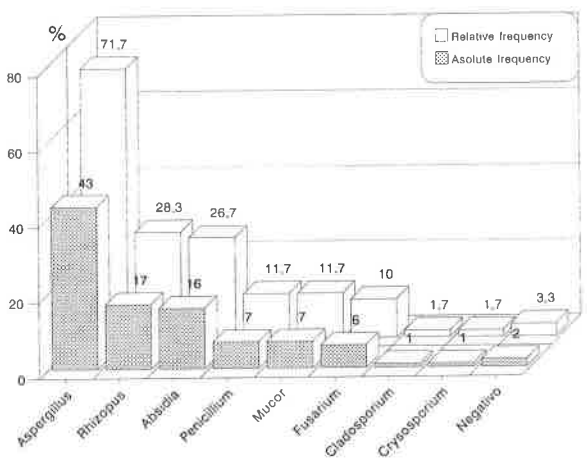

Figure 1. Occurrence of fungi in 60 samples of poultry feedstuffs

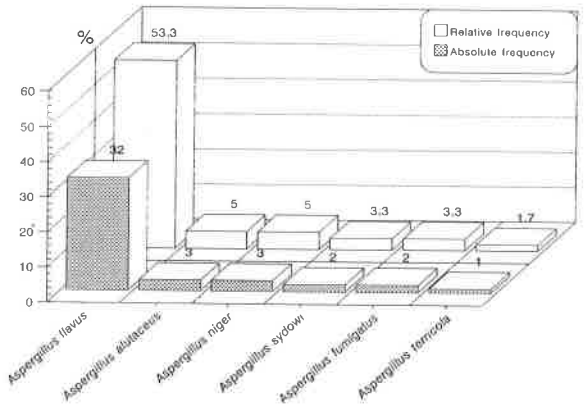

Figure 2. Occurrence of *Aspergillus* species in 60 samples of poultry feedstuffs

Among the *Fusarium* isolates, the species *F. moniliforme* (8.3%) and *F. proliferatum* (1.7%) (Fig. 3) were recovered at moisture content values of 11% to 15%. Likewise, Asevedo *et al.* (3) and Marasas *et al.* (12) detected a prevalence of this genus within a moisture content range of 13 to 15% and at 14.8%, respectively. Concerning water activities (Aw), *Fusarium* species were recovered at values that ranged between 0.79 and 0.61 which are lower than the 0.89-0.86 variation described by Lacey *et al.* (9) as optimal for *Fusarium* growth. This may explain the low frequency of isolation observed for this genus (Tables 1 and 2).

The three most important fungal genera in terms of toxigenicity, namely *Aspergillus*, *Fusarium* and *Penicillium*, presented numbers of colony forming units (CFU/g) that varied from 0.5 to 21.5 x 10^3, 5.5 to 28 x 10^3 and 1.0 to 3.5 x 10^3, respectively. These results fall within the tolerance limits established by

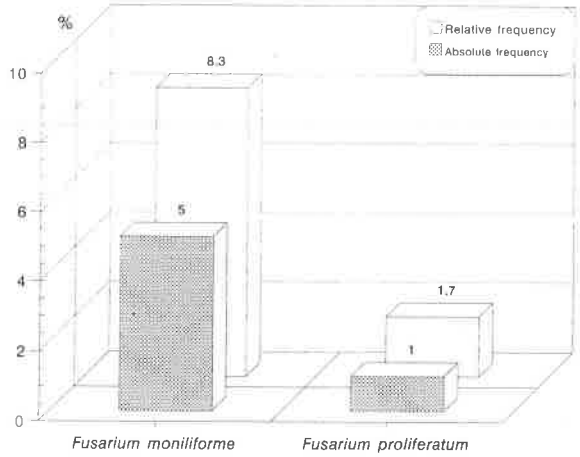

Figure 3. Occurrence of *Fusarium* species in 60 samples of poultry feedstuffs

Table 1. Absolute and Relative frequencies of fungal genera isolated from 60 samples of poultry feedstuffs and range of water activity (aw) values. Sampling period: March to May, 1995.

Genera	range of aw	AF (RF)
	0.91 - 0.80	-----
Aspergillus	0.79 - 0.70	27 (45.0)
	0.69 - 0.66	13 (21.6)
	0.65 - 0.61	3 (5.0)
	0.91 - 0.80	-----
Rhizopus	0.79 - 0.70	8 (13.3)
	0.69 - 0.66	5 (8.3)
	0.65 - 0.61	4 (6.6)
	0.91 - 0.80	-----
Absidia	0.79 - 0.70	13 (21.6)
	0.69 - 0.66	3 (5)
	0.65 - 0.61	-----
	0.91 - 0.80	-----
Penicillium	0.79 - 0.70	2 (3.3)
	0.69 - 0.66	5 (8.3)
	0.65 - 0.61	-----
	0.91 - 0.80	-----
Fusarium	0.79 - 0.70	4 (6.6)
	0.69 - 0.66	1 (1.6)
	0.65 - 0.61	1 (1.6)
	0.91 - 0.80	-----
Mucor	0.79 - 0.70	2 (3.3)
	0.69 - 0.66	4 (6.6)
	0.65 - 0.61	1 (1.6)
	0.91 - 0.80	-----
Cladosporium	0.79 - 0.70	-----
	0.69 - 0.66	1 (1.6)
	0.65 - 0.61	-----
	0.91 - 0.80	-----
Crysosporium	0.79 - 0.70	-----
	0.69 - 0.66	1 (1.6)
	0.65 - 0.61	-----

AF = Absolute Frequencies
RF = Relative Frequencies

the Normas e Padrões de Nutrição e Alimentação Animal (19) for mashed feeds (10^4 to 10^5 CFU/g of feedstuff). *Fusarium* spp, especially *F. moniliforme*, despite a low frequency (10%; Fig. 1), showed the highest level of contamination (28 x 10^3 CFU/g). None of the feed samples studied were contaminated with aflatoxins, which might be due to their low water activity and moisture content. However, 44% of the *A. flavus* isolates were aflatoxins B_1 and B_2 producers at levels that were considered high (40.4 to 10.827 µg/kg and 79.4 to 2.835 µg/kg, respectively), emphasizing the importance of studies on the occurrence of aflatoxins in this type of substrate.

Table 2 - Frequency of *Aspergillus* species, *Fusarium* species and other fungal from 60 samples of poultry feedstuffs, listed with the corresponding moisture content values. Sampling period: March to May, 1995.

Genera and fungal species		Moisture content (%)				
		11 - 13	13 - 15	15 - 17	17 -19	Total
Aspergillus flavus	AF (RF %)	18 (56.2)	11 (34.3)	2 (6.2)	1 (3.3)	32
A. alutaceus	AF (RF %)	3 (100)	-	-	-	3
A. niger	AF (RF %)	2 (66.7)	1 (33.3)	-	-	3
A. sydowi	AF (RF %)	-	2 (100)	-	-	2
A. fumigatus	AF (RF %)	-	1 (50)	-	1 (50)	2
A. terricola	AF (RF %)	1 (100)	-	-	-	1
Fusarium moniliforme	AF (RF %)	2 (40)	3 (60)	-	-	5
F. proliferatum	AF (RF %)	1 (100)	-	-	-	1
Penicillium spp	AF (RF %)	4 (57.1)	2 (21.6)	1 (14.3)	-	7
Rhyzopus spp	AF (RF %)	14 (82.3)	3 (17.7)	-	-	17
Mucor	AF (RF %)	6 (85.7)	1 (14.3)	-	-	7
Cladosporium spp	AF (RF %)	1 (100)	-	-	-	1
Crysosporium spp	AF (RF %)	-	1 (100)	-	-	1

AF = Absolute Frequency
RF = Relative Frequency

RESUMO

Ocorrência de fungos filamentosos e de aflatoxinas em ração destinada a alimentação de aves

Foram analisadas 60 amostras de rações destinadas a alimentação de aves, provenientes de uma fábrica e de quatro granjas situadas em Manaus, Amazonas, para verificar o grau de contaminação fúngica, a potencialidade toxigênica das cepas de *Aspergillus flavus* a influência de fatores abióticos (teor de umidade e atividade de água) e a ocorrência de aflatoxinas neste substrato. O isolamento dos fungos foi efetuado utilizando Ágar Sabouraud Dextrose, acrescido de cloranfenicol e a pesquisa de aflatoxinas foi realizada utilizando-se cromatografia em camada delgada. O gênero *Aspergillus* foi o mais frequente (71,7%), seguido dos gêneros *Rhizopus* (28,3%), *Absidia* (26,7%), *Penicillium* (11,7%), *Mucor* (11,7%), *Fusarium* (10,0%), *Cladosporium* (1,7%) e *Crysosporium* (1,7%), isolados de amostras com teor de umidade variando de 11,0 a 24,6% e atividade de água (Aa) de 0,91 a 0,61. Dentro do gênero *Aspergillus*, a espécie *A. flavus* foi a mais frequente (53,3%). O número de unidades formadoras de colônias por grama (UFC/g), do gênero *Aspergillus* variou de 0,5 a 21,5 x 10^3. Vinte e duas cepas de *A. flavus* (44%) produziram aflatoxinas do grupo B. Análises micotoxicológicas das 60 amostras de rações não revelaram a presença de aflatoxinas.

Palavras-chave: Ração, aves, microbiota fúngica, aflatoxinas

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Revista de Microbiologia (1998) 29:143-148
ISSN 0001-3714

EFFECTS OF PROPOLIS ON *STREPTOCOCCUS MUTANS, ACTINOMYCES NAESLUNDII* AND *STAPHYLOCOCCUS AUREUS*

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Submitted: June 06, 1997; Returned to authors for corrections: October 24, 1997; Approved: March 03, 1998

ABSTRACT

It is known that formation of dental caries is caused by the colonization and accumulation of oral microorganisms and extracellular polysaccharides which are synthesized from sucrose by glucosyltransferases of *Streptococcus mutans*. *Actinomyces naeslundii* and *Staphylococcus aureus* are associated with human root caries and some oral mucosa infections, respectively. In this research *Streptococcus mutans* Ingbritt 1600 exhibiting glucosyltransferase activity was used to test whether different ethanolic extracts of propolis (EEP) inhibit or not the enzyme activity and growth of the bacteria. Antimicrobial activity of EEP against *A. naeslundii* and *S. aureus* was also examined. All EEP from various regions in Brazil inhibited both glucosyltransferase activity and growth of *S. mutans*, *A. naeslundii* and *S. aureus*, but one of propolis from Rio Grande do Sul (RS2) demonstrated highest inhibition of the enzyme activity and growth of the bacteria. It was also found that propolis (RS2) contained highest concentrations of pinocembrin and galangin.

Key words: Dental caries, glucosyltransferase, propolis, antimicrobial activity, *Streptococcus mutans*, extracellular polysaccharides.

INTRODUCTION

A number of investigations have shown a positive correlation between the number of *Streptococcus mutans* in dental plaque and the prevalence of dental caries (9, 17). Substantial numbers of *S. mutans* and lesser numbers of *S. sanguis*, *S. mitis* and species of genus of *Actinomyces* were isolated from cemental surface caries during an extensive survey of oral microbiology (30).

It was found that formation of dental plaque was caused by the colonization and accumulation of oral microorganisms and extracellular polysaccharides which are synthesized from sucrose by glucosyltransferase of *S.mutans* on hard surface of teeth (12). The extracellular polysaccharides consist of glucans containing predominantly α-1,6 bonds that are similar to classical dextrans and of polymers containing more than 50% α-1,3 linkages (12). The latter polysaccharides are highly insoluble and have been termed mutan. Both types of polymers have been detected in samples of human dental plaque (12).

Several studies have shown that species of genus *Actinomyces*, in particular *Actinomyces naeslundii*, are associated with human root caries (15, 26) and gingivitis (22, 27). Staphylococci and aerobic Gram-negative rods are not commonly involved with odontogenic infections, however, *Staphylococcus aureus* is associated with infections of the oral

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mucosa (8). In addition, strains of *S. aureus* have been isolated in periapical lesions and periodontal abscess (1).

It is now clear that microorganisms play an essential role in the pathogenesis of dental caries, gingivitis and some oral mucosa infections and consequently provide a prime target for the prevention of these diseases using antibiotics and vaccine (6, 7). On the other hand, it was reported that mutastein and ribocitrin, which were isolated from the culture supernatants of *Aspergillus terreus* and *Streptomyces sp.*, inhibited the glucosyltransferase of *S.mutans* (10, 24), but did not show antibacterial activity. In the case of the ethanolic extract of propolis, both the growth of *S. mutans* and the activity of glucosyltransferase were inhibited (19). It was also found that propolis has antibacterial activity against *S. aureus* (5, 13, 21). Propolis is a resinous hive product collected by bees from tree buds and mixed with beeswax, which they secrete. The propolis is used by the bees as a glue to seal the opening of the hives (14). It is known that the ethanolic extract of propolis exhibits various pharmacological activities such as antibacterial, antiviral, antifungal, anaesthetic, anti-inflammatory, hypotensive, immunostimulatory and cytostatic properties (3, 11). The objective of this work was to further investigate the use of propolis, collected from various regions of Brazil, for its antibacterial activity and inhibition of glucosyltransferase.

MATERIALS AND METHODS

Microorganisms. Bacterial strains used in this research were *Streptococcus mutans* Ingbritt 1600, *Actinomyces naeslundii* ATCC 12104 and a strains of coagulase positive *Staphylococcus aureus*. The strain of *S. mutans* Ingbritt 1600 was previously selected by its glucosyltransferase activity by both aerobic and anaerobic cultivation, as compared to three others oral microorganisms: *Streptococcus sanguis* ATCC 10556, *Streptococcus sp.* isolated from saliva in our laboratory and *Actinomyces naeslundii* ATCC 12104. *S. sanguis* ATCC 10556 and *Actinomyces naeslundii* ATCC 12104 were donated from Department of Dental Research, University of Rochester, NY, USA. *S. mutans* Ingbritt 1600 was donated from Eastman Dental Research Center, Rochester, NY, USA. A Gram negative bacteria, *Escherichia coli,* was also used for antimicrobial test.

Propolis samples. The propolis samples collected by *Apis mellifera* were obtained from the states of Minas Gerais, São Paulo, Goiás, Mato Grosso do Sul, Paraná and Rio Grande do Sul for this investigation. The specimens of propolis were further dehydrated using low vacuum pump and the extracts of the dried propolis prepared as described by Koo and Park (20). The dried propolis samples were ground into a fine powder and 2 g of the propolis powder mixed with 25 ml of 80% ethanol in a test tube and shaken at 70°C for 30 min. After extraction, the mixture was centrifuged to obtain the supernatants, which were denominated as an ethanolic extract of propolis (EEP).

Production of the enzyme. The strains of *Streptococcus mutans, Streptococcus sanguis, Streptococcus sp.* and *Actinomyces naeslundii* were inoculated into 100 ml conical flasks containing 20 ml of 3% tryptic soy broth and incubated at 37°C for 48 hr with shaking (150 rpm). In the case of anaerobic cultivation, the conical flasks were incubated statically in an anaerobic incubator. After cultivation for 48 hr, the culture media were centrifuged and the supernatans used as crude glucosyltransferase.

Assay of glucosyltransferase activity. Glucosyltransferase activity was determined as described by Smith *et al.* (28). A mixture of 0.9 ml of sucrose 0.25 M in 0.2 M phosphate buffer, pH 7.0 and 0.1 ml of the crude enzyme solution was incubated at 37°C for 2 hr. After incubation, reducing sugars were measured by the method of Somogyi-Nelson (29). The quantitative analysis of fructose was performed by HPLC using a chromatograph equipped with a differential refractometer detector and a Shodex Ionpak KS-802 column, the eluent being water with a flow rate of 1 ml/min. Authentic standards were fructose, glucose and sucrose. One unit of enzyme activity was defined as 1 μmol of fructose/ml of enzyme/hr.

Inhibition of glucosyltransferase activity. The incubation mixture containing 0.9 ml of 0.25 M sucrose in 0.2 M phosphate buffer pH 7.0, 0.1 ml of the crude enzyme solution, 0.005 ml of the ethanolic extract (EEP) (in case of control 0.005 ml of 80% ethanol) and 0.2 M phosphate buffer pH 7.0 to a total volume of 2 ml was incubated at 37°C for 2 hr (19). After incubation, concentration of fructose were determined as described above.

Antimicrobial activity of propolis. Actively growing cultures in tryptic soy broth of the test strains of microorganisms were inoculated onto tryptic soy agar plates with sterile swabs and then the EEP disks were applied on the inoculated plates which were

incubated overnight at 37°C under anaerobic condition. EEP disks were prepared by submerging sterile paper disks (Whatman filter paper nº 3, 5 x 0.5 mm) in EEP solutions, kept drying under low vacuum at room temperature overnight and then incubated at 60°C for 4 hr (4).

High Performance Thin-Layer Chromatography (HPTLC) of EEP. HPTLC precoated plates of silica gel RP-18 $F_{254}S$ were purchased from Merck Co. EEP 5 µl were applied to the lower edge of the plate, and ascending chromatography run using a mobile phase of ethanol:water (55:45, v/v). The detection of flavonoids was carried out by UV-radiation at 366 nm.

Reversed Phase High Performance Liquid Chromatography (HPLC) of EEP. A quantitative analysis of the flavonoids was performed by reversed phase HPLC using a chromatograph equipped with YMC PACK ODS-A column. The mobile phase was acetic acid:methanol:water (5:75:60, by vol.) and the flow rate was 1 ml/min and detection with a diode array detector. Chromatograms were recorded at 254 nm. The quantities of flavonoids in the EEP were calculated by using authentic standards of flavonoids purchased from Extrasynthese A .A . Co., France.

RESULTS AND DISCUSSION

Production of glucosyltransferase. *Streptococcus mutans* produced the highest activity of the enzyme by both anaerobic and aerobic cultivation (9.60 ± 0.12 and 9.00 ± 0.20 units, respectively) as compared to other three strains. *S. sanguis* and *A. naeslundii* produced the lowest activity of the enzyme (0.30 ± 0.06 and 0.48 ± 0.09 units, respectively, for anaerobic cultivation; and 0.30 ± 0.06 and 0.18 ± 0.08, respectively, for aerobic cultivation). *Streptococcus sp.* isolated from saliva also demonstrated lower activity of the enzyme (1.80 ± 0.15 and 2.10 ± 0.28 units for anaerobic and aerobic cultivation, respectively) than *S. mutans*. Therefore, the strain of *S. mutans* was used for further studies.

Effect of propolis on glucosyltransferase activity. The effect of propolis on glucosyltransferase activity from *S. mutans* was determined by incubating the enzymatic reaction mixture as described in the method which contained ethanolic extract of propolis (EEP) at 37°C for one hour. The results are shown in Table 1. All propolis samples inhibited the enzyme activity and propolis sample from Rio Grande do Sul (RS2) state demonstrated highest inhibition of the enzyme activity.

Antimicrobial activity of propolis. Susceptibility of *S. mutans, A. naeslundii* and *S. aureus* to EEP collected from different regions in Brazil were investigated and the results were shown in Table 2. All propolis samples exhibited an inhibitory action on the growth of the bacteria and one of the sample from Rio Grande do Sul state (RS2) demonstrated highest inhibition zone of the bacterial growth. Nevertheless, none of EEPs inhibited the growth of *E. coli*. Earlier studies reported that propolis is more active on Gram positive than on Gram negative bacteria, as *E. coli* (5, 13). It is interesting to note that the propolis from Rio Grande do Sul (RS2) demonstrated both higher antimicrobial activity and inhibition of glucosyltransferase activity. Therefore, analysis of flavonoids in propolis was carried out by using High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) because these two techniques are most often used (16, 31). The results were shown in Figs. 1 and 2. HPTLC and HPLC (Figs. 1 and 2) demonstrated that patterns of chromatograms of propolis from SP, MG1, MG2, GO

Table 1 - Effect of propolis on activity of glucosyltransferase from *S. mutans*.

Place of Collection of Propolis	Glucosyltransferase activity (Unit)*		Inhibition of the enzyme (%)
	whitout propolis	with propolis**	
Paraná State (PR)	2.9 ± 0.25	2.4 ± 0.38	17.2 ± 0.14
São Paulo State (SP)	2.5 ± 0.21	2.3 ± 0.27	8.0 ± 0.25
Rio Grande do Sul State (RS)	3.6 ± 0.30	2.5 ± 0.20	30.5 ± 0.18
Minas Gerais State (MG)	3.0 ± 0.15	2.6 ± 0.22	13.3 ± 0.16
Goiás State (GO)	3.2 ± 0.34	2.7 ± 0.45	15.6 ± 0.27
Mato Grosso do Sul State (MS)	3.6 ± 0.52	2.9 ± 0.31	19.4 ± 0.23

Each value represents the mean ± standard deviation of a triplicate analysis ($p<0.001$)
* One unit was defined as 1 µmol of fructose/hr/ml of enzyme
** Ethanolic extract of propolis (2 g of propolis / 25 ml of 80% ethanol)

Table 2 - Antimicrobial activity of ethanolic extracts of propolis from different regions to *Streptococcus mutans*, *Actinomyces naeslundii* and *Staphylococcus aureus*. (Overnight incubation at 37°C under anaerobic condition)

Regions of propolis collected	Zone of inhibition of microbial growth (mm)		
	S. mutans	*A. naeslundii*	*S. aureus*
Paraná State (PR)	1.0	1.5	1.0
São Paulo State (SP)	1.5	2.0	1.0
Goiás State (GO)	0.5	1.0	0.5
Mato Grosso do Sul State (MS)	1.5	2.5	1.0
Minas Gerais State (MG1)	1.5	2.5	1.0
(MG2)	0.5	2.0	0.5
Rio Grande do Sul State (RS1)	1.5	2.0	1.0
(RS2)	3.0	3.5	2.0
80% ethanol as control	0	0	0

Propolis samples were collected from two (2) different places in Minas Gerais and Rio Grande do Sul States
Ethanolic extract of propolis (2g of propolis / 25 ml of 80% ethanol)

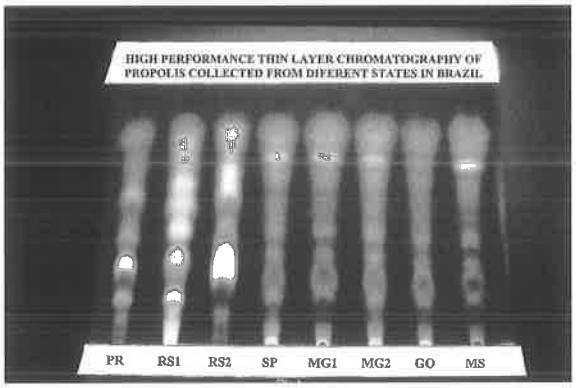

Figure 1. High performance thin layer chromatography of propolis collected from diferent states in Brazil.

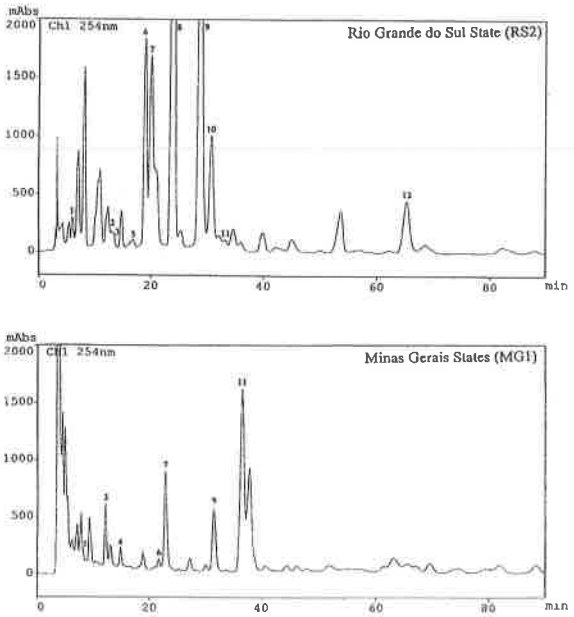

Figure 2. RP-HPLC of ethanolic extract of propolis. (1) quercetin, (2) kaempferol, (3) apigenin, (4) isorhamntin, (5) rhamnetin, (6) pinocembrin, (7) sakuranetin, (8) chrysin, (9) acacetin, (10) galangin, (11) kaempferide and (12) tectocharysin.

and MS (Southeastern and Central Brazil) are entirely different as compared to PR, RS1 and RS2 (Southern Brazil). These different patterns of chromatogram are due to different flavonoids in propolis as reported previously (25). The results of quantitative analysis of flavonoids in propolis were shown in Table 3. It is apparent that propolis RS2 contained highest concentrations of pinocembrin as compared to others. Both RS1 and RS2 contained higher concentrations of galangin than other states. It was already reported that ethanolic extract of propolis showed strong inhibitory activity against various microorganisms *in vitro* test (21). Some flavonoids in propolis are considered to be antimicrobial agents and pinocembrin (32) and galangin (33) were identified as antimicrobial flavonoids. Later, Metzner *et al* (23) also reported that antimicrobial activity of propolis was due to presence of pinocembrin, galangin, pinobanksin, pinobanksin-3-acetate which are flavonoids, and caffeic acid ester. The propolis RS2 also contained higher concentrations of quercetin and mainly chrysin than others. Iio *et al.* (18) showed that quercetin and chrysin inhibited *in vitro* the glucosyltransferase activity and glucan formation.

In conclusion, all the samples of propolis tested showed antimicrobial activity and inhibition of glucosyltransferase activity, however propolis from Rio Grande do Sul-RS2 (Southern Brazil) demonstrated better results than others, propably due its highest concentrations of pinocembrin, galangin

Table 3. Quantitative analysis of propolis from different regions in Brazil.

	Propolis							
Flavonoids	RS1	RS2	PR	SP	MG1	MG2	GO	MS
Quercetin	1.4	2.0	1.3	1.0	0.9	1.2	0.4	0.8
Kaempferol	2.9	0.6	1.1	1.9	2.4	1.2	1.1	1.8
Apigenin	1.8	0.5	1.5	0.5	-	0.5	1.0	2.4
Isorhamnetin	-	-	2.3	-	1.1	-	-	0.2
Rhamnetin	-	0.8	0.5	-	-	-	-	-
Pinocembrin	7.3	16.8	12.6	0.4	1.8	1.3	1.7	9.6
Sakuranetin	12.3	25.0	7.5	15.3	19.6	9.2	1.6	12.6
Isosakuranetin	-	-	-	-	-	0.2	-	5.9
Chrysin	-	21.9	9.7	1.8	-	2.1	1.1	10.7
Acacetin	-	28.8	4.9	13.0	4.3	8.3	3.9	4.9
Galangin	13.5	9.4	7.0	0.6	-	0.5	0.6	1.5
Kaempferide	3.2	1.1	3.2	-	22.2	0.4	0.5	1.1
Tectochrysin	-	3.3	1.0	-	-	-	-	-

All results represent mg/g of propolis

(antimicrobial activity), quercetin and chrysin (inhibition of glucosyltransferase activity and glucan formation).

RESUMO

Efeito da própolis sobre *Streptococcus mutans*, *Actinomyces naeslundii* e *Staphylococcus aureus*

Sabe-se que a cárie dental está relacionada com a colonização e acúmulo de microrganismos e polissacarídeos extracelulares sintetizados a partir da sacarose pelas enzimas glicosiltransferases produzidas pelos *Streptococcus mutans*. *Actinomyces naeslundii* e *Staphylococcus aureus* estão associados, respectivamente, com cárie de raiz e algumas infecções da mucosa oral em seres humanos. Neste experimento, *Streptococcus mutans* Ingbritt 1600 exibindo alta atividade glicosiltransferásica foi usado para analisar extratos etanólicos de própolis (EEP) quanto à possível inibição da atividade enzimática e do crescimento bacteriano. Também foi analisado a atividade antimicrobiana do EEP sobre *A. naeslundii* e *S. aureus*. Todos os EEPs testados, obtidos de diversas regiões do Brasil, inibiram tanto a atividade de glicosiltransferase como o crescimento de *S.mutans, A. naeslundii* e *S. aureus;* entretanto, uma amostra de própolis proveniente do Rio Grande do Sul (RS2) demonstrou maior inibição da atividade enzimática, bem como do crescimento bacteriano. Também foi observado que a própolis RS2 apresentou maiores concentrações de pinocembrina e galangina.

Palavras-chave: Cárie dental, glicosiltransferase, própolis, atividade antimicrobiana, *Streptococcus mutans*, polissacarídeos extracelulares.

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Revista de Microbiologia (1998) 29:149-151
ISSN 0001-3714

SEROPREVALENCE OF HEPATITIS A IN HEALTH CARE STUDENTS FROM A PUBLIC UNIVERSITY OF RIO DE JANEIRO, BRAZIL

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Submitted: September 16, 1997; Returned to authors for corrections: April 9, 1998; Approved: May 4, 1998

SHORT COMMUNICATION

ABSTRACT

The prevalence of antibodies to hepatitis A virus (anti-HAV) was investigated in 116 health care students from a public university of Rio de Janeiro. The overall prevalence for anti-HAV was 31%. This rate is considerably lower than the 54.3% figure observed in a study carried out in the same population in 1986. The decline in the anti-HAV antibodies prevalence reflects a decreasing exposure to HAV infection, which is closely associated with better standards of living.

Key words: Hepatitis A, seroepidemiology , anti-HAV

Hepatitis A virus (HAV) infection is widespread throughout the world. Nevertheless, improvements in general public sanitation have been responsible for a decline on its incidence in several countries (3,7). Former seroprevalence studies conducted in some States from Brazil have indicated a high prevalence of anti-HAV antibodies since the early childhood (1,2,6,8). However, in a recent seroprevalence study carried out in a population group of children and adolescents from Rio de Janeiro, it was demonstrated a low prevalence of anti-HAV antibodies (6.9%) in children under the age of five and an increased proportion of HAV infections being delayed until adolescence (11). The low exposure to HAV infection of younger children in this population could be correlated with improved environmental hygiene and sanitation. In order to identify the magnitude of possible changes in the prevalence of hepatitis A in the State of Rio de Janeiro, other seroprevalence studies should be carried out in people with different socioeconomic levels and from diverse geographical areas of the State.

In the present work, we studied the prevalence of anti-HAV antibodies among health care students from a public university of Rio de Janeiro. The study population was composed by 116 randomly selected students of Medicine, Odontology, Pharmacy, Veterinary, Nutrition and Nursery schools from the Federal Fluminense University, aged 18 to 26 years (average age 21). Blood samples were collected in July 1996. At that time, all students were in the second year of their graduation. Each blood sample was accompanied by a questionnaire recording age, sex, familiar income - number of minimum salaries (U$120 each) received per month - , parents' educational level, previous history of hepatitis and sanitary conditions of housing. Anti-HAV antibodies were determined using a competitive "in-house" EIA

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(10). Probability (p) was determined by chi-squared tests using Yates correction. Differences were regarded as significant when $p \leq 0.05$.

The overall prevalence for anti-HAV antibodies was 31.0% (see Table 1). Familiar income and parents' educational level were negatively associated with the prevalence of anti-HAV, in the sense that as the familiar income increased (from 1-20 to more than 21 minimum salaries) and parents' educational level got higher (from primary to graduate level), the prevalence decreased (p=0.02 and 0.00002, respectively). Previous history of hepatitis was also found to be significantly associated with anti-HAV antibodies prevalence (p=0.0005). The association of anti-HAV antibodies with sex was not significant. Other socioeconomic features that could be observed were that most subjects from this population had middle to high socioeconomic status, in view of their parents' monthly income (63.8% (74/116) received more than 21 minimum salaries) and educational level (51.7% (60/116) had university studies). Moreover, the majority of them (91.4% (106/116)) lived under adequate sanitary conditions, having access to piped water and sewerage system (data not shown).

Hepatitis A viral infection is a problem in areas where the seroepidemiology of the disease is an intermediate endemic type (4). In countries with improving living standards, the infection in children and teenagers is decreasing, thefore making adolescents more susceptible to HAV. The estimated prevalence of 31.0% among the 116 health care students 18-26 years old in our study is considerably lower (p=0.0006) than the 54.3% figure observed in 1986 among 127 health care students from the same university aged 18 to 27 years (5). The decline in the anti-HAV prevalence reflects a decreasing exposure to HAV infection, which is closely associated with better standards of living. Socioeconomic factors also represent important determinants in the spread of this infection, confirmed by the association of anti-HAV prevalence with variables as parents' educational level and familiar income, in agreement with other studies (3,9). This results confirm and extents those obtained in a seroepidemiologic study carried out in a suburb of the western area of Rio de Janeiro among children and adolescents with low socioeconomic status, that showed a possible change in the prevalence of hepatitis A (11). In both studies, most subjects lived under adequate sanitary conditions, in spite of having different socioeconomic status. Improvements in public hygiene increase the number of susceptible persons, raising the probability of future epidemics of HAV in groups previously protected by immunity acquired in early childhood. The group under study could be considered at risk of infection, due the possibility of further direct contact as health care workers with HAV infected persons. Moreover, this new epidemiological pattern has strong clinical and public health implications, since the existence at older ages of a relatively large group of unprotected individuals living in an environmental

Table 1 - Prevalence of anti-HAV by socioeconomic characteristics, previous history of hepatitis and sex among health care students from a public university of Rio de Janeiro, Brazil, 1996.

	anti-HAV antibodies			
	Positive n = 36 (31.0%)	Negative n = 80 (69.0%)	χ^2	p-value
Familiar income[2]				
1 - 20	19 (45.2)	23 (54.8)	5.21	0.02
> 20	17 (23.0)	57 (77.0)		
Parents' educational level				
primary	15 (75.0)	5 (25.0)	21.83	0.00002
secondary	8 (22.2)	28 (77.8)		
graduate	13 (21.7)	47 (78.3)		
Previous history of hepatitis				
yes	9 (81.8)	2 (18.2)		
no	27 (25.7)	78 (74.3)	12.14	0.0005
Sex				
male	10 (31.2)	22 (68.8)	0.04	NS[3]
female	26 (30.9)	58 (69.1)		

Figures in brackets are percentages
[2] Number of minimum salaries (U$120 each) received per month
[3] NS, not significant p-values > 0.05

that is still quite contaminated may give rise to much more severe cases of clinical disease. In this context, the development of strategies for active imunization will become increasingly important in the future.

RESUMO

Soroprevalência da hepatite A em estudantes da área médica de uma Universidade Pública do Rio de Janeiro, Brasil

A prevalência de anticorpos para o vírus da hepatite A (anti-HAV) foi investigada em 116 estudantes da área médica de uma universidade pública do Rio de Janeiro. A prevalência total de anti-HAV foi de 31%, uma taxa significantemente menor do que a verificada em um estudo realizado nesta mesma população em 1986 (54.3%). O declínio na prevalência de anticorpos anti-HAV reflete uma diminuição da exposição a infecção pelo HAV, fato este intimamente associado a melhores padrões de vida.

Palavras-chave: Hepatite A, soroepidemiologia, anti-HAV

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Revista de Microbiologia (1998) 29:152-157
ISSN 0001-3714

AEROMONAS SPECIES ASSOCIATED WITH GASTROENTERITIS IN CHILDREN: PREVALENCE, CHARACTERISTICS AND VIRULENCE PROPERTIES

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Submitted: October 10, 1997; Returned to authors for corrections: February 12, 1998; Approved: March 26, 1998

ABSTRACT

Between January and December 1994, *Aeromonas* species were isolated from 19 (10.91%) of 174 children (1 month to 5 years old) with acute gastroenteritis and from 5 (2.42%) of 207 children without intestinal disturbances (controls), $p \leq 0.01$, in General Hospital in the city of Rio de Janeiro, Brazil. Five (26.31%) coinfections with other common enteropathogens were found among the *Aeromonas* positive children with gastroenteritis. Of the 19 aeromonads isolated, 63.15% were *A. caviae*; 23.31% *A. hydrophila*; 5.26% *A. veronii* biotype *sobria* and 5.26% *Aeromonas* spp.. Four *A. caviae* and one *Aeromonas* spp. were detected in the control group. All the aeromonad isolates were resistant to ampicillin and cephalotin and susceptible to aminoglycosides, second and third-generation cephalosporins (except cefoxitin), chloramphenicol, imipenem and pefloxacin. Examination of the virulence factor production by these species showed that *A. hydrophila* and *A. veronii* biotype *sobria* presented positive results for all assays tested, except for enterotoxin. Neither *A. caviae* or *Aeromonas* spp. produced enterotoxin, cytotoxin and elastase. All strains produced β-hemolysin on washed or unwashed sheep or washed rabbit erythrocytes on blood agar after 48 h of incubation. The results suggest the role of *Aeromonas* as gastrointestinal pathogens in our geographical region.

Key words: *Aeromonas* spp., gastroenteritis, virulence factors.

INTRODUCTION

Gastroenteritis is still a frequent infection among infants, being one of the main causes of morbidity and starvation all over the world. It is the most important cause of child mortality in the developing countries, especially of children under the age of 5. A wide range of microbial pathogens is capable of infecting the gastrointestinal tract and causing disease (34), and, in recent years, the members of the genus *Aeromonas* have been recognized as human pathogens, causing diarrheal disease, especially in small children (18). These organisms have been reported all over the world and the differences in frequency of *Aeromonas* isolation may be related to geographic location, season of collection and temperature or culture media used for isolation (3). The highest incidence of *Aeromonas* diarrhea is frequently associated with contaminated food and water (21). *Aeromonas* gastroenteritis appears to cause a secretory (watery) diarrhea, typical of many other enteric pathogens, and in its worst form resembles shigellosis (3). Clinical material recovered from a variety of extraintestinal diseases also have been *Aeromonas* positive (18). Possible virulence factors produced by *Aeromonas* species include

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mainly cytotoxins, hemolysins and enterotoxins. However, their virulence mechanisms still remain unknown (8,18).

In the present study we describe the occurrence of the genus *Aeromonas* among children under the age of 5, with acute gastroenteric symptons (cases) and asymptomatic patients (controls) from a General Hospital in the city of Rio de Janeiro, Brazil, from January to December 1994. The incidence of *Aeromonas* in feces, the frequency of coinfection with other enteropathogens, the sensitivity to several antibiotics and the virulence factors produced by aeromonads were determined.

MATERIALS AND METHODS

Patient and control population. Between January and December 1994, stool specimens were collected from children, aged 0-5 years old, at General Hospital in the city of Rio de Janeiro, Brazil: 174 specimens were isolated from patients with acute gastroenteritis and 207 from children without intestinal symptoms. Specimens were transported to the laboratory in stool vials and processed within 2 h after collection.

Microbiological methods. Gastrointestinal fecal specimens were cultured for *Salmonella, Shigella,* enteropathogenic *Escherichia coli* (EPEC), *Yersinia, Plesiomonas shigelloides*, *Campylobacter* and Rotavirus by routine microbiological procedures (24). For isolation of *Aeromonas* spp., all fecal samples were inoculated into alkaline peptone water (pH 8.6) and incubated overnight at 37°C. A loopful was subcultured onto ampicillin (10μg/ml)-sheep blood agar (ABA) and onto a selective medium for *Pseudomonas-Aeromonas* agar (GSP-Merck) without antibiotic incubated at 37°C for 24-48 h. Suspect colonies of *Aeromonas* spp. in ABA and GSP were submitted to oxidase test, glucose fermentation, O/129 vibriostatic agent resistance, growth in nutrient broth without NaCl or with NaCl 6% and identified to the species level according to the criteria of Abbott *et al* (1) and Janda *et al* (18). Results were read during 5 days, daily, at 37°C. Antimicrobial susceptibility testing was performed by using agar-disk diffusion method (4).

Extracellular enzime activity. For protease production, bacterial suspensions were cultured, at 37°C for 48-72 h in spots, on Tryptic Soy Broth-TSB (23), (Difco) or Mueller-Hinton (Difco) (E.A. Marques, unpublished data) containing 3% skim milk (Difco) and 2% agar. Elastase, lipase and lecithinase production were determined according to Thorpe and Miller (36) and Janda and Bottone (16). Results were read during 3 days, daily, at 37°C.

Hemolysin assay. Hemolysin production was assayed by three methods. Initially, aeromonads were streaked onto blood agar base (BBL) plates containing 5% rabbit or sheep erythocytes washed three times in phosphate-buffer saline, pH 7.2 or not. Plates were incubated for 72 h at 37°C, and inspected daily. In addition, strains were cultured in TSB for 24h at 37°C and cell-free supernatants were filter sterilized. Hemolytic activity against 1% rabbit and sheep erythrocyte suspensions was tested in microtiter wells (9). A hemolysin titer of > 1: 4 causing lysis of more than 50% of erythocytes at 37°C for 1 h and then at 4°C for 1 h was considered positive. Finally, strains were studied for cell-associated hemolytic assays as described by Janda and Abbott (19) using soft agar suspension (0.65% agar) containing 1% washed sheep erythrocytes.

Cytotoxin and enterotoxin activity. The cytotoxic activity was measured on Vero cells, following the method described by Namdari and Bottone (25) and Neves *et al* (26) after incubation periods of 2, 4, 24 and 48 h. Enterotoxin production using the suckling-mouse test was assayed by Burke *et al* (5) method modified by Neves and Nunes (28).

RESULTS

In the 12 month period (January to December, 1994), stools from 174 children with diarrhea (cases) and 207 without intestinal symptoms (controls), admitted to the hospital, were cultured for *Aeromonas* species and enteric pathogens. *Aeromonas* species were isolated from 19/174 (10.91%) diarrhea samples and from 5 / 207 (2.42%) control children, $p \leq 0.01$ (Table 1). The prevalence of enteropathogens in children with diarrhea is in Table 2. Recognized etiologic agents were found in 39.09% of the children. Fifty-eight (33.33%) specimens yielded only one pathogen and 10 (5.74%) contained one or more (coinfection) pathogens. *Aeromonas* species were associated with enteropathogenic *Escherichia coli* (EPEC), *Salmonella, Shigella* and intestinal parasites in five stools. *Aeromonas* spp. and EPEC were the most frequently identified pathogens, followed by *Shigella*, Rotavirus, *Salmonella* and others. The highest prevalence of *Aeromonas* isolated from diarrheic children was observed during summer (January to March) and autumn (April to July).

Of the 174 patients, 3 were asymptomatic at the moment of the medical check-up, but 1-2 weeks

Table 1. Frequency of *Aeromonas* strains isolated from fecal specimens from children with gastroenteritis (cases) and control children.

	Stools positive for *Aeromonas* No. (%)	Species No. (%)	
CASES (n = 174)	19 (10.91)	*A. caviae*	12 (63.15)
		A. hydrophila	5 (26.31)
		A. veronii biotype *sobria*	1 (5.26)
		Aeromonas spp.	1 (5.26)
CONTROLS (n = 207)	5 (2.42)	*A. caviae*	4 (80)
		Aeromonas spp.	1 (20)

Table 2. Enteropathogens detected in stools of 174 children with gastroenteritis.

Enteropathogen (s)	No. (%)
Aeromonas spp.	19 (10.91)
Aeromonas (sole pathogen)	14 (8.04)
Aeromonas spp. + other	5 (2.87)
A. caviae + Enteropathogenic *E. coli*	1 (0.57)
A. hydrophila + *S. flexneri*	1 (0.57)
A. hydrophila + *Salmonella spp.*	1 (0.57)
A. hydrophila + *S. dysenteriae* + *A. lumbricoides*	1 (0.57)
A. caviae + *G. lambia* + *S. stercoralis*	1 (0.57)
Enteropathogenic *E. coli*	19 (10.91)
Enteropathogenic *E. coli* (sole pathogen)	14 (8.04)
Enteropathogenic *E. coli* + other	5 (2.87)
Rotavirus	8 (4.60)
Shigella spp.	8 (4.60)
Salmonella spp.	4 (2.30)
Giardia lamblia	4 (2.30)
Ascaris lumbricoides	3 (1.72)
Trichuris trichiurus	1 (0.57)
Entamoeba histolytica	1 (0.57)
Campylobacter jejuni	1 (0.57)
TOTAL	
Only one enteropathogen	58 (33.33)
Coinfections	10 (5.74)
Negative cases	106 (60.91)

before these children have been diarrheic, and the ethiological agents isolated were members of the family of *Enterobacteriaceae* or intestinal parasites.

The age distribution of 19 positive cases of aeromonads ranged from 5 to 60 months. Seventeen (89.47%) were aged ≤ 2 years. The sex distribution showed a male: female ratio of 1.4:1. Watery or loose diarrhea (100%) was the most frequent symptom among aeromonads infections. Ten (62.5%) patients presented bloody stools and six (37.5%) faeces with mucous; fever occurred in ten (62.5%), vomiting in seven (43.75%) and nausea in two (12.5%) of the patients, signs of dehydration were observed in 5 patients. The diarrhea frequency ranged from 3-10 evacuations per day, and the duration of illness increased when another enteropathogen was concomitant with *Aeromonas* infection (Table 3). Duration of *A. hydrophila* infection as sole pathogen was longer than with *A. caviae* and *A. veronii* biotype *sobria* (data not shown).

Of the 19 aeromonads isolated from diarrheal group, 63.15% were *Aeromonas caviae*; 26.31% *A. hydrophila*; 5.26% *A. veronii* biotype *sobria* and 5.26% *Aeromonas* spp.. Four (80%) *A. caviae* and 01 (20%) *Aeromonas* spp. were decteted in the control group (Table 1). All the aeromonads isolated were phenotypically compatible with ATCC standard strains, except for two strains which could not be classified to the species level, because of the presence of motility with production of brown water-soluble pigment or 0/129 vibriostatic agent sensitivity.

All the aeromonads isolated were resistant to ampicillin and cephalotin; and 24 *Aeromonas* strains, 41.6%, 45.8% and 58.3% were resistant to tetracycline, sulfonamide and cefoxitin, respectively (Table 4). All microorganisms tested were susceptible to gentamycin, amikacin, netilmycin, chloramphenicol, imipenen, pefloxacin, cefotaxime, cefuroxime, ceftazidime and ceftriaxone.

All cell-free culture supernatants of *A. hydrophila* and *A.veronii* biotype *sobria* had hemolysin titers ranging from 16 to 512 in both erythrocyte suspensions. No significant difference was observed when hemolysin assay was performed using rabbit or sheep erythrocytes. *A. caviae* and *Aeromonas* spp. were negative for this assay. Fourteen of 16 (87.5%) *A. caviae* and 2 (100%) *Aeromonas* spp. were β-haemolytic when soft agar suspensions with 1% washed sheep erythrocytes were used. β-haemolysin on rabbit or sheep blood (washed or not) agar plates was observed after 24 h of incubation for all *A.hydrophila*, *A. veronii* biotype *sobria* and *Aeromonas* spp. None *A. caviae* strain was β-haemolytic on sheep blood unwashed agar after 24h of incubation, 5 (31.25%) strains were haemolytic after 48 h and 12 (75.0%) after 72 h of inoculation at 37°C. β-haemolysin was observed in 1 (6.25%), 14 (87.5%) and 16 (100%) *A. caviae*, respectively to 24, 48 and 72 h of incubation on sheep blood washed agar. All *A. caviae* were β-haemolytic after 24 h on

Table 3. Characteristic features of children with gastroenteritis.

Patient characteristics	No. of patients* with		
	Aeromonas only (n = 12)	Coinfection (n = 4)	Total (%)
Feces			
Watery or loose	12	4	16 (100)
Blood	7	3	10 (62.5)
Mucous	3	3	6 (37.5)
Fever	6	4	10 (62.5)
Vomiting	4	3	7 (43.75)
Nausea	1	1	2 (12.5)
Frequency of diarrhea (per day)	3-10	ND	–
Duration of illness	2-10 days	3-15 days	–

* 03 positive cases were asymptomatic
ND - Not determined

Table 4. Antibiotic susceptibility of twenty four isolated *Aeromonas* strains.

Antibiotics	Number (%) of strains	
	Susceptible*	Resistant
Ampicillin	0	24 (100)
Cephalotin	0	24 (100)
Tetracycline	14 (58.3)	10 (41.6)
Sulfonamide	13 (54.1)	11 (45.8)
Cefoxitin	10 (41.6)	14 (58.3)

* All *Aeromonas* were susceptible to gentamicin, amikacin, netilmicin, chloramphenicol, imipenem, pefloxacin, cefotaxime, cefuroxime, ceftazidime and ceftriaxone.

rabbit blood, washed or not, agar plates. All strains produced lecithinase after 48 h of incubation at 37°C. Only 1 *A. caviae* failed to produce lipase after 5 days. Positive elastase activity was observed exclusively with the *A. hydrophila* and *A. veronii* biotype *sobria* strains. All strains produced protease only when Mueller-Hinton with 3% skim milk was used (data not shown).

Concerning the cytotoxic effects on *Vero* cell monolayers, alteration and intense damage to the monolayers were observed, after 2h of incubation at 37°C, with total detachment of cells from the well-bottom when inoculated with *A. hydrophila* and *A. veronii* biotype sobria. However, no cytotoxic effect was observed when *A. caviae* or *Aeromonas spp.* was inoculated, even after 48 h. Finally, five out of six *A. hydrophila* isolates presented positive results for the suckling mouse test for enterotoxin production. All 19 *A. caviae* isolates and the *A. veronii* biovar. *sobria* strain were not enterotoxigenic, considering the IW/BW ratio ≥ 0.08 as the minimum for enterotoxigenicity (data not shown).

DISCUSSION

In this study *Aeromonas* specimens were more frequently recovered from children with gastrointestinal symptoms (10.91%) than from control children (2.42%), showing significant isolation rates ($p \leq 0.01$) to this pathogen in our geographical area (Table 1). Isolation rates of *Aeromonas* spp., from human feces in different parts of the world are variable. In the USA, Agger *et al* (2) obtained a low percentage: 1.1% in diarrheic patients and no isolation in the controls, although Namdari and Bottone (25) reported 15.31% in children with diarrhea. In Italy (11) no significant difference was observed between symptomatic (3.7%) and asymptomatic (2.1%) children. In Australia (6), 10.8% and 0.7% isolation rates, respectively, of diarrheic and control feces, were found. In Peru a high positive percentage of *Aeromonas* infection (54.4%) in comparison with control cases (8.7%) was reported (29). These contrasts might be a result of different seasonal, water, food and sanitary conditions and may also be influenced by the culture methods or temperature of incubation that were used (10,21).

The most common species of *Aeromonas* isolated in our survey was *A. caviae:* 63.15% from diarrheic stools and 80% from control stools. In Brazil, *A. caviae* is the predominant species isolated from patients (12,14), environment (27) and food (13,31) and in reports conduted in the USA (15,25), Europe (3) and Peru (29). These results suggest some reflection on its ubiquitous presence. Our data support *A. caviae* as a pediatric enteric pathogen, similar to EPEC, a recognized enteropathogen to children (22) (Table 2). *A. hydrophila* (26.31%) and *A veronii* biotype *sobria* (5.26%) were the next group of aeromonads detected. Two *Aeromonas* spp. were phenotypically similar to *A. caviae* (ATCC 15468, HG 4), except for brown-water-soluble pigment and sensibility to agent 0/129. This result suggests the need of molecular methods to classify and identify these atypical *Aeromonas*.

Our isolation rates were higher in children under 2 years (89.47%) in correlation with several investigators research (18) (Table 3). *Aeromonas* was isolated as the sole pathogen in 8.04% and coinfection with other pathogens in 2.87%. Actually, different frequencies of aeromonad isolations and coinfections have been described in some regions of the world, but no significant correlation was reported

(29,35). In the current study, summer and early autumn months also presented a peak for isolation of species of *Aeromonas* (10,17,29).

The typical clinical manifestations of *Aeromonas*-positive patients (sole enteropathogens) were watery or loose diarrhea 12 (100%), blood 7 (58.33%) and fever 6 (50%). All cases of *Aeromonas* associated diarrhea were acute, 10 days being the maximum duration of illness.

One symptomatic child had diarrhea associated with *Salmonella* spp. and had been treated with ampicillin for eight days. Because of continuing symptoms, a second coproculture was made and *A. caviae* grew as a pure culture. After a course of ceftriaxon for 10 days, the patient had no symptoms and the stools were culture negative. These data suggest that *A. caviae* could be an important pathogen in children or indeed could aggravate diarrhea cases after ampicillin therapy.

Our findings of susceptibility of isolates of *Aeromonas* spp. to antimicrobials support previous data reported on *Aeromonas* strains isolated from clinical sources (M.S. Guimarães *et al.*, unpublished data, 12, 32).

Aeromonas strains may produce many different virulence factors, although their production is not necessarily associated with human disease. Hemolysin production by all *Aeromonas* strains was investigated. The best assay to detect hemolysin in *A. caviae* strains was the one based on washed blood agar base for 72 h with daily inspection. Protease activity was closely related to hemolysin, as shown by Cahill (8). Finally, elastase production occurred only in *A. hydrophila* and *A.veronii* biotype *sobria*.

Only 7 (26.92%) of all *Aeromonas* strains isolated were cytotoxic, belonging to *A. hydrophila* and *A.veronii* biotype *sobria* phenospecies. Our findings correlate well to many studies with these species, isolated from several sources and tested on different types of cultured cells (8,18). More significant is the lack of cytotoxicin production by *A. caviae*. Namdari and Bottone (25) observed proven *A. caviae* cytotoxicity, isolated from diarrheic sources. Their method included a glucose-free, double strength-TSB; we also included a glucose-free TSB, but not double-strength. This could explain our negative results for *A. caviae* isolates, although Neves (personal communication) observed positivity with this species when using a conventional TSB.

Enterotoxin production using the suckling-mouse test described by Burke *et al* (5) modified by Neves and Nunes (28) was observed. Among the aeromonads tested, only 5 of the *A. hydrophila* isolates were enterotoxigenic. There was no enterotoxin production by any other species tested, although some authors found it among *A. veronii* biotype *sobria* (3) and *A.caviae* strains (7,25,28). However, considerable differences in culture media, incubation time, shaker speed and final results observation time are reported in these studies (20,30,33). These considerations suggest the need of a better standardization of enterotoxin testing.

In conclusion, our results clearly suggest that *Aeromonas* species are potential enteric pathogens in pediatric patients in the city of Rio de Janeiro, Brazil and the production of virulence factor by these species play an important role in gastroenteritis.

ACKNOWLEDGMENTS

This study was supported by grants from Universidade do Estado do Rio de Janeiro (UERJ, SR-2) and from Conselho Nacional de Pesquisa (CNPq). We thank Dr. M.S. Neves ("in memoriam") and M.P. Nunes by scientific assistance.

RESUMO

Espécies de *Aeromonas* associadas com gastroenterites em crianças: prevalência, características e fatores de virulência.

De Janeiro a Dezembro de 1994 foram isoladas 19 (10,91%) amostras de *Aeromonas* de 174 crianças de até 5 anos de idade, apresentando gastroenterite aguda (casos) e 5 amostras (2,42%) de 207 crianças sem sintomas intestinais (controles), $p \leq 0,01$, atendidas no Hospital Universitário Pedro Ernesto - UERJ, Rio de Janeiro. Cinco (26,21%) casos de coinfecção com outros enteropatógenos foram detectados. Das amostras de *Aeromonas*, *A. caviae* foi isolada em 63,15% dos casos, seguida por *A. hydrophila* 23,31%, *A. veronii* biotype *sobria* 5,26% e *Aeromonas* spp 5,26%. Quatro *A. caviae* e 01 *Aeromonas* spp. foram detectadas no grupo controle. Todas as amostras foram resistentes a ampicilina e cefalotina e sensíveis a aminoglicosídeos, cefalosporina de 2ª. e 3ª. geração (exceto para cefoxitina), cloranfenicol, imipenen e pefloxacin. *A. hydrophila e A. veronii* biotype *sobria* foram produtoras da maioria dos fatores de virulência estudados. Enterotoxina, citotoxina e produção de elastase não foram detectadas entre *A. caviae* e *Aeromonas* spp. Todas as amostras foram β-

hemolíticas em ágar sangue de coelho, lavado ou não, e sangue lavado de carneiro após 48 horas de incubação. Nossos resultados sugerem o importante papel das *Aeromonas* como patógeno gastrointestinal na nossa região.

Palavras-chave: *Aeromonas* spp., gastroenterite, fatores de virulência.

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ERRATUM

VOL. 29(01), 98

Pg. 24-26

Correlation between pellet size and glucoamylase production in submerged cultures of *Aspergillus awamori*

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

Parameter	Experimental run							
C_{spore} (spores/ml)	9.5×10^3		8.8×10^4	9.5×10^4		9.5×10^5		
	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8
X (g/l)	1.20	1.60	2.31	3.22	2.91	4.48	5.09	5.00
pH	2.87	2.80	2.70	2.65	2.50	2.05	2.20	2.00
TRS (g/l)	15.8	16.0	16.2	12.4	14.2	11.0	8.6	9.4
A (U/l)	66	76	182	173	186	442	326	490
D (mm)	1.60	1.60	0.95	0.96	1.14	0.74	0.80	0.59

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

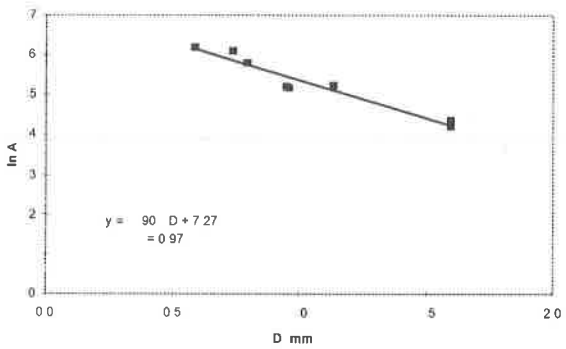

Figure 2. Fit of equation (II) to the experimental data, obtained from the 24-hour shaker cultivations of *Aspergillus awamori.*

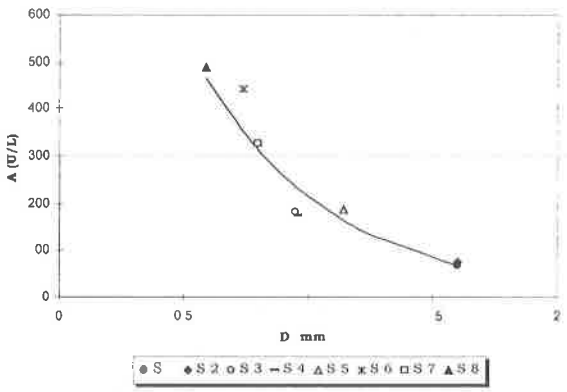

Figure 3. Relationship between glucoamylase activity and pellet size, after 24 hours of *Aspergillus awamori* cultivation in shaker.

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