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RECENT ADVANCES IN THE STUDY OF BIOCORROSION - AN OVERVIEW

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REVIEW

ABSTRACT

Biocorrosion processes at metal surfaces are associated with microorganisms, or the products of their metabolic activities including enzymes, exopolymers, organic and inorganic acids, as well as volatile compounds such as ammonia or hydrogen sulfide. These can affect cathodic and/or anodic reactions, thus altering electrochemistry at the biofilm/metal interface. Various mechanisms of biocorrosion, reflecting the variety of physiological activities carried out by different types of microorganisms, are identified and recent insights into these mechanisms reviewed. Many modern investigations have centered on the microbially-influenced corrosion of ferrous and copper alloys and particular microorganisms of interest have been the sulfate-reducing bacteria and metal (especially manganese)-depositing bacteria. The importance of microbial consortia and the role of extracellular polymeric substances in biocorrosion are emphasized. The contribution to the study of biocorrosion of modern analytical techniques, such as atomic force microscopy, Auger electron, X-ray photoelectron and Mössbauer spectroscopy, attenuated total reflectance Fourier transform infrared spectroscopy and microsensors, is discussed.

Key words: copper, corrosion, steel, sulfate-reducing bacteria, surface spectroscopy

INTRODUCTION

In natural and man-made environments corrosion occurs when materials made of pure metals and/or their mixtures (alloys) undergo a chemical change from the ground state to an ionized species. Corrosion is an electrochemical process consisting of an anodic reaction involving the ionization (oxidation) of the metal (the corrosion reaction), and a cathodic reaction based on the reduction of a chemical species. Many textbooks cover basic corrosion concepts and may be consulted for further details (16, 99). These reactions can be influenced by microbial activities, especially when the organisms are in close contact with the metal surface forming a biofilm (Fig. 1). The resulting metal deterioration is known as biocorrosion, or microbially-influenced corrosion (MIC).

Biofilms consist of microbial cells, their extracellular polymeric substances (EPS), which

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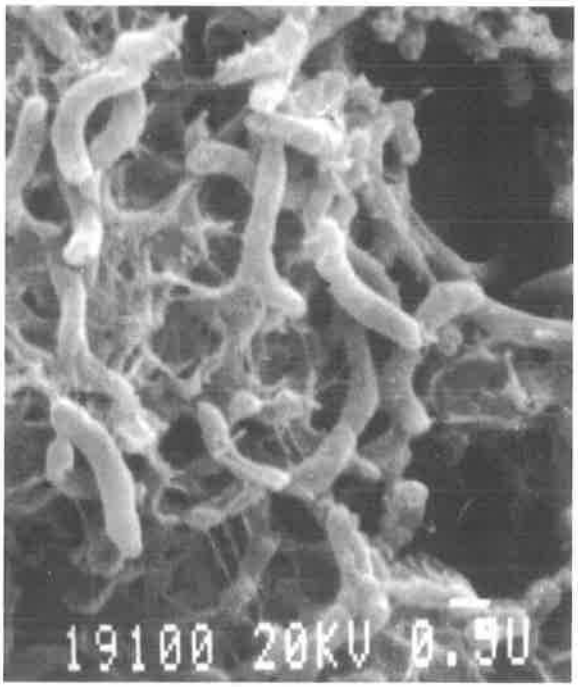

Figure 1 – Biofilm formed by sulfate-reducing bacteria on the surface of mild steel, visualized using SEM.

facilitate irreversible attachment of cells to the surface, inorganic precipitates derived from the bulk aqueous phase and/or corrosion products of the metal substratum. EPS consist of a complex mixture of cell-derived polysaccharides, proteins, lipids and nucleic acids. Microorganisms, and/or products of their metabolic activities, *e.g.* enzymes, exopolymers, organic and inorganic acids, as well as volatile compounds such as ammonia or hydrogen sulfide, can affect cathodic and/or anodic reactions at metal surfaces, thus altering electrochemical processes at the biofilm/metal interface. However, the number of attached microorganisms does not necessarily correlate with the extent of corrosion (6), a fact that has long been known for suspended cells (41). It is the metabolic status of the cells that is believed to be the relevant parameter, but to date no clear consensus has been reached linking specific bacterial metabolic rates to observed corrosion rates.

Economic losses caused by biocorrosion

There are no official figures for the cost of MIC, but some indication of its importance can be gained from individual companies or sectors of industry. Escom, the national power utility of South Africa that provides 90% of power requirements for the country, has detected MIC of carbon steel in cooling water systems in virtually all their power plants. The costs associated with repairs and down time are millions of dollars annually (14). Under-deposit pitting corrosion of heat exchanger tubing in nuclear power generating plants operated by Ontario Hydro of Canada has been estimated to cost the corporation $ 300,000 per unit per day in replacement energy costs (18). Corrosion problems have cost the nuclear utility billions of dollars in replacement costs alone (Jones, 1996). Losses in the oil and gas industry are also substantial; Jack *et al.* (50) estimated that 34% of the corrosion damage experienced by one oil company was related to microorganisms. In the 1950s, MIC-related costs of repair and replacement of piping material used in different types of service in the USA were estimated to be around $ 0.5-2 Billion per annum. Booth (15), in the UK, suggested that 50% of corrosion failures in pipelines involved MIC, while Flemming (40) proposed that approximately 20% of all corrosion damage to metallic materials is microbially influenced. Replacement costs for biocorroded gas mains in the UK were recently reported to be £250 Million per annum. Often, financial losses due to damage of equipment by biocorrosion are combined with those resulting from biofouling. While the two phenomena may be associated, they do not cause the same type of damage. The costs associated with MIC usually include the costs of prevention of both MIC and biofouling; since these are based on a limited understanding of the phenomena, they could be underestimated.

Mechanisms of biocorrosion

MIC does not invoke any new electrochemical mechanisms of corrosion; rather, it is the result of a microbiologically-influenced change that promotes the establishment or maintenance of physico-chemical reactions not normally favoured under otherwise similar conditions. Various mechanisms of biocorrosion, which reflect the variety of physiological activities carried out by different types of microorganisms, have been identified; however, it must be remembered that, in nature, these microbial processes do not act in isolation, but in concert with the chemical and electrochemical forces in the particular environment.

Activities of microorganisms as the driving force for biocorrosion

Microorganisms implicated in biocorrosion of metals such as iron, copper and aluminium and their alloys are physiologically diverse. Their ability to influence the corrosion of many metals normally considered corrosion resistant, in a variety of environments, makes microorganisms a real threat to the stability of those metals.

The main types of bacteria associated with corrosion failures of cast iron, mild and stainless steel structures are sulfate-reducing bacteria (45), sulfur-oxidising bacteria (25), iron-oxidising/reducing bacteria (86 and references therein), manganese-oxidizing bacteria (30), and bacteria secreting organic acids and exopolymers or slime (25, 116). These organisms can coexist in naturally occurring biofilms, often forming synergistic communities (consortia) that are able to affect electrochemical processes through co-operative metabolism not seen in the individual species (34). Much recent research activity has centered on the role of "quorum sensing" molecules, such as acylhomoserine lactones, in control of microbial activities in biofilms (29, 83), with the aim of using this knowledge to reduce problematical biofilm formation in industry (115).

Sulfate-Reducing Bacteria (SRB)

SRB are a group of diverse anaerobes which carry out dissimilatory reduction of sulfur compounds such as sulfate, sulfite, thiosulfate and even sulfur itself to sulfide (4, 74). Although SRB are often considered to be strictly anaerobic, some genera tolerate oxygen (1, 48) and at low dissolved oxygen concentrations certain SRB are able to respire with Fe^{3+} or even oxygen with hydrogen acting as electron donor (32, 94). Excellent reviews on the ecology and physiology of SRB are available in the literature (93, 117, Barton, 1995).

Oil, gas and shipping industries are seriously affected by the sulfides generated by SRB (46 and references therein). Biogenic sulfide production leads to health and safety problems, environmental hazards and severe economic losses due to reservoir souring (increased sulfur content) and the corrosion of equipment. Since the beginning of investigations into the effects of SRB on corrosion of cast iron in 1930s, the role of these bacteria in the pitting corrosion of various metals and their alloys in both aquatic and terrestrial environments, under anoxic as well as oxygenated conditions, has been confirmed. Several models have been proposed to explain the mechanisms by which SRB can influence the corrosion of steel (Table 1) and it is clear that sulfate reducing activity is in some way involved. The product of this activity, sulfide, is corrosive; however, chemically-derived sulfide does not have the same degree of aggressivity (73, 79, 105), demonstrating the importance of bioprocesses and the irrelevance of experiments using abiotic, as opposed to biologically derived compounds. Videla *et al.* (107) used energy dispersion X-ray analysis, X-ray photoelectron spectroscopy, X-ray diffraction, electron microprobe analysis, scanning electron microscopy and atomic force microscopy to demonstrate that the composition and structure of the sulfide films formed on carbon steel in the presence of the SRB, *Desulfovibrio alaskensis*, (biotic sulfides) were different from those formed in sterile, sulfide-containing medium (abiotic sulfides). Recent reviews clearly state that one predominant mechanism may not exist in SRB-influenced corrosion and that a number of factors are involved (47, 60).

Table 1 – Suggested mechanisms of metal corrosion by SRB.

Corrosive process/substance	Reference(s)
Cathodic depolarization* by hydrogenase	von Wolzogen Kühr and van der Vlugt, 1934; Bryant *et al.*, 1991.
Anodic depolarization*	Salvarezza and Videla, 1984; Daumas *et al.*, 1988; Crolet, 1992.
Sulfide	Little et al., 1998.
Iron sulfides	King and Wakerley, 1973.
A volatile phosphorus compound	Iverson and Ohlson, 1983.
Fe-binding exopolymers	Beech and Cheung, 1995; Beech *et al.*, 1996, 1998, 1999.
Sulfide-induced stress corrosion cracking	Edyvean *et al.*, 1998.
Hydrogen-induced cracking or blistering	Edyvean *et al.*, 1998.

* depolarization is an acceleration of the corrosion reaction and may involve removal of cathodic or anodic reactants.

Considerable work has centered on the influence of ferrous ions on SRB action on steel alloys. Obuekwe *et al.* (86) reported extensive pitting of mild steel when ferrous and sulfide ions were being formed concurrently. When only sulfide was produced, corrosion rates first increased and then declined due to the formation of a protective FeS film. High levels of soluble iron prevented the formation of such protective layers. Moulin *et al.* (84) demonstrated that high soluble iron levels could lead to high corrosion rates of piling grade carbon steel and Gubner *et al.* (44) showed that this was linked to a decrease in pH. The hydrogenase of *Desulfovibrio vulgaris* (Hildenborough) has been shown to be regulated by Fe^{2+} availability (20), offering yet another mechanism whereby corrosion may be affected, as assessed by Cheung and Beech (23). Thus the influence of iron ions on SRB-influenced corrosion is a complex phenomenon; this was reviewed by Videla *et al.* (108).

The impact of sulfides on the corrosion of copper alloys has recently received considerable attention. Copper alloys are attacked after only one day in seawater containing 0.01 ppm sulfide. In the presence of sulfide ions, an interstitial cuprous sulfide compound, with the general stoichiometry $Cu_{2-x}S$ ($0 < x < 1$), is formed; copper ions migrate through this layer and react with more sulfide. The result can be the production of thick scale (71).

Specific removal of nickel from 90-10 and 70-30 Cu-Ni has been reported in seawater containing SRB (64, 112). Spalling of the nickel-enriched region of the metal occurs during exposure to flowing seawater, exposing fresh metal and causing further dissolution of the alloy. Welds also exhibit this type of corrosion in the presence of SRB (63).

SRB can induce corrosion of zinc and lead based alloys. The corrosion product on zinc is reported to be sphalerite (ZnS), while the action of SRB on lead carbonates produces galena (PbS), also found as a corrosion product on lead-tin alloys (71).

Metal-Reducing Bacteria (MRB)

Microorganisms are known to promote corrosion of iron and its alloys through reactions leading to the dissolution of corrosion-resistant oxide films on the metal surface. This results in the protective passive layers on *e.g.* stainless steel surfaces being lost or replaced by less stable reduced metal films that allow further corrosion to occur. Despite its widespread occurrence in nature and likely importance to industrial corrosion, bacterial metal reduction has not been seriously considered in corrosion reactions until recently.

Numerous types of bacteria, including those from the genera *Pseudomonas* (86) and *Shewanella* (85) are able to carry out manganese and/or iron oxide reduction and have been shown to influence corrosion reactions. It has been demonstrated that in cultures of *Shewanella putrefaciens,* iron oxide-surface contact was required for bacterial cells to mediate reduction of these metals (85). The rate of reaction depended on the type of oxide film under attack (69).

Metal-Depositing Bacteria (MDB)

Bacteria of the genera *Siderocapsa*, *Gallionella*, *Leptothrix*, *Sphaerotilus*, *Crenothrix* and *Clonothrix* participate in the biotransformation of oxides of metals such as iron and manganese (43). Iron-depositing bacteria (*e.g.*, *Gallionella* and *Leptothrix*) oxidize Fe^{2+}, either dissolved in the bulk medium or precipitated on a surface, to Fe^{3+}. Bacteria of the genera given above are also capable of oxidizing manganous ions to manganic ions with concomitant deposition of manganese dioxide (70).

A role in the corrosion of steels has been proposed for sheathed filamentous bacteria detected by microscopy in naturally formed corrosion deposits (57, 75, 104). These bacteria have been typically associated with formation of tubercles (macroscopic deposits containing microorganisms, inorganic and organic materials) and consequent under-deposit pitting attack on stainless steel. The corrosion resistance of alloys such as stainless steels is due to the formation of a thin passive oxide film. The formation of organic and inorganic deposits by MDB on the oxide surface compromises the stability of this film. Dense accumulations of MDB on the metal surface may thus promote corrosion reactions by the deposition of cathodically-reactive ferric and manganic oxides and the local consumption of oxygen by bacterial respiration in the deposit. However, care must be taken in considering microorganisms in corrosion products to be the causal agent. Some bacteria are known to adhere preferentially to corrosion products and thus will be present in high numbers even when playing no role in the primary corrosion process (72).

MDB have been shown to promote ennoblement of metals (a change to more positive values of pitting

potential) and pitting corrosion. It has been demonstrated that the formation of a surface biofilm containing the sheath-forming, manganese-depositing bacterium, *Leptothrix discophora,* resulted in the ennoblement of 316L stainless steel (31). The biofilm was proposed to be necessary for deposition and electrical contact of cathodically-active MnO_x at the metal surface so that electron transfer from the metal to the MnO_x deposit could occur. The resulting ennoblement, observed under laboratory conditions, mimicked the pattern of ennoblement of stainless steels submerged in natural waters. However, the ennoblement produced in the laboratory study was not accompanied by the characteristic pitting corrosion of the metal, demonstrating the limitations of our current understanding of pit initiation and propagation in steels by MDB.

Slime-producing bacteria

Microorganisms that produce copious quantities of EPS during growth in biofilms have been implicated in localized attack of stainless steels (92). Slime-forming microorganisms that have been recovered from sites of corrosion on stainless steels include *Clostridium* spp., *Flavobacterium* spp., *Bacillus* spp., *Desulfovibrio* spp., *Desulfotomaculum* spp. and *Pseudomonas* spp.

As little as 10 ng cm^{-2} EPS has been reported to provoke the onset of MIC of stainless steel in natural seawater; cathodic protection of the stainless steel, used to prevent corrosion, actually increased the amount of EPS in the biofilm (97). However, the role of EPS in MIC of stainless steel remains obscure. It has been postulated that they are not sufficient to induce biocorrosion of stainless steel unless aided by the presence of a biocatalyst of oxygen reduction (98), which could be oxido-reductase enzymes entrapped in the biofilm (58). EPS has even been suggested to protect metal surfaces from corrosion. A bacterial consortium consisting of a thermophilic *Bacillus sp.* and *Deleya marina* produced metal-binding EPS that reduced the rate of corrosion of carbon steel by 94% (35). Such a mechanism may be responsible for the protection microorganisms afford to mild steel under certain conditions (102).

A case of corroded copper pipework in a drinking water system involved the presence of a film that stained positive with periodic acid- Schiff's reagent (PAS) and alcian blue, suggesting the presence of acidic polysaccharides (2). Scanning electron microscopy showed that copious amounts of biofilm were associated with the pitted sites (55), with the most severely corroded tubes containing the most well-developed biofilm (76). In another case, chemical analysis of the adherent copper corrosion products recovered from failed copper tube suggested an interaction between the inorganic products and biologically-derived organic molecules. Copper corrosion products were located on top of or within a microbial biofilm layer in direct contact with the bare metal surface in areas where the pipe was perforated (38, 39). The biofilm contained linear and/or cross-linked acidic or non-ionic polysaccharides, oligopeptides and N-acetylated derivatives of glucose, mannose and galactose. Corrosion products rich in copper complexes of pyruvate, acetate, and histidine were identified (89). Binding of $[Cu_2Cl_2]_n^{2-}$ ions in the biofilm suggested a mechanism whereby Cl^- sequestration into the pits could promote further ionization of metallic copper (38). Microbiological evaluation of the corrosion deposits showed that while high numbers of bacteria were associated with the pits, the presence of bacteria was not always related to pitting and that the range of cultured bacterial species was quite variable (110, 111).

A correlation has been reported between pitting of copper pipe associated with a black cupric oxide surface layer and the presence of certain bacteria (*Pseudomonas paucimobilis* and *Ps. solanacearum*) or their polysaccharide (2, 21). Davidson *et al.* (28) correlated the production of acidic metabolic products by a biofilm of the bacterium *Acidovorax delafieldii* on a copper surface to an increase in copper concentration in the bulk aqueous phase (i.e., corrosion). The amount of extractable, surface-associated copper was positively correlated with both protein and carbohydrate concentrations in the biofilm. Bremer and Geesey (17) showed a correlation between acidic polysaccharide accumulation in bacterial biofilms on copper films and initiation of copper film dissolution.

Little *et al.* (68) used scanning vibrating electrode microscopy, employing a 20 μm microprobe, to demonstrate the formation of localized anodic areas on copper coupons in the presence of the marine bacterium *Oceanospirillum* and its exopolymer. Fluorescence microscopy with the Live/Dead Backlight Viability Kit® showed that the anodic areas corresponded to those with higher bacterial densities,

but the sequence in which the surface changes occurred was not determined.

The relationship between pitting propensity and the properties of biofilm polymers has been investigated by Siedlarek *et al.* (100). Cyclic voltammetry showed that the artificial biofilms formed by the model polysaccharides, xanthan, alginate and agarose, displayed cation selectivity and exerted considerable influence on the corrosion reaction(s) of a copper surface in contact with an aqueous phase, particularly at the sites where solid corrosion products were precipitated (100, 113). A physicochemical model was developed to describe the pitting corrosion observed on copper piping of potable water systems. The model takes into account membrane properties and heterogeneity, and the distribution of exopolymers on the surface of the pipes (113).

Acid-Producing Bacteria (APB)

Bacteria can produce copious quantities of either inorganic or organic acids as by-products of metabolism. Acidophilic sulfur oxidizing bacteria (SOB), such as *Thiobacillus* spp., oxidize reduced forms of sulfur to sulfate. These microbes can cause severe corrosion damage to mining equipment. Organic acid-producing bacteria were suggested as the primary cause in a case of carbon steel corrosion in an electric power station; they were the only group of culturable microorganisms whose abundance was correlated positively with corrosion (103). Acetic, formic and lactic acids are common metabolic by-products of APB. Little *et al.* (62) showed that an aerobic, acetic acid-producing bacterium accelerated the corrosion of cathodically protected stainless steel. Protective calcium-rich deposits formed during cathodic polarization were destabilized or dissolved by artificially applied acetic acid. Little *et al.* (65) also provided examples of acids synthesized in the Krebs Cycle, common to most aerobic microorganisms, which can contribute to MIC; however, the intermediate metabolites of the Krebs cycle are generally retained within the microbial cells. A culture of *Streptococcus* released high amounts of copper from a Cu-Zn-Al-Ni odontological alloy (91), showing that lactic acid released by these bacteria can participate in corrosion reactions.

The mechanism of action of acids on corrosion of mild steel is well established in the metallurgical literature (99), but the acids produced and their concentrations are rarely monitored under MIC conditions. Acids produced by slime-producing microorganisms are concentrated at the metal surface; hence the bulk aqueous phase pH (most frequently measured by investigators) may be an entirely irrelevant parameter. Microsensors have been used to probe the pH gradients within 1mm thick microbial biofilms growing on corroded mild steel surfaces (59). pH values increased from 7.5 at the bulk fluid-biofilm interface to 9.5 at the metal surface in cathodic areas and ranged from 5 to 7 at the surface of the tubercle in anodic areas.

Slime-producing microorganisms that excrete acidic extracellular polysaccharides during biofilm formation on metal surfaces may influence corrosion. Carboxylic acid groups of matrix polysaccharides such as alginic acid, produced by the biofilm-forming bacterium *Pseudomonas aeruginosa,* have been calculated to be of the order of 6 Angstroms apart, and thus highly concentrated at the metal-biofilm interface (52). It is virtually impossible to concentrate dissolved low molecular weight acids to such a high level. These ionizable acidic groups may therefore be very important in corrosion when the pH of the biofilm is low.

Fungi

Fungi are well-known to produce organic acids, and are therefore capable of contributing to MIC. Much of the published work on biocorrosion of aluminum and its alloys has implicated fungal contaminants of jet fuel, *Hormoconis* (previously classified as *Cladosporium*) *resinae, Aspergillus spp., Penicillium spp.* and *Fusarium spp.* The fungus *H. resinae* utilizes the hydrocarbons of fuel to produce organic acids. Surfaces in contact with the aqueous phase of fuel-water mixtures and sediments are common sites of attack (95). The large quantities of organic acid by-products excreted by this fungus selectively dissolve or chelate the copper, zinc and iron at the grain boundaries of aircraft aluminum alloys, forming pits which persist under the anaerobic conditions established under the fungal mat. Growth of this and other fungi in diesel fuel storage tanks can produce large quantities of biomass (13) and this may provoke crevice attack on the metal (37). Grease-coated wire rope wound on wooden spools stored in a humid environment has been reported to be corroded by *Aspergillus niger* and *Penicillium spp.* Both fungal species are

known to produce citric acid (67), which may be involved in the attack.

Iron-reducing fungi have been isolated from tubercles in a water distribution system (36), suggesting another mechanism whereby corrosion may be accelerated by this group of microorganisms.

Microbial consortia

Microorganisms are almost never found in nature as pure species and, while laboratory studies on isolated pure cultures are essential to the understanding of MIC, the role of microbial consortia is becoming increasingly recognized.

The acids produced by APB serve as nutrients for SRB and methanogens and it has been suggested that SRB proliferate at sites of corrosion due to the activities of APB (103). Dowling *et al.* (33) compared corrosion of C1020 pipeline steel in the presence and absence of the acetogenic bacterium, *Eubacterium limosum*, and mixed SRB populations (*Desulfovibrio sp.* and *Desulfobacter spp.*). *E. limosum* alone had little effect on the corrosion rate compared to sterile controls, but when inoculated with the *Desulfovibrio sp.*, a significantly higher rate of corrosion was found. It was proposed that by-products of *E. limosum* supported *Desulfovibrio* sp. growth and sulfide production.

The interactions between microbial species are complex. Gaylarde and Johnston (42) showed that anaerobic corrosion of mild steel was enhanced in pure cultures of *Desulfovibrio vulgaris*, but reduced to below control levels by pure *Vibrio anguillarum*; in the presence of both species, corrosion rates were the highest of all. On the other hand, a second facultatively anaerobic bacterium, probably of the genus *Citrobacter*, had little effect on corrosion rates, except in triple cultures, where it apparently modified the action of the other species (Fig. 2). It was suggested that *V. anguillarum* produced a strongly-bound, protective film on the metal surface in pure cultures, but that this film incorporated SRB cells when *D. vulgaris* was present, turning it into a highly aggressive biofilm. The incorporation of the third organism into this biofilm would reduce the SRB population, thereby ameliorating its effects.

Consortia of MDB and SRB often exist as biofilms on corroding metal surfaces. It has been proposed that oxygen consumption by MDB creates redox conditions favorable for the growth of SRB (106) and the joint action of MDB and SRB may promote the breakdown of the passive film on stainless steel (61).

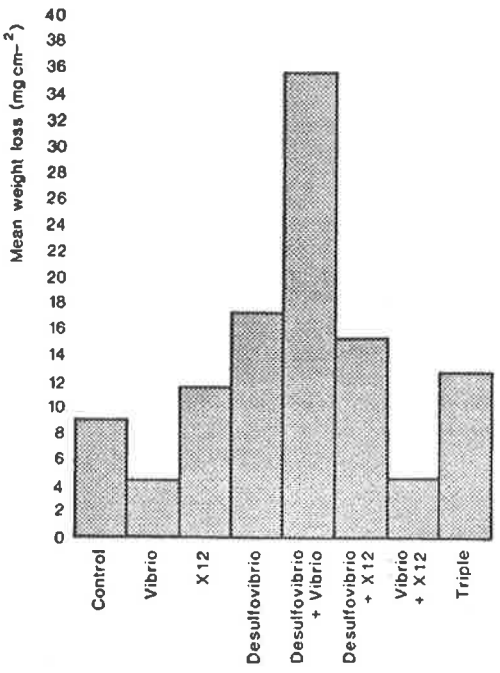

Figure 2 – Weight loss of mild steel exposed to pure and mixed bacterial cultures after 3 weeks incubation in Postgate Medium B at room temperature (approx. 22 °C). X12 = presumptive *Citrobacter*.

A bacterial consortium was shown to be necessary for the maintenance of corrosion current of pitted 304L stainless steel in seawater under anaerobic conditions (3). SRB were present on the cathode, leading to high charge transfer resistance, while the consortium on the anode decreased charge transfer resistance. These results were stated to support the involvement of cathodic depolarization in the anaerobic biocorrosion of stainless steel.

A number of microorganisms isolated from corroding copper pipework in Auckland, New Zealand, attached to and grew on copper surfaces in a simulated potable water medium (114). The four most numerous culturable bacterial species were identified by 16s rRNA gene sequence analysis as *Sphingomonas capsulata* (European Bioinformatics Institutes (EMBL) Nucleotide sequence database # AJ223450), *Staphylococcus warneri* (EMBL #AJ223451), *Erythrobacter longus* (EMBL # AJ223452) and *Methylobacterium sp.* (EMBL #AJ223453). A yeast, identified as a *Candida sp.*, was also recovered from the copper surface. Biofilms

containing these isolates were shown to promote release of copper corrosion by-products in subsequent laboratory reactor experiments (114).

Techniques for the Study of Biocorrosion

The forms of corrosion which can be promoted by the interaction of microorganisms with metals are numerous, including general pitting, crevice attack, stress corrosion cracking, enhancement of corrosion-fatigue, intergranular stress cracking and hydrogen embrittlement and cracking. Most cases of MIC are associated with localized attack (Fig. 3). The complexity of MIC reactions means that a broad range of techniques must be employed to relate the corrosion processes to the microbial activities at surfaces.

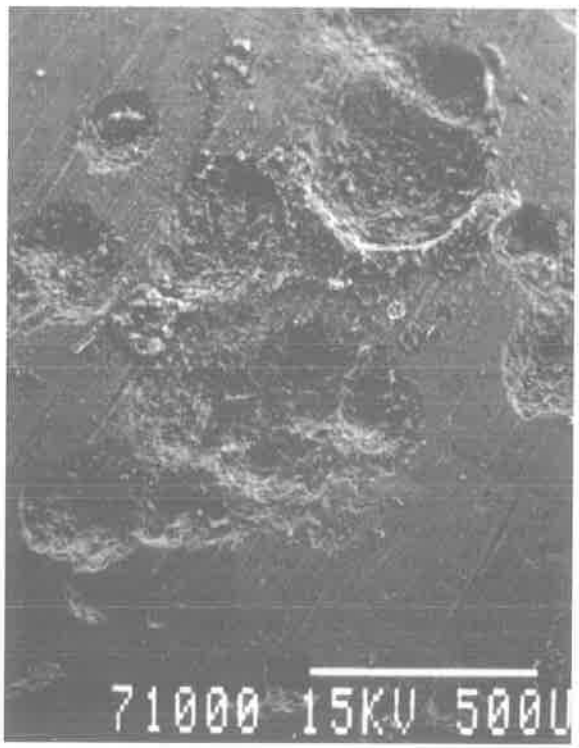

Figure 3 – SEM micrograph of mild steel surface, showing localized attack, following exposure to mixed population of *Pseudomonas* spp and sulfate-reducing bacteria.

Qualitative and semi-quantitative evaluation of MIC

The contribution of microorganisms to corrosion has been assessed using a variety of optical and electron microscopy techniques. Recently, environmental scanning electron microscopy (ESEM), atomic force microscopy (AFM; Fig. 4) and confocal laser scanning microscopy (CLSM) have been employed to study biofilms and biocorrosion phenomena (10). Microscope techniques provide information about the morphology of microbial cells and colonies, their distribution on the surface, the presence of EPS (Figs. 1 and 4) and the nature of corrosion products (crystalline or amorphous; Fig. 5a and b). They can also reveal the type of attack (*e.g.* pitting or uniform corrosion) by visualizing changes in microstructure and surface features after removal of the biofilm and corrosion products (Fig. 6). CLSM and AFM allow the examination of hydrated biofilms and yield clean, three-dimensional images of living biofilms in real time. CLSM has shown that 75 to 95% of the volume of bacterial biofilms is occupied by the matrix, and cells may be concentrated in only 5-25% of the lower or upper layers (24). ESEM studies of biocorrosion and protective coatings have also been reported (54, 112). However, the detection of microorganisms, in itself, should not be the sole basis on which their involvement in the corrosion process is implicated. To confirm MIC, specific activities of the microbes at the site where corrosion is occurring should be demonstrated. Microscopic and culture techniques alone rarely provide such evidence.

Chemical spectroscopy at surfaces offers information on the nature of the accumulated

Figure 4 – Atomic force microscopy image of a single bacterial cell and its associated EPS on a surface of AISI 316 stainless steel.

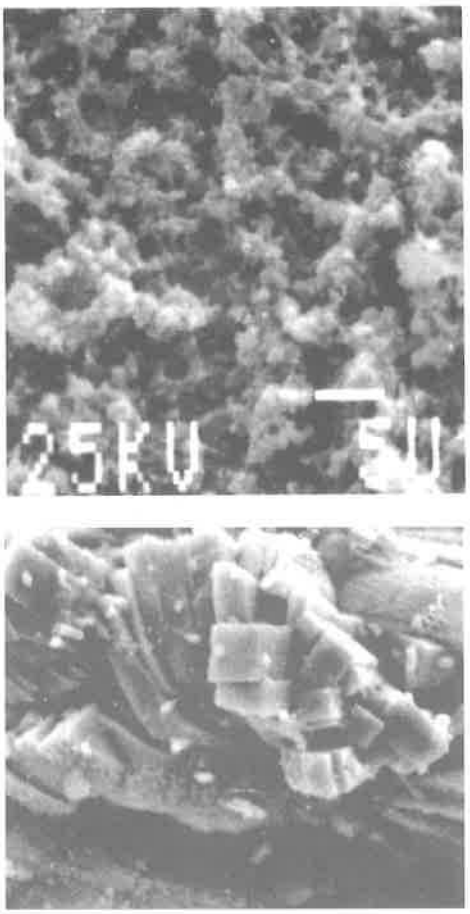

Figure 5 – SEM micrograph of amorphous (a) and crystalline (b) biocorrosion products on a mild steel surface.

corrosion products, which can be specifically associated with microbial activities. Spatially resolved surface chemistry obtained by spectroscopy must be related to the spatially resolved microbiology at the same location. Surface chemical analysis provides information on the chemical composition of the corrosion products and microbiological deposits, and thus gives the opportunity to gain insight into the electrochemical reactions involved in the corrosion process. X-ray diffraction (XRD) and energy dispersive X-ray analysis (EDAX) have been widely used to obtain elemental information on corrosion products on metal surfaces (82). Auger electron spectroscopy (AES) allows mapping of corrosion products across a metal surface that has experienced localized attack. It has been used to investigate biocorrosion in condenser tubes (22). X-ray photoelectron spectroscopy (XPS) can resolve the oxidation state of the elements present, facilitating prediction of corrosion product chemistry and, to some extent, chemistry of the associated microbial biofilm (90). It has also been used to determine the influence of a biofilm on the structure of the passive layer formed on AIS 316 stainless steel (11). AES and XPS are suitable only for evaluating the composition of thin scaling deposits, but laser Raman spectroscopy (LRS), coupled with optical microscopy, can be used to analyze thicker (above

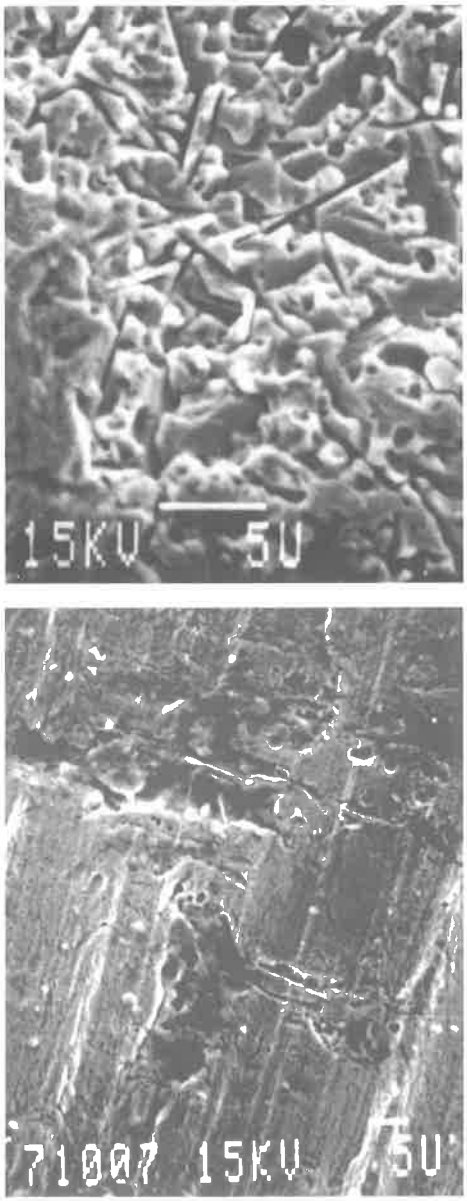

Figure 6 – SEM image of a mild steel surface after the removal of bacterial biofilm, revealing changes in surface characteristics.

1 μm) deposits (101) and offers an interesting technique for future biocorrosion studies. Mössbauer spectroscopy can be applied to iron-containing compounds. It has been used to detect "green rust 2" among corrosion products of steel exposed to marine sediments containing SRB (88) and subsequent, controlled laboratory studies showed that this corrosion product was exclusively associated with SRB-induced corrosion (83).

Machado *et al.* (80) used XRD, Mössbauer spectrophotometry and EDAX to show that the surface film formed on mild steel in the presence of a consortium of *H. resinae* and SRB was mainly composed of magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), goethite (α-FeOOH) and lepidocrocite (γ-FeOOH). Under the experimental conditions used, this film was protective.

The presence of mackinawite and greigite among corrosion products of iron is generally evidence that SRB participated in the corrosion reaction (51, 77, 79). Under alternating reducing and oxidizing conditions, the partially oxidized iron oxide magnetite is often produced, along with lepidocrocite and goethite (51). These mineral signatures of MIC have been detected, using XRD and EDAX, as corrosion products on many oil and gas pipeline systems (51). Amorphous iron sulfide is also often detected by EDAX at pipeline corrosion sites. Little is known about its subsequent crystallization, although biomineralization around SRB colonies or within biofilms may be a key process.

Characteristic copper sulfides, chalcocite (Cu_2S), covellite (CuS_{1-x}) and djurleite ($Cu_{31}S_{16}$) are formed during corrosion of copper and its alloys in the presence of SRB (77, 78). The formation of thick, non-adherent layers of chalcocite or the formation of hexagonal chalcocite is indicative of SRB-induced corrosion of copper and copper alloys.

Quantitative assessment of MIC

Corrosion rates are commonly determined by electrochemical methods, such as potentiodynamic polarization, zero-resistance ammetry, electrochemical impedance spectroscopy (EIS) and electrochemical noise (ECN), in addition to classical weight loss measurements. A detailed review of these techniques is given by Mansfield and Little (81).

Microsensors, which are largely electrochemically-based, offer the resolution that is needed for studying the localized corrosion processes induced by microorganisms. They have been applied to characterize the chemical gradients within biofilms on corroding metal surfaces. Microsensors were employed to show depletion of oxygen within tubercles formed on corroding mild steel surfaces (59) and at anodic areas of the surface covered by a 1mm-thick biofilm. This spatially resolved surface chemical approach enabled these investigators to demonstrate the existence of differential oxygen concentration cells and their role as the driving force for the corrosion reaction.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR/FTIR) was used to quantify the rate of corrosion of a thin metal film deposited as an internal reflectance element, which is exposed to either flowing or stagnant aqueous media. The method is based on the observation that water absorbance in the infrared increases as the thin film decreases in thickness as a result of corrosion. Changes in film thickness corresponding to a few atomic layers can be detected and the measurements can be obtained non-destructively in real time. Quantitative changes in water absorbance are expressed as a corrosion rate. ATR/FTIR has been used to demonstrate the participation of a microbial biofilm in the localized attack of copper films and the relation between onset of corrosion and the production of polysaccharide during biofilm formation (17). It has also been used to demonstrate the influence of the exopolysaccharide produced by the marine bacterium *Pseudoalteromonas* (*Pseudomonas*) *atlantica* on the corrosion of copper (53).

It is indisputable that both qualitative and quantitative approaches are necessary to investigate the role of microorganisms in corrosion processes. Increasingly sophisticated techniques are being employed to study corrosion, microbial activities in biofilms and the types of microorganisms present. The information from the use of molecular gene probes, demonstrating that the majority of microorganisms in the natural environment are unculturable, means that our understanding of MIC is extremely limited. This multi-disciplinary subject, with its important practical applications, is certain to be an area of intense research activity in the future.

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RESUMO

Avanços recentes no estudo da biocorrosão: uma revisão

Processos de biocorrosão na superfície de metais são associados com microrganismos ou com os seus produtos metabólicos, tais como: enzimas, exopolímeros, ácidos orgânicos e inorgânicos, e compostos voláteis como amônio ou sulfeto de hidrogênio. Todos estes produtos podem afetar reações catódicas e/ou anódicas, alterando processos eletroquímicos na interface biofilme/metal. Esta revisão discute diversos mecanismos de biocorrosão causados pelos diferentes atividades fisiológicas associadas com microrganismos e os conhecimentos mais recentes. Estudos modernos da corrosão microbiologicamente influenciada focalizam problemas em ligas de ferro e de cobre. Microrganismos especialmente enfocados são as bactérias redutoras de sulfato e bactérias que depositam metais, destacando aquelas que depositam manganês. A importância de consórcios microbianos e o papel de substâncias poliméricas extracelulares na biocorrosão são enfatizados nesta revisão. Considera-se a contribuição de técnicas analíticas modernas, tais como microscopia de força atómica, espectroscopia Auger, espectroscopia de raio-X, espectroscopia Mössbauer, espectroscopia de infra-vermelho de reflectância total com transformação de Fourier e microsensores.

Palavras-chave: Aço, bactérias redutoras de sulfato, espectroscopia de superfície, cobre, corrosão.

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ANTAGONISM OF YEASTS TO *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* ON CABBAGE PHYLLOPLANE IN FIELD

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ABSTRACT

Twenty yeast isolates, obtained from cabbage phylloplane, were evaluated for antagonistic activity against *Xanthomonas campestris* pv. *campestris*, in field. Plants of cabbage cv. Midori were pulverized simultaneously with suspensions of antagonists and pathogen. After 10 days, plants were evaluated through percentage of foliar area with lesions. Percentage of disease severity reduction (DSR%) was also calculated. Yeast isolates LR32, LR42 and LR19 showed, respectively, 72, 75 and 79% of DSR. These antagonists were tested in seven different application periods in relation to pathogen inoculation (T_1=4 d before; T_2=simultaneously; T_3=4 d after; T_4=4 d before + simultaneously; T_5=4 d after + simultaneously; T_6=4 d before + 4 d after; T_7=4 d before + simultaneously + 4 d after). The highest DSRs were showed by LR42 (71%), LR42 (67%), LR35 (69%) and LR19 (68%) in the treatments T_7, T_4, T_5 and T_6, which significantly differed from the others. The same yeast antagonists were also tested for black rot control using different cabbage cultivars (Fuyutoyo, Master-325, Matsukaze, Midori, Sekai I and Red Winner). The DSRs varied from 58 to 61%, and there was no significant difference among cultivars.

Key words: *Xanthomonas campestris* pv. *campestris*, cabbage, biocontrol, yeasts

INTRODUCTION

Xanthomonas campestris pv. *campestris* (Pammel) Dowson (Xcc) causes black rot of crucifers, one of the most destructive diseases of cruciferous crops worldwide. It is present in all crucifer-producing regions where yield and quality losses may be very high (31). Black rot can appear on plants at any growth stage. On young plants, margins of cotyledons turn black and may drop off. On mature leaves, symptoms appear along leaf margins as yellow, V-shaped lesions, with the base of the V usually directed along a vein. As the lesions expand toward the base of the leaf, the tissue wilts and eventually becomes necrotic. The infection may move down to the vascular tissue of the petiole and spread up or down the stem of the plant and into roots. The presence of black veins in yellow lesions

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along leaf margins is diagnostic of black rot (13). Disease control usually consists of using resistant cultivars, certified seeds, hot-water treatment of seeds followed by application of antibiotics or protectant fungicides, crop rotation, control of weeds and insects, and destruction of infected plants and debris in the field (13, 24). Nowadays, the use of resistant cultivars is the most economical method of control.

In addition, several microbial antagonists have been studied to improve control alternatives (1, 6). The use of yeasts as control agents of plant diseases is a strategy with great potential (11, 18, 20, 21, 28), mainly because of their ability to compete for nutrients (5, 9) and colonization sites (22).

Despite some research on the biological control of *X. campestris* pathovars in citrus (25, 19), clusterbean (26), cotton (30), mungbean (8,15) rice (14), and soybean (29), very little information exists on the biocontrol of black rot in crucifers (2, 3, 4). This work aims to study the potential of yeasts to control black rot on cabbage *(Brassica oleracea* var. *capitata* L.) in field.

MATERIALS AND METHODS

Yeast isolates

The epiphytic yeast isolates used in this study were obtained from leaves of cabbage collected from three nurseries in the State of Pernambuco, Brazil. From each plant, samples with five leaves were randomly taken. A total of 15 plants were sampled per nursery. The isolation followed the methodology of Melo *et al.* (20) with modifications. Ten discs of 12 mm diameter were removed per leaf, placed in a tube containing 10 ml of sterile tap water (STW), and sonicated for 10 min. Serial dilutions were made in STW and aliquots (0.1 ml) were plated on potato-dextrose-agar medium (PDA) supplemented with 250 mg.L^{-1} tetracycline. Plates were incubated for 48 h at 25°C, and one representative of each yeast colony morphology was transferred to a fresh PDA plate to obtain pure cultures, which were stored on the same medium at 4°C. Suspensions of antagonist yeast candidates were prepared in sterile distilled water (SDW) using 48 hour-old culture grown at 25°C on PDA (0.05% Tween 80 was added to the suspension).

The pathogen strain was obtained from the Bacterial Collection of Plant Bacteriology Laboratory, Agronomy Department/Federal Rural University of Pernambuco. Bacterial suspensions (10^8 CFU.ml^{-1}) were prepared in SDW using 48 hour-old culture grown at 30°C on NYDA medium (27).

Preliminary screening of yeast antagonistic to cabbage black rot

Twenty epiphytic yeast isolates were tested for their ability to reduce the severity of cabbage black rot under field conditions.

Seven week-old cabbage plants (cv. Midori) were simultaneously treated with antagonist candidates (10^6 cell.ml^{-1}) and inoculated with Xcc suspension (10^8 CFU. ml^{-1}). Sprays were performed until run-off. Controls were inoculated with Xcc but not treated with yeasts. Field conditions at the time of the inoculation were: temperature at 32±2°C and 87±3% of relative humidity.

The percentage of disease severity (DS%) was evaluated 10 days after inoculation by estimating the percentage of leafs with lesions areas. The percentage of disease severity reduction (DSR%) was calculated according to Edginton *et al.* (10): DSR(%) = [(DSc - DSt)/DSc)] x 100, where DSc = leaf area with lesions on the control plants and DSt = leaf area with lesions on the treated plants.

Influence of the application period of antagonistic yeasts on the control of cabbage black rot

In order to determine the optimum time for application of the antagonist, the four best black rot biocontrol agents were selected and tested as in the previous trial. Antagonist cell suspensions (10^6 cell.ml^{-1}) were sprayed at seven different periods in relation to inoculation with the pathogen suspension (10^8 CFU.ml^{-1}). The treatments were: T_1 = 4 days before; T_2 = simultaneously; T_3 = 4 days after; T_4 = 4 days before and simultaneously; T_5 = 4 days after and simultaneously; T_6 = 4 days before and 4 days after; T_7 = 4 days before, simultaneously and 4 days after.

Influence of the cabbage cultivar on the control of black rot by antagonistic yeasts

The four best black rot biocontrol agents were also tested as in previous trials, using six cabbage cultivars: Fuyutoyo, Master-325, Matsukaze, Midori, Sekai I and Red Winner. The concentrations of antagonist and pathogen suspensions were 10^6 cell.ml^{-1} and 10^8 CFU.ml^{-1}, respectively.

Statistical Analyses

In all experiments, four leaves per replicate were

used. Each treatment was replicated six times. The results were submitted to analysis of variance (ANOVA), and means were compared by Scott-Knott and Tukey's tests (P=0.05).

RESULTS AND DISCUSSION

As a result of the isolation procedure, 20 yeast isolates were obtained from cabbage. The low number of yeast isolates shows that plants in field are submitted to a high amount and diversity of pesticides, which reduce the epiphytic population of microorganisms. The same fact was observed by Michereff *et al.* (23) when studying yam phylloplane populations. According to Ghini (12), alterations on leaf surface and its environment could be caused by application of pesticides, hormones, fertilizers and also by pollution.

In the initial screening, disease severity reduction ranged from 24.2 to 78.6% for all 20 yeast isolates. Ten isolates had efficiency varying from 24.2 to 35.6%, four from 38.8 to 47.0, three from 57.7 to 60.2 and three from 72.3 to 78.6%. Among the isolates tested, LR19, LR42 and LR35 showed, respectively, 78.6, 75.5 and 72.3% of DSR. These data point the viability of using yeasts for Xcc biocontrol. Few complete studies had shown yeast activity against other plant pathogenic bacteria, such as, *Erwinia carotovora* subsp. *carotovora* on bell pepper fruits (20) and *E. amylovora* on pear flowers (21). The success of an antagonist introduced into the phyllosphere is function of many factors including resistance to an adverse environment, microbiota and plant metabolites, migration ability, competition for space, water and nutrients as well as production of lytic enzymes or antibiotics (1, 5, 16). The resident yeast population has the special ability to compete for nutrients and/or space which enable them to act as biocontrolers (22).

The best period for antagonist application was T_7 (4 days before, simultaneously and 4 days after, in relation to inoculation with the pathogen suspension) however without significantly difference from T_4, T_5 and T_6. The highest DSRs were showed by LR42 in T_7 treatment (71%), LR42 in T_4 treatment (66.7%), LR35 in T_5 treatment (69.5%) and LR19 in T_6 treatment (68.3%), which differed from the others (Fig. 1). The good results obtained with the treatments applied before inoculation are in agreement with Boudreau and Andrews (7), Michereff *et al.* (23) and Reis *et al.* (28). This possibly means that the antagonists are able to inhibit the pathogen establishment probably by colonizing the infection sites and competing for nutrients (5, 9, 23).

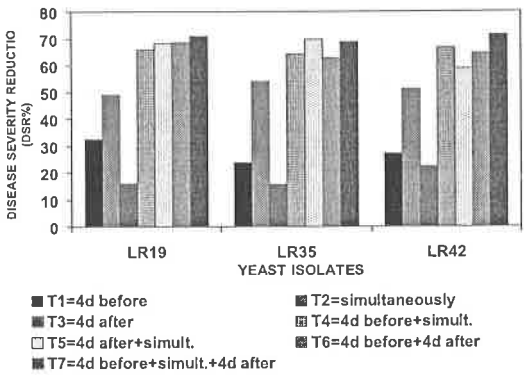

Figure 1: Influence of application period of antagonistic yeasts on the cabbage black rot control.

When the three antagonists were tested against Xcc, using six cabbage cultivars, DSR ranged from 61.3 to 57.9%, without significant difference among cultivars (Fig.2). There was high uniformity among the reaction of the cultivars to Xcc and also to the treatment with antagonists. These cultivars are Japanese hybrids and two of them, "Midori" and "Fuyutoyo", are the most frequent in Pernambuco.

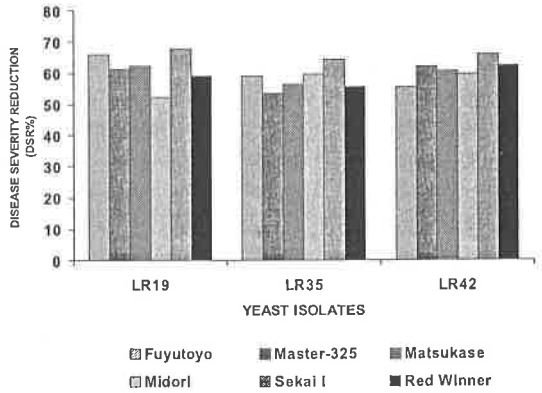

Figure 2: Influence of cabbage cultivar on the black rot control by antagonistic yeasts.

As conclusion, one can say that an integrated control program for the black rot should include resistant cultivar, besides biological control and cultural practices. In the biological control, yeasts should be used due to their efficient mechanisms such

as competition for nutrients and infection sites and, furthermore, because they are easy to grow and formulate.

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RESUMO

Antagonismo de leveduras a *Xanthomonas campestris* pv. *campestris* no filoplano de repolho em condições de campo

Vinte isolados de leveduras, obtidos a partir do filoplano de repolho foram avaliados pela atividade antagônica contra *Xanthomonas campestris* pv. *campestris*, em condições de campo. Plantas de repolho cv. Midori foram pulverizadas simultaneamente com suspensões do antagonista e do patógeno. Após 10 dias, as plantas foram avaliadas através da porcentagem de área foliar infectada. A porcentagem de redução da severidade da doença (DSR%), também foi calculada. Os isolados de leveduras LR32, LR42 e LR19 apresentaram, respectivamente, 72, 75 e 79% de DSR. Estes isolados foram testados em sete diferentes períodos de aplicação dos antagonistas em relação a inoculação do patógeno. (T_1=4d antes; T_2=simultaneamente; T_3=4 d após; T_4=4 d antes + simultaneamente; T_5=4 d após + simultaneamente; T_6=4 d antes + 4 d após; T_7=4 d antes + simultaneamente + 4 d após). As maiores DSRs foram obtidas por LR42 (67%), LR35 (69%) e LR19 (68%), respectivamente nos tratamentos T_7, T_4, T_5 e T_6, que diferiram significativamente dos demais. As melhores estirpes de levedura também foram testadas para controle da podridão negra das crucíferas usando diferentes cultivares de repolho (Fuyutoyo, Master-325, Matsukaze, Midori, Sekai I and Red Winner). As DSRs variaram de 61 to 58%, embora não tenha havido diferença significativa entre as cultivares.

Palavras-chave: *Xanthomonas campestris* pv. *campestris*, repolho, biocontrole, leveduras.

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BACTERIA AND PROTOZOA POPULATIONS IN GROUNDWATER IN A LANDFILL AREA IN SÃO CARLOS, SP

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ABSTRACT

The microbial populations of groundwaters were analyzed in a region under the influence of a landfill (piezometer L12) in the town of São Carlos, São Paulo, Brazil, and in an area not influenced by the landfill (piezometer L5). Heterotrophic bacteria were counted by spread plate method and the number of protozoa was estimated by the most probable number method. There was a larger number of organisms in well L12, with a mean value of 15.76×10^4 CFU/ml for bacteria and 9.7 MPN/ml for protozoa, whereas the mean values for piezometer L5 were 2.88×10^4 CFU/ml for bacteria and 3.4 MPN/ml for protozoa. The greater abundance detected in piezometer L12 may be related to the influence of the leachate through the landfill on the microbial populations, also demonstrated by deoxygenation and by the high conductivity values (3530 μS/cm) compared to piezometer L5 (2.47 mg/L dissolved oxygen and 42 μS/cm conductivity). The most commonly detected protozoa were amoebae and flagellates. The density of flagellate protozoa determined under microaerophilic conditions was 10 times higher than that determined under aerobic conditions.

Key words: bacteria, protozoa, groundwater, landfill.

INTRODUCTION

Countries undergoing an accelerated process of industrialization face serious problems of pollution of underground waters that affect or will affect the drinking water supply for important segments of the population. Urban refuse, accumulated in large amount in cities, is often dumped on open surfaces or disposed of in landfills, with a potential risk of groundwater pollution (8, 19).

Because of the importance of groundwaters, it is essential to understand the behaviour of chemical and biological pollutants in the subsoil, the processes that govern this compartment and the interactions between microorganisms and abiotic factors. In this way it will be possible to prevent or relieve environmental and sanitary problems and to expand the knowledge needed for an integrated management of land systems, underground and surface waters.

The microbiology of groundwater is little known compared to that of surface waters and was studied on a small scale before 1970 (7). Bacterial populations predominate in the subsoil, accompanied by a smaller number of eukaryotic microorganisms such as the dormant forms of protozoa and fungi (13). Flagellates and amoebae are the major protozoa

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found in groundwaters (20, 25), although ciliated protists have also been detected in aquifers (6, 13). Protozoa are widely distributed in nature, are important components of aquatic ecosystems and are used as indicators of water quality. These characteristics show the potential of these organisms and the need of their ecologic study (22), with few reports, apparently only twenty publications (23), being available about protozoa in groudwaters.

In the present study we investigated the microorganisms populations and some abiotic variables in an area inside and outside the influence of a land fill within the boundaries of the towr of São Carlos in order to determine the possible effect of the leachate on the populations of bacteria and protozoa.

MATERIALS AND METHODS

Study area. The landfill is located in the rural region of São Carlos, in the upper part of the basin of the Ribeirão do Feijão stream, State of São Paulo. at 47°45'-47°55' longitude West and 22°00'-22 °15' latitude South (24).

Sampling. Samples were collected monthly from May 1993 to April 1994 from two piezometers installed according to technical regulations (4). Piezometer L12 is 8.0 m deep and is under the action of the landfill, and piezometer L5 is 9.0 m deep and is located in an area outside the action of the landfill. After the water level was measured, the piezometer was depleted with a PVC tube (3" in diameter and 2 m long) containing a valve at the lower end (3), in order to guarantee that stagnating water was replaced with freshly formed water. To avoid water contamination by external agents, a protective blanket was placed around the piezometer and the material used in the procedure of water depletion was disinfected with alcohol. The samples for physical and chemical analysis (pH, water temperature, conductivity and dissolved oxygen - Winker) and for microbiological control were collected with a bacteriologic sampler (3) containing glass flasks and sterile glass flasks, respectively. One sampler was used for piezometer L5 and another for piezometer L12.

Heterotrophic bacteria. The number of heterotrophic bacteria was estimated by inoculating plates containing P medium of the following composition: 1.0 g/L peptone, 0.1 g/L glucose, 0.1 g/L K_2HPO_4, 0.02 g/L $FeSO_4$, 15.0 g/L agar, pH 5.0 (17). The material was incubated at 25°C for 15 days.

Protozoa. The density of protozoa was estimated by a modification of the technique described by Harris and Sommers (11). Six dilutions were used, with eight replication per dilution, for a total of 48 flasks (10 ml) per well. A previously autoclaved unhulled kernel of rice was placed in each flask together with water from the well. The flasks were incubated at 25°C under conditions of aerobiosis and protected with a plastic bag in order to minimize evaporation. In April, the samples from piezometer L12 were also incubated under conditions of microaerophilia produced by a combination of iron, copper and sodium bicarbonate (15) and analyzed after 13 days of incubation. The material in the flasks was examined after 3, 6, 9 and 12 days for piezometer L5 and after 4, 7, 10 and 13 days for piezometer L12. Microdrops (10 μL) were analyzed for the presence or absence of protozoa and the results were quantified according to the table of Harris and Sommers (11).

RESULTS

The variation in density of heterotrophic bacteria during different periods of incubation was performed in order to determine the most adequate time of incubation. An increase in bacterial density was observed up to the 15th day of incubation, with nonsignificant changes occurring thereafter.

Fig. 1 illustrates the bacterial counts in piezometer L5 and L12. In piezometer L12, under the influence of the landfill, the densities observed were of the order of 2.2×10^4 to 4.98×10^4 CFU/ml ($X = 2.88 \times 10^4$ CFU/ml). Fig. 2 shows the counts of protozoa for the two piezometers, with mean values of 9.7 MPN/ml for L5 and 3.4 MPN/ml for piezometer L12.

Fig. 3 compares the groups of protozoa detected during the study period. Amoebae were detected in both piezometers throughout the period of collection, while flagellates occurred only in March in piezometer L5 and throughout the collection period in piezometer L12. Piezometer L5 and L12 presented on average 1.50 and 4.47 MPN/ml amoebae and 0.96 and 1.47 MPN/ml flagellates, respectively. Ciliated microorganisms were observed on some occasions but could not be quantified by the method adopted.

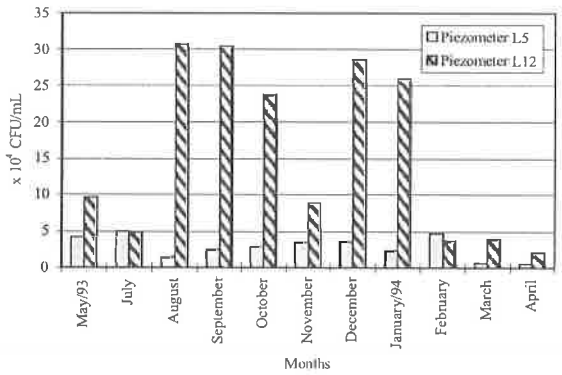

Figure 1. Counts of heterotrophic bacteria in groundwater (15 days of incubation at 25°C under conditions of acrobiosis). CFU= Colony forming units in P medium

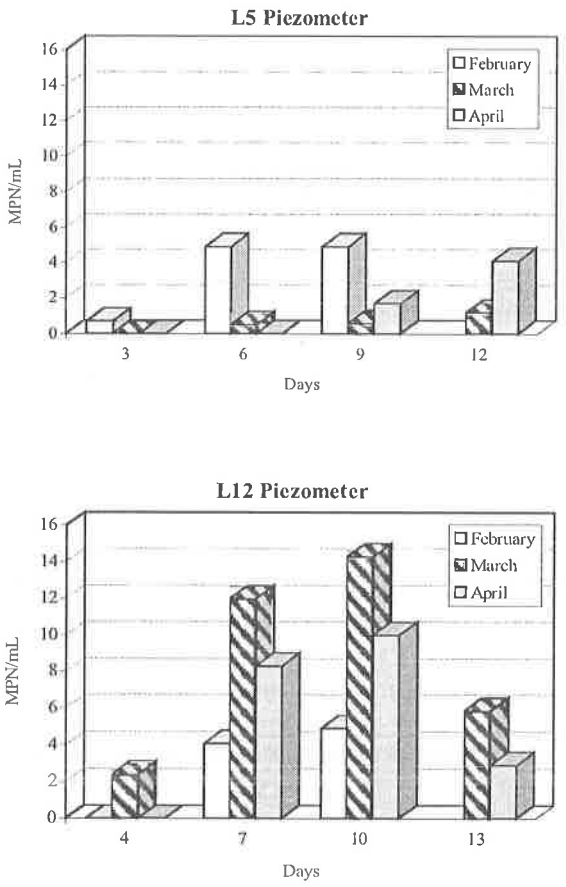

Figure 2. Counts of protozoa in groundwater. Incubation at 25°C under conditions of acrobiosis.

Fig. 4 compares the counts of protozoa in piezometer L12 after incubation under conditions of aerobiosis and microaerophilia. While the density of amoebae was similar for the two conditions, the density of flagellates was 10 times higher under conditions of microaerophilia than under conditions of aerobiosis. Different types of protozoa and cysts were observed, but only *Colpoda steinii* and *Bodo lens* were identified.

Table 1 shows air temperature and precipitation data and a comparison of the physical and chemical variables analyzed during the study period for piezometers L5 and L12. Air temperature ranged from 16.9°C to 24.5°C and accumulated monthly rainfall ranged from 6.2 to 282.0 mm. Piezometer L5 presented a mean water temperature of 23°C, acid pH (3.74), 32.2% saturation with dissolved oxygen, conductivity of 42 µS/cm and a variation of the water column of 82.0 to 140.0 cm. Piezometer L12 presented a mean water temperature of 25.1°C, pH 6.7, 0.3% saturation with dissolved oxygen, conductivity of 3530 µS/cm, and variation of the water column from 71.0 to 123.0 cm.

DISCUSSION

The organic compounds essential for the survival of heterotrophic bacteria in groundwater mainly originate from secondary organic compounds that percolate from the surface, and their quantity and quality depend directly on the reloading rate of the water table (7) and on the leaching of soil organic matter (2).

The greater abundance of organisms in piezometer L12 may be related to the influence of the leachate of the landfill on the populations since this material contains nutrients of fundamental importance for bacterial growth. An increase in biomass and in bacterial activity observed in oligotrophic groundwaters was attributed to the proximity to a source of pollution, suggesting the influence of organic contaminants (12). In a study of the leachate of the landfill in question, Gomes (9) showed that the material contained 203 mg/L ammonium nitrogen, 147 mg/L organic nitrogen and 3.8 mg/L total phosphorus. A study previously conducted by Gonçalves *et al.* (10) at the same site indicated the possible occurrence of infiltration of leaching material into the deep water table due to the direction of the phreatic flow associated with the high permeability of the local substrate. This

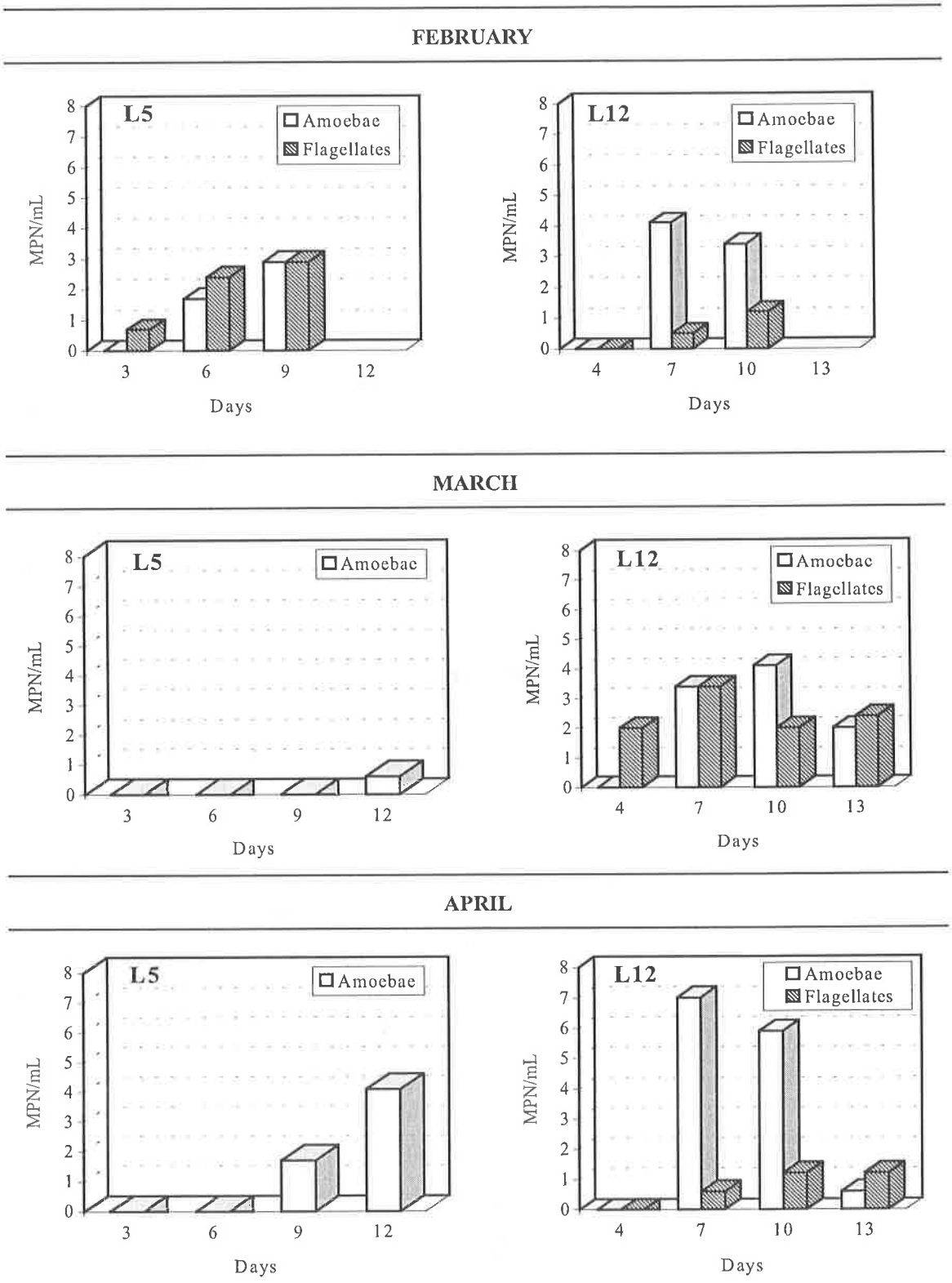

Figure 3. Counts of amoebae and flagellates in groundwater during the study period. Incubation at 25°C under conditions of acrobiosis.

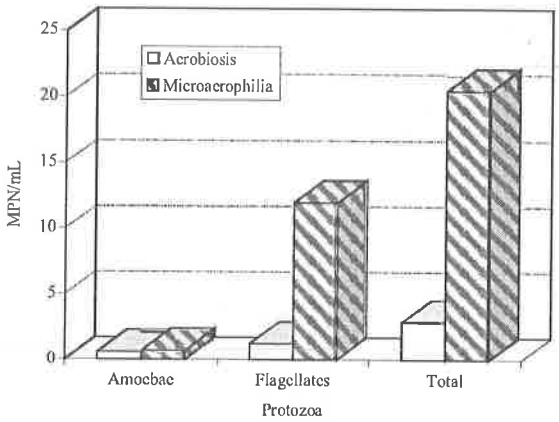

Figure 4. Counts of protozoa in piezometer L12 on the 13th day of incubation at 25°C under conditions of acrobiosis and microacrophilia in April 1994.

possibility is emphasized by the high indices of bacteriologic indicators of fecal pollution in underground water in the area in question (3).

The results obtained for piezometer L5 are of the same order of magnitude as those observed by Harvey *et al.* (12), who detected 4.1 $\pm$ 0.3 x 10^4 bacteria/ml and 4.2 $\pm$ 0.5 x 10^4 bacteria/ml in two noncontaminated water tables, respectively. The small number of organisms detected in piezometer L5 compared to piezometer L12 may be related to the nutritional scarcity of the environment. This fact may limit microbial abundance in water tables, especially with respect to sources of organic carbon, nitrogen and phosphorus, with no limitation occurring for sulphur and other minerals (7). The influence of the leachate on piezometer L12 was also observed on the basis of conductivity, which was on the average 84 times higher than in piezometer L5, and on the basis of the concentration of dissolved oxygen, which was not detected in the samples, with the exception of one sampling date.

By comparing the present data with those obtained by Bossolan (3) in a study carried out at the same site and using the same culture medium, the bacterial density observed in piezometer L12 was twice higher and the density observed in piezometer L5 was 1.5 times lower.

The development of bacterial colonies in P medium, which is nutritionally poor, was slow and the largest number of colonies was obtained after 15 days of incubation. Jannasch and Jones (14) stated that, since many colonies only become visible after 72 h of incubation, the culture of bacteria in more diluted media should last more than one week, thus guaranteeing the formation of a larger number of colonies.

The higher density of protozoa in piezometer L12 supports the statement made by Sinclair *et al.* (25)

Table 1 - Chemical and physical variables in L5 and L12 piezometers.

	Precipitation (mm)	Air (°C)	Water (°C)		Water column (cm)		pH		Dissolved oxygen (mg/L)		Oxygen saturation (%)		Conductivity (µS/cm)	
Month/ Piezometer			**L5**	**L12**	**L5**	**L12**	**L5**	**L12**	**L5**	**L12**	**L5**	**L12**	**L5**	**L12**
May/93	59.1	20.2	23.0	25.5	128.0	113.0	3.95	6.81	2.74	0	35.6	0	35.0	2700.0
July	62.1	16.9	22.0	24.0	140.0	112.0	3.66	6.64	2.37	0.26	30.3	3.44	44.0	3300.0
August	6.2	17.3	23.0	25.0	121.0	98.0	3.64	6.53	1.7	0	22.1	0	42.0	3700.0
September	43.6	17.6	22.0	23.5	109.0	96.5	3.54	6.55	-	0	-	0	44.0	3900.0
October	140.8	20.0	24.0	26.0	104.0	92.0	3.28	6.53	1.97	0	26.1	0	46.0	3400.0
November	111.6	22.2	23.5	25.0	94.0	80.0	3.33	6.55	4.46	0	58.4	0	45.0	3600.0
December	128.9	24.0	23.0	25.0	85.0	75.5	4.21	6.69	2.23	0	33.0	0	40.0	3700.0
January/94	198.7	19.8	21.5	24.5	82.0	74.0	4.31	6.81	1.94	0	24.6	0	36.0	3500.0
February	282.0	24.5	25.0	27.0	97.0	80.0	3.27	6.78	2.87	0	38.6	0	40.0	3700.0
March	89.3	22.8	23.0	25.5	106.0	123.0	4.33	6.85	2.71	0	35.3	0	47.0	3700.0
April	89.6	21.7	23.0	25.0	92.50	71.0	3.62	6.92	1.37	0	17.8	0	44.0	3600.0
Average	110.17	20.64	23.0	25.1	105.0	92.3	3.74	6.7	2.47	0.02	32.1	0.30	42.0	3530.0

- No data

about the probable greater abundance of protozoa at sites with organic contamination of the subsoil, with possible important ecologic roles for these organisms in these environments.

It is possible that the protozoa counted by the method employed were underestimated. Since this is an estimate based on the presence and absence of protozoa, even though the organisms were detected in 1, 2 or 3 flasks, the density would have been zero on the basis of the table used. An underestimate of the number of protozoa in the sediment may occur with the use of this method since not all organisms develop in enriched cultures due to culture conditions or feeding incompatibility (25). Another problem detected during the enumeration of protozoa was the occurrence of temporary encysting for nuclear reorganization and multiplication (18), which may occur for *Colpoda steinii*, or the possible occurrence of a resistance cyst in unfavourable environmental situations (21). This fact may have led to an underestimate of ciliated organisms, since, depending on the time when counts were performed, some organisms may have been encysted.

It is difficult to determine precisely whether or not a microorganism is native to the subsurface environment. The protozoa detected may reach the water table through hydrologic flow and may become encysted when they do not find conditions favourable to development.

Incubation of samples from piezometer L12 under microaerophilic conditions showed that the protozoa may be metabolically active under anaerobic and/or microaerophilic conditions, and that they may become encysted under aerobic conditions.

In conclusion, the microorganisms observed were mainly flagellates and amoebae while ciliates were observed in much lower numbers. *Colpoda steinii* was observed both encysted and as trophic cells in different periods of the same day. This observation shows that care is needed to avoid estimation errors as well as the urgency of studies related to encysted versus trophic cells (23).

The high numbers of bacteria and protozoa detected in the contaminated piezometer may play a key role in the self-purification processes of the aquifer. The bacteria may be using different organic pollutants as substrate and protozoa, witch are the main consumers of bacteria in nature, may control the bacteria populations (16), stimulate the bacterial activity and reduce the number of pathogens (5) present in the system. On the other hand, careful considerations should be given to the fact that protozoa may harbour pathogenic bacteria. It has been shown that *Legionella pneumophila* has the ability to survive and replicate within protozoa, improving its resistance to physical and biochemical agents used in bacterial eradication and therefore may contribute to the increase of bacteria in the environment (1).

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RESUMO

Populações de bactérias e protozoários em águas subterrâneas na área de um aterro controlado em São Carlos, SP

Foi feita a análise das populações microbianas na água subterrânea da região sob a influência do aterro controlado da cidade de São Carlos, São Paulo, Brasil (poço L12) e sem a influência do mesmo (poço L5). As bactérias heterotróficas foram enumeradas pelo método de espalhamento em placa e o número de protozoários foi estimado pelo método do número mais provável. Observou-se um maior número de organismos no poço L12, com uma média de bactérias de 15,76 x 10^4 UFC/ml e 9,7 NMP/ml de protozoários. No poço L5 foi detectada uma média de bactérias de 2,88 x 10^4 UFC/ml e 3,4 NMP/ml de protozoários. A maior abundância encontrada no poço L12 pode estar relacionada à influência do chorume do aterro controlado sobre as populações microbianas, evidenciada também pela desoxigenação e pelos altos valores de condutividade (3530 µS/cm) quando comparados ao poço L5 (2,47mg/L de oxigênio dissolvido e 42 µS/cm de condutividade). Os protozoários mais comumente observados foram as amebas e os flagelados. A incubação de protozoários flagelados em condições microaerofílicas foi mais favorável, dando uma densidade 10 vezes maior do que em aerobiose.

Palavras-chave: bactérias, protozoários, água subterrânea, aterro controlado.

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QUANTITATIVE EVALUATION OF ACIDITY TOLERANCE OF ROOT NODULE BACTERIA

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ABSTRACT

Quantification of acidity tolerance in the laboratory may be the first step in rhizobial strain selection for the Amazon region. The present method evaluated rhizobia in Petri dishes with YMA medium at pH 6.5 (control) and 4.5, using scores of 1.0 (sensitive, "no visible" growth) to 4.0 (tolerant, maximum growth). Growth evaluations were done at 6, 9, 12, 15 and 18 day periods. This method permits preliminary selection of root nodule bacteria from Amazonian soils with statistical precision. Among the 31 rhizobia strains initially tested, the INPA strains 048, 078, and 671 presented scores of 4.0 at both pHs after 9 days of growth. Strain analyses using a less rigorous criterion (growth scores higher than 3.0) included in this highly tolerant group the INPA strains 511, 565, 576, 632, 649, and 658, which grew on the most diluted zone (zone 4) after 9 days. Tolerant strains still must be tested for nitrogen fixation effectiveness, competitiveness for nodule sites, and soil persistence before their recommendation as inoculants.

Key words: rhizobia, tolerance to pH, Amazon.

INTRODUCTION

The majority of Amazon Basin soils are acid and have low fertility. Soil pH is usually 4.0-4.5, and P, N, Ca, K, Mg are deficient in most plateau soils of the Amazon. It is estimated that P and N are deficient in 90% of the regional soils (4). Agricultural, forestry and agroforestry yields are negatively affected by these constraints. Liming and fertilization are practiced by only a few landowners, because these amendments are expensive and difficult to obtain in the region, especially for small farmers (14).

Nitrogen fixation by legume-rhizobia symbioses may supply nitrogen to the ecosystem, but plants and bacteria must tolerate soil constraints, such as acidity and Al toxicity. Legume nodulation under natural soil fertility is sparse or absent (3,7), except when soil constraints are eliminated (6). *Bradyrhizobium* strains have been selected for their tolerance to low pH and low soil fertility (1,2,16,17) for practical use in agriculture and forest systems. However, these methods do not quantify the rhizobia's tolerance to acidity.

Colony counting of rhizobia grown in acid YM liquid medium is a possibility, but it is very difficult and expensive in terms of material and time for screening a large number of strains or isolates. To facilitate strain selection, a fast and easy method is necessary to evaluate a large number of isolates, as well as to quantify bacterial tolerance/sensitivity to

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these soil constraints. The objective of the present study was to test a laboratory method for this purpose as the first step in selection of root nodule bacteria for acid soils. The goal is to eliminate those strains that are acid sensitive, thus decreasing the number of isolates for other tests, such as root infection, host effectiveness, nodule competitiveness, and soil persistence (5,8,9,10,11), which will be done only for those which present high tolerance to acidity. This procedure may shorten evaluation time and decrease costs in the process of rhizobia selection.

MATERIALS AND METHODS

The present method evaluated several root nodule bacteria strains in Petri dishes with YMA medium (15,18) using an adaptation of a method for streaking rhizobia (15) at two pHs, 4.5 and 6.5 (control). These rhizobia were isolated from different legume plant species and Amazonian soil conditions (Table 1). For adequate solidification, YMA acid medium (pH 4.5) was prepared with 25 g of agar/L, instead of the usual 15 g. The media were not buffered, so as to find strains which are able to modify the pH. This bacterial ability may be important under soil conditions, where all tolerance mechanisms may help their survival and soil colonization. Rhizobia isolates were streaked from a mother Petri dish with YMA (pH 6.5, where bacteria were growing during a five day period) with a platinum loop. Four replicates were used for each rhizobia isolate in each pH medium. Fig. 1 presents the general streaking procedure, which used only one loop per replication from the mother Petri dish. A dilution factor occurs when looping is done from one zone to the other, so zone 4 is the most diluted. Fig. 2 presents the scoring system: 1.00 - "no" growth (no visible growth); 1.25 - some growth only in zone 1; 2.00 - maximum growth in zones 1 and 2; 3.00 - maximum growth in zones 1 to 3; 4.00 - maximum growth in all four zones. Intermediate scores were also given at intervals of 0.25. Growth evaluations were done until score stabilization, which occurred 6-18 days after streaking in the plates. Statistical analyses of growth were done at 6, 9, 12, 15, 18 days after streaking, using the F test and Tukey at 5% for mean comparisons. This method also permits a less rigorous analysis, which consisted of the interpretation given in Table 2, with the best strains presenting scores higher than 3.00. A total of 31 strains were tested by this method. A second test consisted of adding green bromocresol solution to the acid medium to verify possible pH modification during bacterial growth. A visual change may be seen when pH changes from the initial value of 4.5 (bromocresol changes to yellow color at pHs below 4.5, and becomes green/blue at higher pHs).

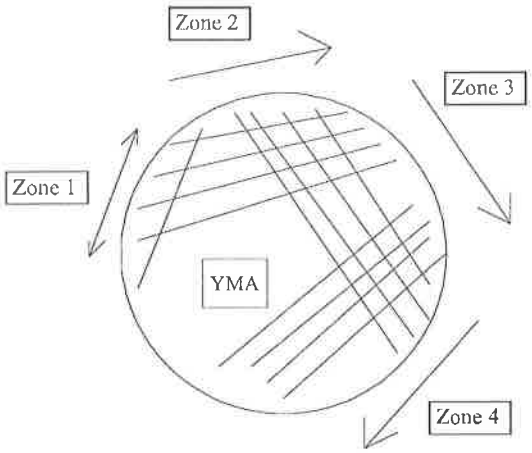
Table 1. Host species and procedence of rhizobia tested on the YMA medium.

STRAINS*	HOST SPECIES	PROCEDENCE
INPA 029	*Vigna unguiculata*	Várzea Ariaú/Solimões
INPA 044	*Vigna unguiculata*	FUCADA/Ultisol
INPA 046	*Vigna unguiculata*	FUCADA/Ultisol
INPA 048	*Vigna unguiculata*	Várzea Ariaú/Solimões
INPA 055	*Vigna unguiculata*	Várzea Ariaú/Solimões
INPA 078	*Vigna unguiculata*	FUCADA/PVA
INPA 511	*Ormosia excelsa*	INPA/CPCA
INPA 520	*Pithecellobium saman*	Maracá/RR
INPA 522	*Clitoria* sp.	Maracá/RR
INPA 526	*Platymiscium paraensis*	Maracá/RR
INPA 550	*Inga edulis*	Calado Lake/AM
INPA 558	*Pithecellobium latifolium*	Anavilhanas/AM
INPA 562	*Pithecellobium latifolium*	Anavilhanas/AM
INPA 563	*Acacia multipinnata*	Maracá/RR
INPA 565	*Rhynchosia minima*	Ariaú/AM
INPA 568	*Dalbergia inundata*	Anavilhanas/AM
INPA 576	*Galactia jussiaeana*	Maracá/RR
INPA 602	*Enterolobium maximum*	Anavilhanas/AM
INPA 609	*Swartzia laevicarpa*	Anavilhanas/AM
INPA 624	*Swartzia laevicarpa*	Anavilhanas/AM
INPA 630	*Pithecellobium latifolium*	INPA/CPCA
INPA 632	*Centrolobium paraensis*	INPA/CPCA
INPA 641	*Pithecellobium inaequale*	INPA/CPCA
INPA 642	*Cassia mimosoides*	Anavilhanas/AM
INPA 649	*Dalbergia inundata*	Anavilhanas/AM
INPA 650	*Clitoria amazonum*	Ponta Negra/AM
INPA 657	*Entada polyphylla*	INPA/CPCA
INPA 658	*Entada polyphylla*	INPA/CPCA
INPA 671	*Inga edulis*	INPA/CPCA
INPA 673	*Abrus tenuiflorus*	Maracá/RR
INPA 678	*Andira riveriana*	INPA/CPCA

* Source: Soil Microbiology Laboratory - CPCA/ INPA

Table 2. Tolerance score ranges for evaluating rhizobia growing on YMA medium.

TOLERANCE	SCORE RANGES
Sensitive	1.00 - 2.00
Moderate tolerance	2.06 - 3.00
Tolerant	3.06 - 4.00



Platinum loop must be sterilized for each streaking zone.
Zone 1. One line. Streaking from a loop, several times in both directions indicated by the arrow.
Zone 2. Four lines streaked in only one direction (indicated by the arrow). Streaking once per line.
Zones 3 and 4. As for zone 2.

Figure 1. Streaking procedure in Petri dishes with YMA medium for rhizobia growth evaluation.

RESULTS AND DISCUSSION

Tolerant rhizobia (INPA 078, Fig. 3A) presented scores of 4.00 at both pHs at 6, 9 or 12 days after streaking in the Petri dishes, while sensitive strain (INPA 641, Fig. 3B) never reached this score. INPA 642 (Fig. 3C) presented a moderate tolerance to pH 4.5.

The method was successful for quantitative evaluation of rhizobia tolerance to acidity. It was possible to separate rhizobia strains statistically, and with low variation among the four replicates for each strain at each pH (Table 3). The standard deviations in general were very low, with the majority being between 0.00 and 0.10. Only five of the 31 strains presented standard deviations higher than 0.10. At 18 days of growth, 15 of the 31 strains presented high growth at pH 4.5, being statistically similar or

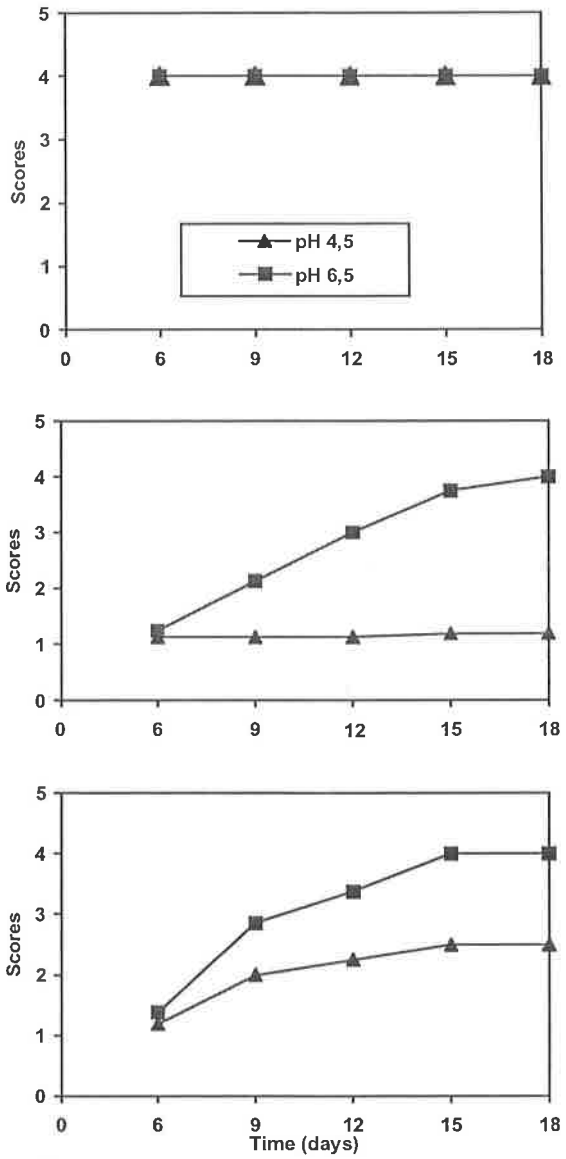


Figure 3. Different degrees of acidity tolerance of rhizobia grown at pH 4.5 and 6.5 during 18 days on YMA medium. A. tolerant strain INPA 078; B. sensitive strain INPA 641; C. moderately tolerant strain INPA 642.

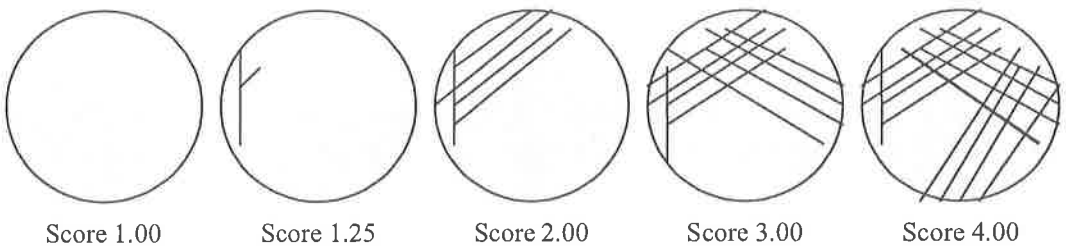


Figure 2. Scores for rhizobia growth in Petri dishes with YMA medium.

Table 3. Mean (sd)[2] growth of root nodule bacteria on YMA medium at pH 4.5 and 6.5. Means of four replications.

Strains INPA	Scores[1]/days of growth									
	6 days		9 days		12 days		15 days		18 days	
	pH 4.5	pH 6.5	pH 4.5	pH 6.5	pH 4.5	pH 6.5	PH 4.5	pH 6.5	pH 4.5	pH 6.5
29	1.25(.00)a	1.25(.00)a	2.00(.00)a	2.00(.00)a	3.50(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
44	1.75(.00)a	1.75(.00)a	2.00(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
46	1.25(.00)a	1.31(.02)a	1.63(.02)b	2.56(.10)a	2.06(.02)b	2.56(.10)a	3.00(.00)b	3.19(.06)a	3.50(.00)b	3.75(.00)a
48	2.00(.00)a	2.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
55	1.00(.00)b	2.00(.00)a	2.75(.00)b	3.00(.00)a	2.25(.00)b	3.50(.00)a	3.25(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a
78	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
511	2.00(.00)a	2.00(.00)a	2.25(.00)a	3.19(.02)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
520	1.00(.00)a	1.00(.00)a	1.25(.00)b	2.00(.00)a	2.00(.00)b	3.50(.00)a	2.75(.00)b	4.00(.00)a	3.50(.00)b	4.00(.00)a
522	1.25(.00)b	1.75(.00)a	2.00(.00)b	3.00(.00)a	3.25(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
526	1.50(.00)b	2.00(.00)a	1.75(.00)b	2.75(.13)a	2.06(.02)b	3.38(.02)a	3.88(.06)b	4.00(.00)a	2.88(.06)b	4.00(.00)a
550	1.38(.06)b	1.75(.04)a	2.44(.14)a	2.56(.06)a	3.19(.02)b	3.56(.06)a	3.25(.00)b	4.00(.00)a	3.75(.00)b	4.00(.00)a
558	1.00(.00)b	1.75(.00)a	1.94(.02)b	2.44(.10)a	2.75(.04)b	3.38(.06)a	3.25(.00)b	3.75(.00)a	3.25(.00)b	3.75(.00)a
562	1.25(.00)b	1.56(.02)a	1.31(.02)b	2.00(.00)a	2.56(.14)b	2.75(.00)a	3.00(.00)b	3.75(.00)a	3.00(.00)b	3.75(.00)a
563	1.25(.00)b	2.00(.04)a	2.13(.02)b	2.48(.02)a	3.25(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a
565	2.00(.00)b	2.19(.02)a	3.13(.02)a	3.25(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
568	1.81(.02)a	1.50(.08)b	2.00(.00)b	2.63(.02)a	2.00(.00)b	2.63(.02)a	2.00(.00)b	3.13(.02)a	2.00(.00)b	3.38(.02)a
576	2.25(.00)a	2.25(.00)a	3.25(.00)b	3.69(.02)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
602	1.00(.00)a	1.00(.00)a	1.00(.00)b	1.50(.00)a	2.25(.00)b	2.75(.00)a	2.75(.00)b	3.50(.00)a	3.50(.00)b	3.75(.00)a
609	1.00(.00)b	1.25(.00)a	2.00(.00)b	3.00(.00)a	2.75(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a	3.75(.00)b	4.00(.00)a
624	1.25(.00)a	1.25(.00)a	1.75(.00)b	2.75(.00)a	2.25(.00)b	3.00(.00)a	3.00(.00)b	3.50(.00)a	3.50(.00)a	3.50(.00)a
630	1.19(.02)b	1.44(.02)a	1.38(.02)b	2.00(.00)a	1.98(.02)b	2.75(.00)a	2.13(.02)b	3.94(.02)a	2.13(.02)b	4.00(.00)a
632	2.25(.00)a	1.88(.02)b	3.25(.00)a	2.63(.02)b	4.00(.00)a	3.50(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
641	1.13(.02)a	1.25(.00)a	1.13(.02)b	2.13(.10)a	1.13(.02)b	3.00(.00)a	1.19(.06)b	3.75(.00)a	1.19(.06)b	4.00(.00)a
642	1.81(.02)a	1.38(.02)b	2.00(.00)b	2,86(.02)a	2.25(.00)b	3.38(.02)a	2.50(.00)b	4.00(.00)a	2.50(.00)b	4.00(.00)a
649	2.25(.00)b	3.06(.02)a	3.25(.00)b	4.00(.00)a	3.25(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
650	1.25(.00)b	3.00(.00)a	2.50(.08)b	3.25(.00)a	3.25(.00)b	3.75(.00)a	3.75(.00)b	4.00(.00)a	3.75(.00)b	4.00(.00)a
657	2.00(.00)a	2.00(.00)a	3.00(.00)a	3.00(.00)a	3.50(.00)b	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
658	2.00(.00)a	2.00(.00)a	3.25(.00)a	3.13(.02)a	3.75(.00)a	3.88(.02)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
671	2.00(.00)a	2.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a	4.00(.00)a
673	1.00(.00)a	1.00(.00)a	1.75(.00)a	1.88(.10)a	2.31(.31)b	3.69(.02)a	2.25(.08)b	3.88(.02)a	2.25(.08)b	3.88(.02)a
678	2.19(.64)b	2.69(.02)a	2.69(.43)b	3.75(.04)a	3.50(.08)b	4.00(.00)a	3.63(.19)b	4.00(.00)a	3.63(.19)b	4.00(.00)a

1 - Scores: 1.00 = "no visible" growth; 4.00 = maximum growth.
2 - (sd) = standard deviation.
3 - Means with the same letter in a line (inside each date) are not statistically different (Tukey test at 5%).

equal to growth at pH 6.5: INPA 029, 044, 048, 055, 078, 511, 522, 565, 576, 624, 632, 649, 657, 658, and 671.

This method also allows selection of strains without statistical analysis, by choosing those which grow well on the medium (scores > 3.00). Using this less rigorous criterion of evaluation (without statistical analysis, Table 2), it is possible to include in this tolerance group the strains INPA 046, 520, 550, 558, 563, 602, 609, 650, and 678, since they presented scores higher than 3.00 at pH 4.5 at 18 days of growth.

Growth stabilization occurred from day 6 to day 15 after streaking the plates. Consequently the time of growth may also be used for selection, since those strains that present higher scores with less time of growth indicate they are more tolerant to low pH. Thus, at 9 days of growth, it was possible to verify, statistically, that the strains INPA 048, 078, and 671 presented 4.00 scores at both pHs. The less rigorous evaluation, using the Table 2 as reference, also includes in this highly tolerant group the strains INPA 511, 565, 576, 632, 649, and 658, which presented scores higher than 3.00 at this time of evaluation.

When bromocresol green was used to evaluate visual changes of medium pH, only strain INPA 046 presented the ability to decrease medium pH. All the other strains presented no visual pH changes in the medium. The ability to decrease the pH may have affected in some way the tolerance of the strain INPA 046, which reached scores higher than 3.00 only after 18 days of growth. None of the strains presented a visual change of the medium pH, which could be a mechanism to neutralize the acidity, as observed by other authors (2, 13, 19).

The method proved useful for quantitative evaluation of rhizobia tolerance at low pH. It is very easy and fast for screening a large number of rhizobia isolates under laboratory conditions. Statistical analysis may be used when accuracy is necessary. This method also evaluated fastgrown rhizobia (*Rhizobium leguminosarum* bv. *phaseoli*) (Oliveira, L.A. and Graham, P.H., unpublished results) for this purpose, as well as for evaluating tolerance to other soil constraints, such as aluminum toxicity (12). However, it is only the first step in rhizobia selection. Tests for nitrogen fixation effectiveness, competitiveness for nodule sites and soil persistence (1,5,8,9,10,11) must be done, before recommendation of these tolerant strains as inoculants.

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RESUMO

Avaliação quantitativa da tolerância de rizóbios à acidez.

A quantificação da tolerância à acidez em testes de laboratório pode ser o primeiro passo na seleção de estirpes de rizóbios para a Amazônia. O presente método avaliou isolamentos de rizóbios em placas de Petri contendo meio YMA com pHs 6,5 (controle) e 4,5, usando notas de 1,0 (sensíveis, sem crescimento visual), até 4,0 (tolerantes, máximo crescimento). As avaliações foram realizadas aos 6, 9, 12, 15 e 18 dias de crescimento. O método permite selecionar preliminarmente, rizóbios isolados de solos da Amazônia, com precisão estatística. Entre as 31 estirpes inicialmente testadas, as estirpes INPA 048, 078 e 671 apresentaram notas iguais a 4,0 em ambos os pHs testados após os 9 dias de crescimento. Ao se analisar as estirpes usando um sistema menos rigoroso (nota de crescimento acima de 3,0), foi possível incluir também neste grupo, as estirpes INPA 511, 565, 576, 632, 649 e 658, que cresceram na zona mais diluída (zona 4) após 9 dias. As estirpes tolerantes devem ser testadas para eficácia na fixação de nitrogênio, competição por sítios de nódulos e persistência no solo antes de serem recomendadas para o uso em inoculantes comerciais.

Palavras-chave: Rhizobia, tolerância ao pH, Amazônia

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ALGAE AND CYANOBACTERIA ON PAINTED SURFACES IN SOUTHERN BRAZIL

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ABSTRACT

Algae and cyanobacteria disfigure the external surfaces of buildings and may cause their physico-chemical deterioration. Even though the climate in Brazil is humid, there is no published literature on this problem. The objective of this work was to identify the major phototrophs present on Brazilian constructions in residential, urban and rural sites. The algal and cyanobacterial types present on discolored surfaces of painted buildings in nine different municipalities in Brazil, all lying between latitudes 19° South and 30° South, were examined. A total of 816 different organisms was detected in 58 sites. Approximately 63% were single-celled or colonial organisms. The cyanobacterial genus, *Synechocystis*, was the most biodiverse and frequently comprised the major biomass. It was present in 63.4% of sites. Second and third most frequently detected were *Oscillatoria* and the algal genus, *Chlorella*, respectively. The latter organism showed the most widespread occurrence (72.4%). Cyanobacteria were the most important colonizers, especially at urban sites, where over 62% of the organisms detected belonged to this class. Filamentous phototrophs were found in smaller numbers than non-filamentous at all locations.

Key words: algae, biodeterioration, Brazil, cyanobacteria, paint films

INTRODUCTION

The growth of phototrophic microorganisms, algae and cyanobacteria, on the external surfaces of buildings can cause discoloration and physico-chemical deterioration. The main consequences of such growth are disfigurement, retention of water (5), encouragement of colonization by macroorganisms and, in some cases, corrosion caused by organic acids (9). Such problems are particularly important in humid climates (1). Nevertheless, there is no published information on algae growing on buildings in Brazil, or, indeed, in Latin America. Information on algae present on building surfaces in countries with humid climates is restricted to Singapore (19) and India (11). However, these countries differ from Brazil in many respects and it is important to know the types of organisms present in such ecosystems in Brazil, if rational procedures of control are to be applied.

Two books have been recently published on algae in Southern Brazil. Garcia-Baptista (7) identified the algae present on a beach in Northern Rio Grande do Sul. The major psammic colonizers were diatoms, but 11 cyanobacterial and eight algal genera were also identified. Francesschini (6) studied various freshwater sites in Porto Alegre – RS, and identified 48 cyanobacterial species, 65 euglenophyta, 2

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pyrrhophyta, 25 chromophyta (excluding diatoms) and 213 chlorophyta. The main aquatic chlorophyta were *Chlamydomonas, Volvox, Botryococcus, Coelastrum, Scenedesmus, Pediastrum, Klebsormidium, Ulothrix, Uronema, Microspora, Bulbochaete* and *Oedogonium*. Obviously, these data cannot be extrapolated to the environment of external building surfaces, where sub-aerial algae are the colonizing phototrophic microorganisms.

The present investigation aimed to show whether there is any similarity between water and sand phototrophic microorganisms and those present on painted walls in Southern Brazil, and to provide information on the types present in the latter ecosystem.

MATERIALS AND METHODS

Sampling. Samples were taken aseptically from the external surfaces of selected buildings in the Brazilian states of Rio Grande do Sul (RS), Santa Catarina (SC), Mato Grosso do Sul (MS) and São Paulo (SP). Samples were obtained from urban sites in Porto Alegre, RS; Lages, SC; Florianopolis, SC; Corumba, MS and São Paulo, SP, from residential locations in Porto Alegre, RS; Gravatal, SC; Lagoa de Conceicão, SC; Bonito, MS and São Paulo, SP, and from rural sites in Porto Alegre, RS; Atlantida, RS; Porto Belo, SC and Pereque, SC. All these lie between latitudes 19° South and 30° South. Sampling techniques used included the adhesive tape method which has been much used for fungal sampling (15), the contact plate method, scraping with a sterile scalpel and removal of flaking paint with sterile forceps, depending on the state of the painted surface. Buildings with obvious lichen growth were not sampled.

Detection and identification techniques. Samples were incubated, under standard, low-light, conditions, on solid media for algae (Modified Knop's medium – MKM; 8) and cyanobacteria (BG11; 16). Adhesive tape and paint flake samples were examined directly, with low power binocular and high power optical microscopes, in addition to incubating on solid media. After growth, subcultures were made on solid media and in liquid MKM. Identification was by microscopic examination (light and fluorescence microscopy), the iodine test for starch, pigment extraction with methanol followed by fluoroscopy, and growth on nitrogen-free media. Cyanobacteria were principally classified according to Bergey's Manual (10).

The algae were classified according to Belcher and Swale (3) and Prescott (13), with additional reference to Smith (17), Prescott (14) and Bicudo and Bicudo (4). The identification of all organisms was to genus level, where possible. Some families, such as the *Trentepohliaceae*, were not divided into genera, since the development of the morphological characteristics required for identification, even at the genus level, requires prolonged unialgal culture under various conditions.

A total of 58 sites were sampled during the months of February to July (RS, SC and MS) and in December (SC), and analyzed.

RESULTS AND DISCUSSION

Culture methods

After the first two months the use of BG11 was discontinued for the following reasons:

1. BG11 allowed the growth of considerably more groups of microorganisms than the algal medium. Apart from algae and cyanobacteria, relatively good growth of fungi (basidiomycetes, ascomycetes, aquatic and terrestrial phycomycetes and slime molds), gliding bacteria and actinomycetes was noted.
2. The range of algae and cyanobacteria appearing on MKM was equal to or greater than that on BG11 and growth appeared earlier on MKM than on BG11.

Organisms were identified to genus level, where possible, based on their morphological characteristics. Very few organisms were obtained in pure culture, owing to the complexity of the biofilms on these painted surfaces. Not only algae and cyanobacteria, but also protozoa, fungi, slime molds, actinomycetes and other bacterial groups were observed. These organisms frequently kill many of the algae and blue-green bacteria in laboratory cultures, and for this reason morphological identification in short-term (1, 2, 3 and 4 day) culture was adopted, followed by continued regular examination up to 4 weeks. The large variety of types found in this study in comparison with other published data (11; 19) is almost certainly due to this strategy.

Observations showed that a succession of protozoa, bacteria and fungi occurs in these cultures. Protozoa may the most important components, grazing on bacteria, algae and fungi. Few other cell types remained in some cultures after 2-3 weeks, but protozoa were not present at all sites. Ciliates were often the dominant protozoa in the first 48h of culture, but were normally replaced later by other types. Competition and succession were observed on almost all plates, indicating that only sequential observations can reliably detect the range of organisms reported here. Traditional culture techniques result in the detection of many fewer types from each sample. Filamentous cyanobacteria, *Chlorella* and *Trentepohlia* were more frequent in late cultures and, in the worst cases, all other phototrophic genera seen in the initial growth were lost from the plates or liquid cultures after extended incubation. For example, in a sample which yielded 15 genera after 48h incubation, only *Chlorella* was detected after two weeks, and yet this genus was not seen when the sample was examined after 48h incubation.

Table 1 – Analysis of phototrophs detected on painted surfaces at 20 residential, 14 urban and 24 rural sites in Brazil.

	Total	Residential	Urban	Rural
Total number of different types	816	270	211	335
Mean N° per site	14.07	13.5	15.07	13.96
Mean N° of algae per site	6.28	6.8	5.71	6.17
Mean N° of cyanobacteria per site	7.79	6.7	9.38	7.79
Mean N° of non-filamentous cyanobacteria per site	4.59	3.95	5.07	4.83
Mean N° of non-filamentous algae per site	4.86	5.35	4.79	4.5
Mean N° of filamentous cyanobacteria per site	3.21	2.75	4.29	2.96
Mean N° of filamentous algae per site	1.41	1.45	0.93	1.67

Microorganisms detected

Table 1 shows the numbers of different phototrophic microorganisms detected at all sites. A total of 816 different organisms was detected in the 58 sites. Of these, approximately 63% were single-celled or colonial algae and cyanobacteria. Around 1.5 times as many cyanobacteria of Bergey's Groups 1 and 2 (10) were detected as of the other (filamentous) groups and for the algae the difference was even greater, 3.5 times as many single-celled or colonial forms as filamentous algae.

The preponderance of non-filamentous cyanobacteria is somewhat surprising, in view of previously published data. Joshi and Mukundan (11) showed that cyanobacteria were the dominant photosynthetic organisms present on surfaces painted with acrylics, cement-based coatings and oil-based enamels in India and that filamentous cyanobacteria were the most frequent isolates. *Plectonema* (stated in Bergey (10) to be of uncertain classification, either Group 3 or Group 4) was found in 60% of samples, with *Lyngbya* and *Nostoc* being the next most abundant groups. Although Wee and Lee (19) reported that *Anacystis*, now classified as *Synechocystis* (10), was present on over 50% of walls and buildings in Singapore, the most frequent phototroph occurring was *Trentepohlia*. They found *T. odorata* on 66% of walls sampled and this genus has since been accepted as the sole standard organism for use in the Singapore Standard test method for algicidal paints (2). The results of our survey suggest that this is not an appropriate organism for a single-organism test in Brazil, as the *Ulotrichaceae* are the most commonly occurring filamentous green algae in our samples (Table 2). *Trentepohliaceae* were isolated from 16 of 58 sites (27.6% as compared with 48.3% for *Ulotrichaceae*). In only one site containing *Trentepohliaceae* was the pained surface stained orange or red, although this is regarded as typical of the surface growth of this group (12). Black, gray, green, or occasionally brown staining was present. The first three are the predominant colors on soiled painted surfaces in S. Brazil. The other, rare, examples of orange discoloration seen proved to be mineral or fungal in origin, or associated with the presence of pigmented sheathed filamentous cyanobacteria, mainly *Scytonema*, and with unidentified coccoid cyanobacteria with heavily pigmented capsules.

The genus which showed highest diversity in our samples was *Synechocystis*, with 143 detections. This was present at 63.4% of sampled sites and was often

Table 2 – Major types of phototrophs detected on painted surfaces at 20 residential, 14 urban and 24 rural sites in Brazil.

Type	Total	Residential	Urban	Rural
Total cyanobacteria	**452**	**134**	**131**	**187**
Synechocystis	143	42	30	71
Oscillatoria	58	17	19	22
Lyngbya	44	11	17	16
Synechococcus	35	12	15	8
Gloeocapsa	33	10	6	17
Gloeothece	29	13	8	8
Nostocaceae	23	9	3	11
Total algae	**364**	**136**	**80**	**148**
Chlorella	55	19	14	22
Ulotricaceae	51	16	10	25
Chlorococcum	37	13	9	15
Eustigmatos	28	8	10	10
Trentepohliaceae	26	9	2	15
Trebouxia	20	10	2	8
Bacilliarophyta	15	7	4	4
Xanthophyta	13	7	0	6

the major biomass; hence it could be an appropriate genus from which to select an organism for standard tests. It must be pointed out, however, that a very wide range of morphological types of *Synechocystis* was seen in our biofilms, indicating many different species. Classical botanical classification divides this group into different genera (*Aphanocapsa*, *Microcystis*, etc.), but Stanier *et al.* (18) emphasized the inconsistencies of this approach and it has not been adopted here.

The only green algae isolated by Joshi and Mukundan (11) from acrylic painted surfaces in India were *Trebouxia* and *Chlorella* and the latter organism was our most frequent phototrophic genus (Table 2), present in 72.4% of sites. Wee and Lee (19) found only 6% occurrence of this algal genus in Singapore, once again emphasizing the differences between these two humid climates.

There is little relationship between the phototrophic microorganisms detected on these painted walls and those found in water or on sand in S. Brazil (7; 6), indicating that this is a completely different ecosystem, governed by its own specific factors. The most important of these may be frequent desiccation and rehydration of the biofilm. Microorganisms on walls have to withstand such variations, in addition to very high temperatures in the summer months. The factors determining the biodiversity of this unusual and very understudied ecosystem deserve more attention.

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RESUMO

Algas e cianobactérias em superfícies pintadas no Sul do Brasil

Algas e cianobactérias produzem coloração nas superfícies externas de construções e podem causar a sua deterioração físico-química. Apesar a clima úmida do Brasil, não existe no pais uma literatura sobre este problema. O objetivo deste trabalho foi identificar os microrganismos fototróficos mais importantes nas superfícies de construções, em áreas residenciais, urbanas e rurais do Brasil. Foram avaliados os tipos de algas e cianobactérias presentes em superfícies pintadas coloradas, em nove municípios do Brasil localizados entre 19° Sul e 30° Sul. Aproximadamente 63% destes foram células simples, ou organismos coloniais. O gênero, *Synechocystis*, foi o organismo que mostrou-se o mais diverso e, frequentemente, compõe a maior parte da biomassa, foi detectado em 63,4% das amostras. Outros organismos frequentemente detectados foram os gêneros *Oscillatoria* e *Chlorella*. Este último se destacou como o organismo de maior ocorrência (72,4%). As cianobactérias foram muito comuns, especialmente em locais urbanos, sendo que, nestas amostras, maior que 62% dos organismos detectados pertenceu a este classe. Organismos fototróficos filamentosos foram detectados em menor número do que os não filamentosos em todas as amostras.

Palavras-chave: algas, biodeterioração, Brasil, cianobactérias, tintas

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BACTERIA ISOLATED FROM A SUGARCANE AGROECOSYSTEM: THEIR POTENTIAL PRODUCTION OF POLYHYDROXYALCANOATES AND RESISTANCE TO ANTIBIOTICS

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ABSTRACT

In this investigation, a sugarcane agroecosystem at a coastal tableland, in the northeast of Brazil, was screened to obtain bacteria strains able to synthesize poly-β-hydroxyalkanoates (PHA), using sucrose as the main carbon source. The potential to synthesize PHA was tested qualitatively by Sudan Black staining of colonies growing in different carbon sources: sucrose, glucose, fructose, propionate and cellulose. In a typical sugarcane crop management system, the plantation is burned before harvesting and vinasse, a by-product of alcohol production, is used in a fertirrigation system causing, probably, selective pressures on the microbiota of natural environments. Eighty-two bacteria strains, belonging to 16 different genera and 35 different species, were isolated. The data showed that 11 strains (*ca* 13%), nine of which belonging to the genus *Pseudomonas*, presented a strong Sudan Black staining in several carbon sources tested and, simultaneously, showed multiple resistance to antibiotics. Resistance to antibiotics is an advantageous feature for the biotechnological production of PHAs. The total number of isolates with multiple resistance to antibiotics was 73, and 38% of them belong to the genus *Pseudomonas*. Among the isolates, *ca* 86% and 43% grew in the presence of 10-100 U/ml of penicillin and/or 100-300 mg/ml of virginiamycin, respectively. These antibiotics are utilized in the alcohol distillery we investigated. The results suggest that some agroecosystem environments could be considered as habitats where bacteria are submitted to nutritional unbalanced conditions, resulting in strains with potential ability to produce PHAs, and also, to an increase in the microbial diversity.

Key words: soil bacteria, poly-β-hydroxyalkanoate, PHA, resistance to antibiotics, sugarcane agroecosystem, vinasse

INTRODUCTION

Poly-β-hydroxyalkanoic acids (PHA) are biodegradable polymers that accumulate in the citosol of microbial cell, as granules, under unbalanced growth conditions with high C:N ratio in the medium. C_4 to C_{18} hydroxyalkanoates can be the monomers of different polymers. The first and more abundant PHA found in the microbial biomass was poly-β-hydroxybutirate (PHB) which under limiting environmental conditions may constitute as much as 90% of the dry cell weight (11, 27). A biodegradable plastic with the registered trade name BIOPOL is already industrially produced by Imperial Chemical

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Industries (ICI), of England, as the copolymer poly-3HB/poly-3HV which is less brittle then PHB and has been used as packaging material. The ICI obtain copolymers from mutant strains of *Alcaligenes euthrophus* H16, using glucose as main carbon source (5). However, glucose is an expensive substrate for producing biodegradable plastic in industrial scale. *Alcaligenes latus* is able to produce PHB from sucrose, a cheaper substrate, but in smaller amount than *A. eutrophus*. Thus, other carbon sources like sodium propionate, fructose, valerate, octanoate, etc have been tested for PHA production, as well as the expression of the structural genes for the key enzymes of PHA synthesis from *Alcaligenes eutrophus* in other organisms, such as *E. coli* and *S. cerevisiae* (2,14,25).

Antibiotic is considered, in ecological terms, a natural product of microorganisms, synthesized as a secondary metabolite, when excess substrate concentration is available. They are produced mainly by actinomycetes and they are widespread, though evidences of their production in the environments are considered to be limited (33), but they are forthcoming (9, 17, 29). Besides the production of antibiotics by the microbiota in natural environments, it is quite impressive nowadays the general use of antibiotics in many activities leaded by man, as animal husbandry, agriculture, hospitals and prophylaxis. This extensive and careless usage of antibiotics has encouraged growth of resistant strains leading to an imbalance in prior relationships between susceptible and resistant bacteria (6, 13). The resistance strategies used by bacteria can be either by mutation or by plasmid acquisition and the seletive pressure exerted by the presence of the antibiotic will induce resistance to antimicrobial agents among bacteria. Efficient transfer of R plasmids between bacteria of diverse origin under simulated natural conditions, even in the absence of antimicrobial agents, demonstrate that R plasmids can spread among bacterial strains of humans, animal, and fish origins that are unrelated either evolutionarily or ecologically (10). Transformation and transduction are, besides conjugation, also important mechanisms of gene transfer in the environment (31). Data confirm that the more frequent antibiotic-resistant strains correspond to the commonest and largest amounts of antibiotics produced and used commercially (16). It was suggested (32) that, in tropical soils, resistant microbial population to antibiotics may be selected from the natural soil microbiota upon contact with these compounds. The resistance to antibiotics would be an advantage for PHA industrial production, once the bacterium that produces this compound could be cultivated free of competitors.

In the northeast of Brazil, the sugarcane is an extensive cash crop plantation spread over many diverse soil ecosystems. One of the most utilized of such ecological systems is the coastal tableland (or 'tabuleiro', as it is named in this part of Brazil). In a typical sugarcane crop management system, every plantation is burned before harvesting, for economical reasons. The vinasse generated as a by-product of alcohol distillery (for every liter of alcohol produced, 11-15 liters of vinasse are discharged to wastewater stabilization ponds), is used in a fertirrigation system, where 300-600 m^3 of the wastewater are spread per hectare, before sugarcane crop renovation. Some soil, chemical, physical and ecological implications of this process have been commented elsewhere (15, 28).The distillery here studied, produced 56 million liters of alcohol, from August/1992 to March/1993, which means that at least 616 millions liters of vinasse should be recycled in the sugarcane agroecosystem. In the cultivated area of the Usina Japungu (*ca* 13 thousands hectares), 3 thousands hectares are fertirrigated with wastewater containing vinasse, once a year, before sugarcane crop renewal. In the fermentors of the distillery (with capacity of 350,000-550,000 liters), variable quantities of antibiotics are used for controlling bacteria populations that compete with *Saccharomyces cerevisae* for the sucrose of the sugarcane juice. The crop management and processes above described, we believe, contribute to likely selective pressures on the microbiota of natural environments, with differing consequences on microbial populations involved in biogeocycling of essential nutrients to sugarcane plantation. The poor tablelands soils, whose acid pH (5.0 to 5.5) and low nutrients contents are limiting factors to productivity, may be particularly affected by this crop management system, once many hectares of these ecosystems have been abandoned as unproductive, when yield decreases to less than 40 Mg of sugarcane.$ha^{-1}.yr^{-1}$. Physical and chemical soil properties seem not to change over some time of cropping; and vinasse, used for fertirrigation, showed to benefit the microbial biomass, in one year observation (15). However, intensive sugarcane cultivation and vinasse effect on soil properties, in a long-term basis, lack further investigations. The possible relation between environments under stress or strong perturbation and

the biodiversity increase, is another ecological parameter that has been investigated by some authors (4, 30) and is also considered in the present study.

In the present investigation, a screening of bacteria living in environments under anthropic pressure in the sugarcane agroecosystem was performed, searching for **a**) strains with potential to produce PHA using different carbon sources, mainly sucrose; **b**) strains with multiple resistance to antibiotics and **c**) microbial diversity of the isolates in an attempt to use these parameters as bioindicators of anthropic pressure on natural microbiota of diverse environments, in the sugarcane agroecosystem.

MATERIALS AND METHODS

Environment selected. The investigations were performed over two years (1992-94) in the Usina Japungu, an alcohol distillery situated in the municipality of Santa Rita, Paraíba State, Brazil. The local climate type is As' of Köppen classification, hot and humid, and the annual mean rainfall is 1,640 mm. The soil here studied is an oxisol (15). A large and a small stabilization ponds were investigated. The large one receives vinasse from the fermentors and water used for washing the sugarcane before being processed. The small one receives vinasse only. The temperature of the wastewater in the stabilization pond, measured at 10:30, in May 23rd, 1994, was 37°C. The vinasse as an effluent, coming out straight from the fermentors, reaches a temperature between 80 and 90°C, BOD_5 20,000 to 35,000 mg/L, pH 3.6 to 4.0 and total solids 21.8 g/L. Vinasse applied to soil (*ca* 195 $m^3.ha^{-1}$) supplies twice more K than the amount required by sugarcane plantation, therefore, a serious threat to soil salinization (15). The samples collected (**Group A**) and the field experiments carried out in the area we investigated (**Group B**) are organized in distinct types as described. **Group A**: samples collected from soil, from the borders of the wastewater stabilization pond and from the wastewater itself, numbered from I to VII. I) Soil amended with sugarcane bagass (20 cm depth); II) soil amended with sugarcane bagass and other organic residues from the distillery (10 cm depth); III) soil amended with the wastewater (10 cm depth); IV) soil from the borders of the wastewater stabilization pond, in a place with a mixture locally named '*gelose*', which means hydrogel or agar; V) soil from the borders of the stabilization pond, in a place covered temporarily by the wastewater; VI) vinasse collected straight from the fermentors; VII) wastewater from the stabilization pond. **Group B**: field experiments, where small pieces of sugarcane into nylon bags (45 μm mesh size) were buried in several soil environments or submersed in the large and in the small stabilization ponds. These samples were numbered from VIII to XII. VIII) samples from the borders of the large stabilization pond (20 cm depth), in a place covered temporarily by the wastewater; IX) samples from the borders of the large stabilization pond (10 cm depth), in a place covered permanently by the wastewater; X) samples from the soil of sugarcane plantation (10 cm depth), not burned before harvesting; XI) samples from the soil of sugarcane plantation (10 cm depth), burned before harvesting; XII) samples submersed in the small stabilization pond.

Samples processing and bacteria identification. Soil samples (2 g) and sugarcane pieces contained in the nylon bags (2 g), were processed as follow (19): they were mixed in 250 ml of water (6,000 r.p.m.) for 2 min. Wastewater and vinasse samples were mixed manually for 2 min. Serial dilutions were performed using buffers with the same pH of the samples collected from the environments (7). Soil pH was determined in water (1:2.5 v/v), according to Allen *et al.* (1). Aliquots from each dilution were transferred to nutrient broth (NB) and incubated at 30°C and 37°C. After 24 h the cultures were streaked in agar nutrient and incubated to form isolated colonies (19). The colonies were characterized morphologically and then replicated for further biochemical tests, for identifying the isolated species (8, 26). Standards strains from the collection of the Departamento de Antibióticos, Universidade Federal de Pernambuco were used for comparison: *Bacillus subtilis* (DAUFPE-16; Waksman, USA); *B. anthracis* (DAUFPE-09; Escola Nacional de Ciências Biológicas, México), *B. cereus* (DAUFPE-11; Departamento de Antibióticos, UFPE), and *Pseudomonas aeruginosa* (DAUFPE-39; Instituto Tecnológico-2633).

Detection of potential PHA production. Colonies were grown in minimum unbalanced agar medium (MUA): $NH_2HPO_4.7H_2O$, 6.7 g/L; KH_2PO_4, 1.5 g/L; $(NH_4)_2SO_4$, 0.5 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $CaCl_2.2H_2O$, 0.01 g/L; Fe(III)NH_4 citrate, 0.06 g/L and trace element solution, 1 ml/L. Trace element solution contained: H_3BO_3, 0.3 g/L; $CoCl_2.6H_2O$, 0.2 g/L; $ZnSO_4.7H_2O$, 0.1 g/L; $MnCl_2.4H_2O$,

0.03 g/L; $NaMoO_4.2H_2O$, 0.03 g/L; $NiCl_2.6H_2O$, 0.02 g/L and $CuSO_4.5H_2O$, 0.01 g/L. The carbon source (sucrose, glucose, fructose, sodium propionate or carboxy methyl cellulose) was added at 5.0 g/L (22, modified). Agar was added at 15 g/L. Potential PHA production was detected after five days incubation at 30 or 37°C, by Sudan Black (SB) staining of the colonies. Briefly: a 0.02% SB solution in ethanol 96% was spread over the colonies for 30-60 min, discarded and washed with ethanol 96% (24). Dark blue stained strains were compared to standards strains of *Alcaligenes eutrophus* DSM 545 and *A. latus* 1023 (from IPT-Instituto de Pesquisas Tecnológicas, São Paulo).

Antibiotics screening procedure. Plate dilution technique, in nutrient agar (NA), was used for determining the levels of the strains resistance to the following antibiotics and respective concentrations (23): kanamycin sulphate, km, 20 µg/ml (Sigma); tetracyclin, tc, 10 µg/ml (Briston); ampicillin, amp, 50-100-150-200 µg/ml (Wyeth); streptomycin sulphate, sm, 20 µg/ml, (Inlab); nalidixic acid, nal, 20 µg/ml (Sigma). The antibiotics virginiamycin, virg, and penicillin, pen, from SmithKline Beecham, were a gift from Usina Japungu. The maximal concentration used in the alcohol distillery, for all antibiotics, was 20 µg/ml. In this work, the final concentrations for virginiamycin were 100-150-300 µg/ml and for penicillin were 1-10-100 U/ml.

RESULTS AND DISCUSSION

Bacteria strains with potential PHA production. Table 1 shows the bacteria strains (**Group A**) isolated from the environments studied, their ability to grow in different carbon sources and their multiple resistance antibiotic phenotype. From the 40 isolates, 27 strains grew well in sucrose, 32 in glucose, 31 in fructose, 22 in propionate and 7 in cellulose. Samples from sites IV and VII presented the highest number of strains, belonging to 10 and 8 different species respectively. 36% (site IV) and 67% (site VII) of the strains grew well in sucrose, glucose, fructose, and propionate. Positive Sudan Black (SB) staining in the distinct carbon sources is shown in Fig. 1, except for cellulose, because bacteria growing in this carbon source showed very weak staining. Although 85% (34 strains) of the isolates in Group A can be SB positive, a wide range of tint can be observed and only 25% (10 strains) were in the

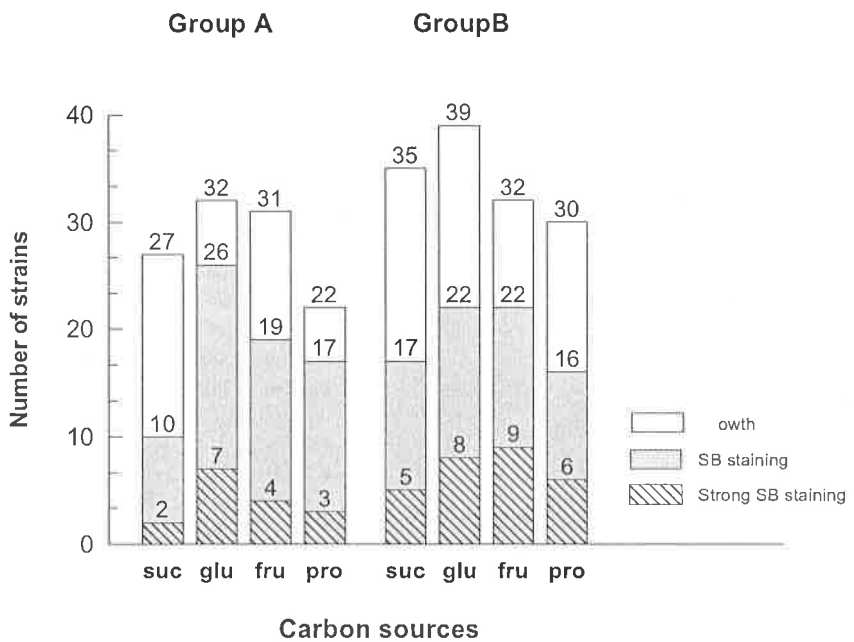

Figure 1 – Growth and intensity Sudan Black (SB) staining of 82 strains growing in minimum unbalanced agar medium plus sucrose, glucose, fructose or sodium propionate (5.0 g/L), stained with a 0.02% ethanolic SB solution (the numbers on the top of the bars indicate the number of strains).

Table 1 – Group A strains (types I to VII) isolated from the sugar cane agroecosystem: growth in different carbon sources and resistance to antibiotics

Types	Strains	Species	Growth in Carbon Sources[1]					Resistance to antibiotics (μg/ml)[2]						
			suc	glu	fru	pro	cel	amp	virg	pen	sm	km	tc	nal
I	AS1	*Enterobacter cloacae*	+	+	+	–	–	150	300	100	–	–	–	–
	AS2	*Enterobacter cloacae*	+	+	+	–	–	150	300	100	–	–	–	–
	AS3	*Enterobacter cloacae*	+	+	+	–	–	150	300	100	–	20	–	–
	AS4	*Enterobacter cloacae*	+	+	+	w	w	150	300	100	–	20	–	–
	AS11	*Pseudomonas sp.*	–	w	+	w	–	150	–	100	–	–	10	20
II	AS5	*Enterobacter cloacae*	+	+	+	–	–	100	300	100	–	–	–	–
	AS7	*Pseudomonas vesicularis*	+	+	+	+	–	50	–	100	–	–	–	–
	AS10	*Pseudomonas picketii*	–	+	+	+	–	150	150	100	20	20	10	–
III	AS12	*Pseudomonas picketii*	+	+	+	–	–	150	–	100	20	20	–	–
	AS12A	*Pseudomonas picketii*	–	+	+	+	–	50	300	100	20	–	–	–
	AS13	*Bacillus anthracis*	+	+	+	–	–	–	–	–	–	–	–	–
	AS13A	*Sporosarcina ureae*	+	+	w	–	–	–	–	–	–	–	–	20
	AS14	*Pseudomonas picketii*	w	w	+	+	w	150	300	100	20	20	10	20
	AS14A	*Bacillus sphaericus*	w	w	+	–	+	–	–	1	20	20	–	20
IV	AS15	*Pseudomonas delafieldii*	+	+	+	+	w	150	–	100	20	–	–	20
	AS15A	*Pseudomonas syringae*	+	+	+	+	–	150	150	100	20	20	–	–
	AS16	*Serratia marcescens*	+	+	+	+	w	50	300	100	–	20	10	–
	AS16A	*Burkholderia cepacia**	–	+	–	w	–	150	–	100	20	20	10	–
	AS17	*Citrobacter freundii*	w	w	+	+	w	50	150	100	–	–	–	–
	AS17A	*Pseudomonas picketii*	–	+	–	+	–	–	–	–	–	–	–	–
	AS17B	*Pseudomonas alcaligenes*	–	w	w	–	–	150	150	10	–	–	10	–
	AS17C	*Pimelobacter simplex*	+	+	+	w	+	–	–	10	–	–	–	20
	AS18	*Pseudomonas aeruginosa*	+	+	+	+	–	150	300	100	20	20	10	20
	AS18A	*Burkholderia cepacia*	+	+	w	+	–	150	300	100	20	20	10	20
	AS18B	*Pseudomonas pseudoalcaligenes*	–	+	+	+	–	150	–	10	20	–	–	–
V	AS76	*Serratia. marcescens*	+	+	+	+	+	150	150	100	20	–	10	–
	AS76A	*Citrobacter freundii*	w	w	+	+	–	150	–	100	–	–	–	–
	AS77	*Pseudomonas alcaligenes*	–	+	w	+	–	150	300	100	20	20	10	20
	AS78	*Agromyces ramosus*	w	w	+	w	–	100	–	10	–	–	–	–
	AS79	*Pseudomonas picketii*	+	+	+	+	w	150	300	100	20	20	10	–
VI	AS81	*Bacillus pasteurii*	+	+	+	+	w	–	–	10	–	–	–	–
VII	AS44	*Proteus vulgaris*	+	+	+	+	w	150	–	100	20	20	–	–
	AS44A	*Aeromonas salmonicida* subsp. *salmonicida*	+	+	+	+	+	200	100	100	20	20	–	–
	AS45	*Aeromonas caviae*	+	+	+	+	+	150	–	100	–	–	–	–
	AS46A	*Pseudomonas succharophila*	+	w	+	+	w	200	–	100	20	–	–	–
	AS46B	*Burkholderia cepacia*	+	+	+	+	+	200	100	10	–	–	10	–
	AS47	*Pseudomonas pseudoalcaligenes*	+	+	–	w	–	50	–	10	–	–	–	20
	AS47A	*Pseudomonas pseudoalcaligenes*	+	+	–	w	–	50	–	100	–	–	–	–
	AS47B	*Aeromonas sobria*	+	+	+	w	+	200	–	100	–	–	–	–
	AS48	*Kurthia gibsonii*	+	+	–	w	w	–	–	–	–	–	–	20

* Formerly, *Pseudomonas cepacia*

[1] suc, sucrose; glu, glucose; fru, fructose; prop, propionate; cel, celulose. Strong growth (+); weak (w) and no growth (–);

[2] amp, ampicillin; virg, virginiamycin; pen, penicillin; sm, streptomycin; km, kanamycin; tc, tetracyclin; nal, nalidixic acid; (–) no growth in the range of antibiotic concentration used.

superior limit of the range (++ or +++). Among the strains growing in sucrose, glucose, fructose, and propionate, *ca* 7%, 22%, 13%, and 14%, respectively, are strong SB stained. These strains were found in the sites II, III, IV and VII, from which, four of them having the highest staining, could be potential PHA producers: two *Pseudomonas picketii*, one *Pseudomonas delafieldii*, and one *Burkholderia* (formerly *Pseudomonas*) *cepacia* (Table 3). It is noteworthy that *P. delafieldii* is strong SB stained when growing either on sucrose, glucose, fructose or propionate. The environments IV and VII are related, directly or not, to vinasse and, therefore, they are likely under stress.

In **Group B** (field experiments using sugarcane pieces), from the 42 strains isolated, 35 grew well in sucrose, 39 in glucose, 32 in fructose, and 30 in propionate (Table 2). The highest number of different species was found in samples from sites XI and XII. Also in Group B, 30 strains (71%) were SB positive in the different carbon sources tested and *ca* 30% (12 strains) was in the superior limit of the range. Among the strains growing in sucrose, glucose, fructose, and propionate, *ca* 14%, 20%, 28%, and 20%, respectively, are strong SB stained (Figura 1). Seven of these strains, from field experiments IX, X, XI and XII, could be potential PHA producers: *Pseudomonas picketii* (3 strains) using propionate as the main C source; *Pseudomonas delafieldii* (3 strains) using sucrose as the main C source and glucose, fructose or propionate; and *Burkholderia cepacia* (1 strain) using glucose and fructose (Table 3). Interestingly, from the 10 strains identified in the field experiment XI (sugarcane plantation burned before harvesting), 5 (50%) were strong SB stained. In the sample from not burned plantation, 25% of the isolated strains were strong SB stained.

In both Groups A and B, eleven strains (5 *P. picketii*, 4 *P. delafieldii*, and 2 *Burkholderia cepacia*) presented the strongest Sudan Black staining intensity (+++) in the indicated C source, and simultaneously, showed multiple resistance to antibiotics (Table 3), which may represent an advantage for biotechnological PHB production. The environments III, IV, VI, VII, IX, XI, and XII could be considered as habitats where bacteria are submitted to unbalanced nutritional growth condition, caused by the introduction of vinasse utilized in the fertirrigation and the use of fire in the sugarcane plantation. These environmental conditions could exert a positive selective pressure for potential PHA producers strains. From the 11 strongest SB staining strains, 8 came from these environments (*ca* 73%). Four *P. delafieldii* strains (AS15, AS60, AS61A, and AS64) were highly SB stained in sucrose, a cheaper C source, and also, multiresistant to antibiotics, which may represent a good potential for biotechnological purpose. Production of PHAs by *P. delafieldii* has not yet been reported in the literature although synthesis of PHAs is a common feature of pseudomonads when grown on hydrocarbons, and expression of PHA synthase gene from *P. aeruginosa* in *E. coli* leads to PHA accumulation in the cells growing in LB medium containing fatty acids (21).

Bacteria strains antibiotics-resistant. A total number of 35 strains with multiple resistance to antibiotics was isolated from all the environments investigated in **Group A**. Among these strains, *ca* 46% belong to genus *Pseudomonas* (Table 1). The sets of samples collected from the stabilization pond, IV ('*gelose*') and VII (wastewater), show high species variability (16 different species) with multiple resistance to antibiotics. These environments, as pointed out in the previous item, are related to vinasse and are possibly under stress. But from the vinasse collected straight from the fermentors (temperature between 80 and 90°C), only a strain of *Bacillus pasteurii* was isolated, a typical endospore-forming bacterium. The majority of the bacteria strains were resistant to penicillin (90%) and ampicillin (*ca* 82%) and thirty-three strains were simultaneously amp/pen-resistant. The percentage of isolates resistant to virg and sm was *ca* 47% and 42% respectively. The percentage of isolates resistant to km, tc or nal is lower than 40% in each case. From the 22 soil strains related to the large stabilization pond (III, IV, V), *ca* 45% showed multiple resistance to five or more antibiotics.

A total amount of 38 strains with multiple resistance to antibiotics was isolated from all the environments investigated in **Group B**. Among these strains, 34% belong to genus *Pseudomonas* (Table 2). Variable numbers of strains with multiple resistance were isolated either from restricted environments of the stabilization pond or from soil of burned and unburned sugarcane plantations. Nine strains showed multiple resistance to 5-7 antibiotics, and 7 of them originated from samples X (not burned) and XI (burned). The majority of the bacteria strains was ampicillin-resistant (*ca* 97%) and penicillin-resistant (*ca* 95%). Thirty-six strains were simultaneously amp/pen-resistant. Forty-two percent

Table 2 – Group B strains (types VIII to XII) isolated from the sugar cane agroecosystem: growth in different carbon sources and resistance to antibiotics

Types	Strains	Species	Growth in Carbon Sources[1]					Resistance to antibiotics (μg/ml)[2]						
			suc	glu	fru	pro	cel	amp	virg	pen	sm	km	tc	nal
VIII	AS49	*Klebsiella pneumoniae subsp. pneumoniae*	+	+	+	+	+	200	300	100	–	20	–	–
	AS50	*Pseudomonas stutzeri*	+	+	w	w	–	200	–	100	20	20	–	–
	AS50A	*Pseudomonas delafieldii*	+	+	+	+	–	150	100	100	–	–	–	–
	AS51	*Klebsiella. pneumoniae subsp. pneumoniae*	+	+	+	+	+	200	300	100	–	–	–	–
	AS51A	*Kluyvera cryocrescens*	+	+	w	w	–	200	–	100	–	–	–	–
	AS51B	*Pseudomonas pseudoalcaligenes*	w	+	w	w	w	200	150	100	–	–	–	20
IX	AS68	*Escherichia hermannii*	+	+	+	+	–	150	–	100	–	–	–	–
	AS68A	*Bacillus cereus*	+	+	+	+	+	100	–	100	–	–	–	–
	AS69	*Bacillus sphaericus*	+	+	w	+	w	–	–	–	–	–	–	20
	AS69A	*Pseudomonas stutzeri*	–	w	w	+	–	50	100	100	20	20	–	–
	AS70	*Bacillus cereus*	+	+	+	+	+	150	–	100	–	–	–	–
	AS70A	*Escherichia hermannii*	+	+	+	+	w	100	–	100	–	–	–	–
	AS70B	*Pseudomonas picketii*	+	+	+	+	+	100	150	100	20	20	–	–
	AS71	*Escherichia hermannii*	+	+	+	w	–	150	–	100	–	–	–	–
	AS71A	*Bacillus sphaericus*	+	+	+	+	w	–	–	–	–	–	–	20
X	AS64	*Pseudomonas delafieldii*	+	+	+	w	–	150	300	100	–	–	10	20
	AS64A	*Serratia marcescens*	+	+	+	+	+	150	150	100	–	–	10	–
	AS65	*Pseudomonas delafieldii*	w	+	+	w	–	150	–	100	–	–	10	20
	AS66A	*Escherichia coli*	+	+	+	+	w	100	–	100	–	–	–	–
	AS67	*Bacillus cereus*	+	+	+	+	+	150	–	100	–	–	–	–
	AS67A	*Serratia marcescens*	+	+	+	w	+	150	150	100	20	–	10	–
	AS67B	*Pseudomonas picketii*	+	+	–	+	w	150	300	100	20	20	–	20
	AS67C	*Bacillus cereus*	+	+	+	+	+	150	–	100	–	–	–	–
XI	AS72	*Burkholderia cepacia**	+	+	+	+	–	150	300	10	20	–	10	20
	AS72A	*Aeromonas salmonicida subsp. smithia*	+	+	+	+	w	150	–	–	–	–	–	20
	AS72B	*Bacillus larvae*	+	+	+	+	+	150	–	10		–	–	
	AS72C	*Enterobacter(Erwinia) nimipressuralis*	+	+	+	w	+	150	–	100	–	–	–	–
	AS73	*Burkholderia cepacia*	+	+	+	+	–	150	300	100	20	–	10	20
	AS74	*Bacillus cereus*	+	+	+	+	+	150	–	100	–	–	–	–
	AS74A	*Enterobacter(Erwinia) nimipressuralis*	+	+	+	w	+	50	–	100	–	–	–	–
	AS74B	*Enterobacter cloacae*	+	+	+	w	+	50	–	10	–	–	–	–
	AS75	*Pseudomonas picketii*	+	+	w	+	–	150	300	100	20	20	10	20
	AS75A	*Bacillus alcalophilus*	+	+	+	–	+	–	–	–	–	20	–	20
XII	AS60	*Pseudomonas delafieldii*	+	+	+	+	+	150	–	100	20	–	–	20
	AS60A	*Pseudomonas syringae*	w	+	–	–	–	150	–	100	20	–	–	20
	AS61	*Escherichia hermannii*	w	w	w	+	–	150	100	100	–	–	–	–
	AS61A	*Pseudomonas delafieldii*	+	+	+	+	+	150	–	100	–	–	–	20
	AS61B	*Arthrobacter amescens*	+	+	+	+	+	–	–	–	–	–	–	–
	AS62	*Klebsiella pneumoniae subsp. pneumoniae*	+	+	+	+	+	150	300	100	–	–	–	–
	AS62A	*Bacillus cereus*	+	+	+	+	+	50	–	100	–	–	–	–
	AS62B	*Bacillus coagulans*	w	+	+	+	w	–	–	–	–	–	–	20
	AS63	*Pseudomonas aeruginosa*	0	w	w	+	0	150	300	100	20	20	10	20

* Formely *Pseudomonas cepacia*

[1] suc, sucrose; glu, glucose; fru, fructose; prop, propionate; cel, celulose. Strong growth (+); weak (w) and no growth (–);

[2] amp, ampicillin; virg, virginiamycin; pen, penicillin; sm, streptomycin; km, kanamycin; tc, tetracyclin; nal, nalidixic acid; (–) no growth in the range of antibiotic concentration used.

Table 3 – Bacteria strains isolated from the sugarcane agroecosystem, with their respective potential for producing polyhydroxyalcanoates (Sudan Black stained) in the carbon sources tested, and their resistance to antibiotics.

Strains Group A*	Source	Species	Carbon Source				Antibiotics
			suc	glu	fru	pro	
AS10	II	*P. picketii*	–	+++	–	++	Amp/Tc/Sm/Km/Nal/Virg/Pen
AS12	III	*P. picketii*	–	++	++	–	Amp/Sm/Km/Pen
AS12A	III	*P. picketii*	–	++	–	+++	Amp/Sm/Virg/Pen
AS15	IV	*P. delafieldii*	+++	+++	+++	+++	Amp/Sm/Nal/Pen
AS15A	IV	*P. syringae*	–	–	++	–	Amp/Sm/Km/Virg/Pen
AS16A	IV	*B. cepacia**	–	++	–	–	Amp/Tc/Sm/Km/Virg/Pen
AS17C	IV	*P. simplex*	++	–	–	–	Nal/Pen
AS18A	IV	*B. cepacia**	–	+++	–	–	Amp/Tc/Sm/Km/Nal/Pen
AS44A	VII	*A. salmonicida*	–	++	–	–	Amp/Sm/Km/Virg/Pen
AS46A	VII	*P. saccharophila*	–	–	++	–	Amp/Sm/Pen
Group B*			Suc	Glu	Fru	Pro	
AS50A	VIII	*P. delafieldii*	–	++	++	++	Amp/Nal/Virg/Pen
AS60	XII	*P. delafieldii*	+++	+++	+++	+++	Amp/Sm/Nal/Pen
AS61A	XII	*P. delafieldii*	+++	++	++	+++	Amp/Nal/Pen
AS64	X	*P. delafieldii*	+++	+++	+++	–	Amp/Tc/Sm/Nal/Pen
AS65	X	*P. delafieldii*	–	++	++	–	Amp/Tc/Nal/Virg/Pen
AS67B	X	*P. picketii*	–	–	–	+++	Amp/Sm/Km/Nal/Virg/Pen
AS70B	IX	*P. picketii*	–	++	–	+++	Amp/Sm/Km/Virg/Pen
AS72	XI	*B. cepacia**	++	+++	+++	–	Amp/Tc/Sm/Nal/Virg/Pen
AS73	XI	*B. cepacia*	–	–	++	–	Amp/Tc/Sm/Nal/Virg/Pen
AS74A	XI	*E. (Erwinia) nimipressuralis*	–	–	++	–	Amp/Pen
AS74B	XI	*E. cloacae*	–	–	++	–	Amp/Pen
AS75	XI	*P. picketii*	++	++	–	+++	Amp/Tc/Sm/Km/Nal/Virg/Pen

++/+++/ = Sudan Black staining, in the carbon sources tested: sucrose, Suc; glucose, Glu; fructose, Fru; sodium propionate, Pro.
*Formerly *Pseudomonas cepacia*.
Amp, ampicillin; virg, virginiamycin; pen, penicillin; sm, streptomycin; km, kanamycin; tc, tetracyclin; nal, nalidixic acid; (–) no growth in the range of antibiotic concentration used

of the isolates were resistant to virg and/or nal. The percentage of isolates resistant to sm, km or tc is lower than 30% in each case.

Among the 73 isolates with multiple resistance to antibiotics we emphasize the multiple resistance to seven antibiotics of *P. picketii* (AS14), *P. aeruginosa* (AS18), *P. alcaligenes* (AS77) and *B. cepacia* (AS18A) in Group A and *P. picketii* (AS75) and *P. aeruginosa* (AS63) in Group B. More than 95% of the isolates are resistant to amp or pen in the concentration range tested and about 48% are resistant to virg. The unknown proportions and specificity of antibiotics used in the fermentors of the alcohol distillery (sometimes they use a mixture called by them of 'cocktail'), do not allow in the present study to establish any plausible relation between the use of antibiotics in the distillery and the high numbers of antibiotics-resistant strains we isolated. Despite the personal information from technicians of the distillery chemical laboratory on greater use of penicillin, the possibility of some sort of selective pressure of this antibiotic on the microbiota environments here studied, deserves a deeper investigation; otherwise, several strains with multiple resistance to antibiotics, were also isolated from the soil under unburned sugarcane plantation (Table 2, site X, a place in a similar condition to a natural vegetation), and from all the other sites from groups A and B. Although there is a general agreement that the pool of resistance genes in the environment is amplified by the use of antimicrobial agents (12, see *Science*, 264: 359-393, 1994), the mechanisms of gene transfer between bacteria of different origins can spread the resistance genes

efficiently in the microbial world, even in the absence of antimicrobial agents (10).

Microbial diversity and environmental bioindicators. Eighty-two bacteria strains were isolated from the sugarcane agroecosystem. In microbial diversity terms, 16 different genera and 35 different species of bacteria were isolated from the two groups of samples. Group A samples presented 12 different genera and 24 different species and Group B presented 10 genera and 21 species. The microbial diversity showed to be greater in samples related to the wastewater from the stabilization ponds (IV, 10 species; VII, 8 species; XII, 8 species) and the soil sample from the sugarcane plantation burned before harvesting (XI, 8 species). Odum's (18) classical observation that communities with low energy cost for maintaining the entropy (high respiration: biomass ratio), divert their energy supply into diversity, may be happening to the microbiota of the environments here investigated. A high respiration: biomass ratio observed in unproductive soil irrigated with vinasse, in the tableland soil of the Usina Japungu (15) gives support to this theory.

Discussion about biodiversity and environmental stress is controversial. Atlas (1984) pointed out diversity changes in response to environmental stress, showing both tendencies: (a) increase in diversity by selective toxicity, eliminating dominant organism; (b) diversity decreases by elimination of many species due to toxicity or increase in particular populations. Torsvik *et al.* (30) observed diversity decrease in perturbed soil due to agriculture, as compared to undisturbed environments. A molecular analysis of microbial diversity in Amazonia soils, based on PCR amplification of small-subunit rRNA, showed microbial population shifts related to deforestation in the Amazonian forest, with predominance of *Bacillus* and high G+C gram-positive-like sequences in pasture and predominance of *Clostridium* and unclassified bacteria in the forest (4). *Bacillus* seems to be a 'natural indicator' of inhospitable environmental conditions and their endospore forming characteristic certainly explains their occurrence in these situations.

Among the isolates in Group A, sporulating bacteria are present only in site III, soil amended with vinasse. That is the case for the highly virulent animal pathogen *B. anthracis*, with high tolerance range to temperature (-5 to 75 °C), to pH acidity (from 2 to 8) and salt (up to 25% NaCl) (20). It is noteworthy to remind that vinasse causes salinization of soils. In Group B, sporulating bacteria were found in all samples, except for VIII. Nevertheless, the highest percentage of sporulating strains was found in the borders of the large stabilization pond, in a place pemanently covered by wastewater (site IX). Bacteria strains with special ability to degrade recalcitrant compound, like *Burkholderia cepacia* (16), were found in the environments under greater selective pressure (sites IV, VII and XI). These altered conditions, of anthropic origin, may have contributed to an increase in the bacteria diversity and may also explain the greater occurrence of *Bacillus*, an endosporing bacterium. Atlas (3) conclusions are still certainly valid, as he said that though diversity measurements are a reflection of the dynamic status of an ecosystem, they do not show a cause and effect relationship between a particular level of stress and a particular species composition of the community.

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RESUMO

Bactérias isoladas de um agrossistema de cana-de-açúcar: produção potencial de polihidroxialcanoatos e resistência a antibióticos

Neste trabalho, um agrosistema de cana de açúcar em tabuleiro litorâneo do nordeste do Brasil, foi rastreado para obtenção de bactérias capazes de sintetizarem polihidroxialcanoatos (PHA) usando sacarose como principal fonte de carbono. O potencial para sintetizar PHA foi testado

qualitativamente por coloração, com Sudan Black, de colônias crescendo em diferentes fontes de carbono: sacarose, glicose, frutose, propionato e celulose. Num sistema de manejo típico do cultivo da cana-de-açúcar, a plantação é queimada antes de cada colheita e a vinhaça, subproduto da produção de álcool, é utilizada num sistema de fertirrigação causando, provavelmente, pressões seletivas sobre a microbiota dos ambientes naturais. Oitenta e duas linhagens de bactérias, pertencentes a 16 diferentes gêneros e 35 diferentes espécies foram isoladas. Os dados mostraram que 11 linhagens (13%), 9 das quais pertencentes ao gênero *Pseudomonas*, apresentaram intensa coloração por Sudan Black em algumas das fontes de carbono testadas e mostraram, simultaneamente, múltipla resistência a antibióticos. Resistência a antibióticos, é uma característica vantajosa à produção biotecnológica de PHAs. O número total de isolados com múltipla resistência a antibióticos foi 73, dos quais, 38% pertencentes ao gênero *Pseudomonas*. Entre os isolados, 86% e 43% cresceram na presença de 10-100 U/ml de penicilina e 100-300 µg/ml de virginiamicina. Estes antibióticos são utilizados na Usina em estudo. Os resultados sugerem que alguns ambientes do agrossistema podem ser considerados como habitats onde as bactérias estão submetidas a condições nutricionais desbalanceadas, contibuindo para estabelecimento de linhagens com habilidade potencial de produzir PHAs e, também, para o aumento da diversidade microbiana.

Palavras-chave: poli-β-hidroxialcanoato, PHA, resistência de bactérias a antibióticos, agrossistema da cana-de-açúcar, vinhaça.

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FLOCCULATION OF FINE FLUORITE PARTICLES WITH *CORYNEBACTERIUM XEROSIS*

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ABSTRACT

The treatment of fine particles dispersed in liquids is common in several industries and especially important in mineral processing. The efficiency of settling operations can be substantially increased by flocculation. The aim of this work was to study the flocculation of fine fluorite particles by the bacterium *Corynebacterium xerosis*. Flocculation tests, microelectrophoresis measurements and optical microscopy were used to evaluate flocculation. The results showed that *C. xerosis* cells adhere to the fluorite surfaces promoting the aggregation of the particles. High quality flocs can be obtained rapidly at pH 7.0 using a cell concentration of 40 mg/l, considerably lower than previously reported in the literature. The results are discussed with reference to the surface characteristics of the mineral and of the microorganism.

Key words: adhesion, *Corynebacterium xerosis*, flocculation, fluorite.

INTRODUCTION

At various stages during the processing of minerals it is necessary to separate the aqueous suspensions into their component solid and liquid phases. Typical examples of this are thickening of flotation concentrates, recovery of pregnant leach liquors, and dewatering of tailings. In many cases, the mineral particles settle out of suspension very slowly, so that the liquid-solid separation is slow and incomplete.

The efficiency of such gravity settling operations can be substantially increased by aggregation of the mineral particles. Methods currently used for aggregation and settling of finely divided solids in slurries include pH adjustment, increasing ionic strength, introduction of inorganic coagulants such as aluminum and ferric salts, and introduction of organic flocculants such as starch and long chain synthetic flocculants (10,15,16). These methods, in addition to increasing costs, can also lead to the release of polluting waste materials.

Finely divided mineral particles can also be flocculated by microorganisms. An isolate of *Arthrobacter* has been shown to promote the settling of fines in manganese ore washings (5). The bacterium *Mycobacterium phlei* (7,14) and the yeast *Candida parapsilosis* (12,13) improved settling rates in mineral suspensions, but flocculation was not as good as that obtained with commercial long chain flocculants. Most microorganisms adhere to solid surfaces if the charge and hydrophobic interactions between the cells and the solid surface are favorable, but do not always promote flocculation of mineral

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particles. The mechanism of this flocculation appears primarily to be cell adhesion and bridging between the particles.

Van Loosdrecht *et al.* (17,18) showed that cells of *M. phlei* and *Corynebacterium xerosis* were highly hydrophobic and negatively charged. Since previous work has shown that the former microorganism does not effectively promote flocculation of fluorite (8), we investigated the potential of the latter, *C. xerosis,* to flocculate fine fluorite particles. The effect of settling time, cell concentration, and pH of the suspension on solids settling was studied. The results are discussed in terms of the surface properties of the mineral and of the microorganism.

MATERIALS AND METHODS

Fluorite

Fluorite crystals (99% CaF_2) were obtained from a mining company (Criciúma - Santa Catarina, Brazil). The crystals were ground in a laboratory mill. The particle size distribution of the sample used for flocculation tests was determined by laser difraction in a Malvern Particle Size Analyzer Model 3601 (Table 1).

Table 1. Particle size distribution of fluorite.

Size range	Weight (%)
larger than 40 μm	3
40 - 30 μm	9
30 -20 μm	18
20 - 10 μm	29
10 - 5 μm	18
5 - 1 μm	21
smaller than 1 μm	2

Microorganisms

The bacterium *Corynebacterium xerosis*, from Caroline Biological Supply Company - USA, was grown for 72 hours in nutrient broth. Cultures were produced in 300 ml flasks, continuously shaken at 37°C. Cells were harvested by centrifugation, washed twice in distilled water and finally suspended in water to give a 2 g/l suspension. This stock cell solution was kept at 5°C and used in flocculation studies within 48 hours, either neat, or at various dilutions.

Flocculation tests

Flocculation studies were carried out using a standard "Jar Test" apparatus. Settling tests were performed by preparing 1000 ml suspensions containing 10 g/l of fluorite. The pH of the medium was adjusted as required and the microorganisms added to the mineral suspension. The suspensions were mixed by stirring for 1 min at 250 rpm to promote a uniform distribution of the cells. The stirrer speed was reduced to 50 rpm for 2 min, to create low shear conditions and allow floc formation. Particles were then allowed to settle under gravity and samples were withdrawn at set time intervals from 10 cm below the water surface.

The flocculation efficiency was evaluated by the following parameters:

- naked eye and microscope observation of floc quality;
- residual turbidity in nephelometric units of turbidity (NTU);
- solids removal from water.

The solids concentration was determined gravimetrically. The solids removal percentage (or flocculation efficiency) was calculated using the relationship.

$$E(\%) = 100 \times \frac{(Ci - Cf)}{Ci}$$

where: Ci = initial concentration of solids
Cf = concentration of solids after time t

Zeta potential measurements

The electrokinetic properties were studied as a function of pH using a Rank Brothers Ltd microelectrophoresis apparatus. *C. xerosis* cells and fluorite particles were suspended in water containing $1x10^{-3}$ M $NaNO_3$ and the pH was adjusted by addition of NaOH and HNO_3. For each pH value 20 readings were taken and averaged. The zeta potential was calculated from electrophoretic mobility using the Smoluchowsky equation (9).

RESULTS

Fig. 1 shows photomicrographs demonstrating the flocculation of fluorite by *C. xerosis*. Fig. 1a shows dispersed fluorite particles in the absence of the microorganisms. In the presence of *C. xerosis* cells, particles are agglomerated (Fig. 1b). These fluorite/cell aggregates settle very rapidly in water allowing an almost complete solids removal and a high clarification of the water.

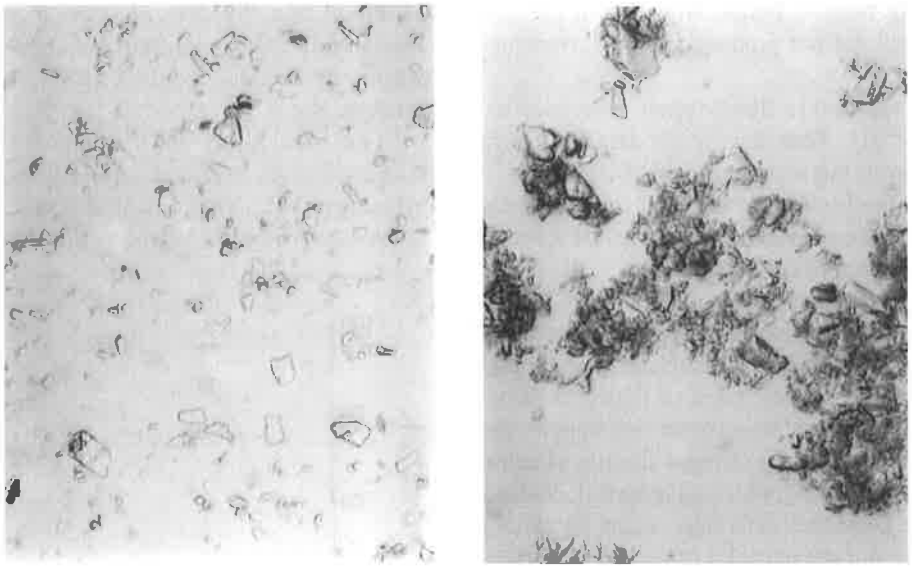

Figure 1. Photomicrographs of fluorite particles suspended in water (a) and aggregated in the presence of *Corynebacterium xerosis* (b). Magnification 100x.

The effect of settling time on flocculation efficiency in the presence of 40 mg/l *C. xerosis* cells at pH 7.0 is shown in Fig. 2. The result is compared to a plot obtained in the absence of flocculant. In the presence of bacteria, near maximum flocculation is obtained within 30 s after the beginning of settling. The same test procedure was used to evaluate the effect of settling time on residual turbidity, which was decreased from 130 NTU to 20 NTU in approximately 5 min in the presence of *C. xerosis* (Fig. 3).

Fig. 4 shows the flocculation results as a function of cell concentration. Good flocculation is obtained at a microorganism dosage of 20 mg/l and maximum flocculation at 40 mg/l. About 96 % of the solids present in the suspension were removed

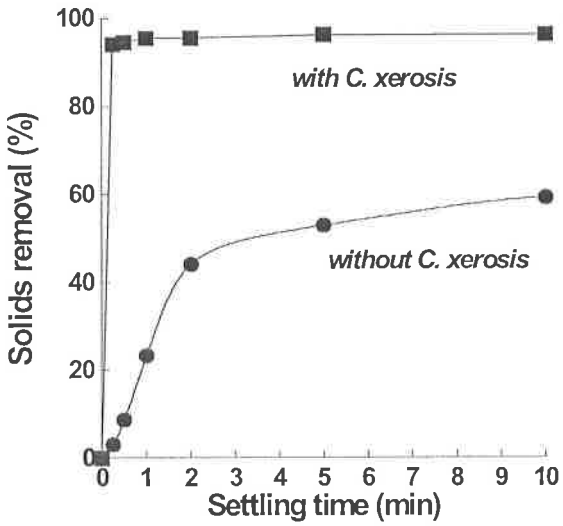

Figure 2. Solids removal efficiency of fluorite suspensions as a function of settling time in the presence or absence of 40 mg/l of *Corynebacterium xerosis* (pH 7.0 +/- 0.2).

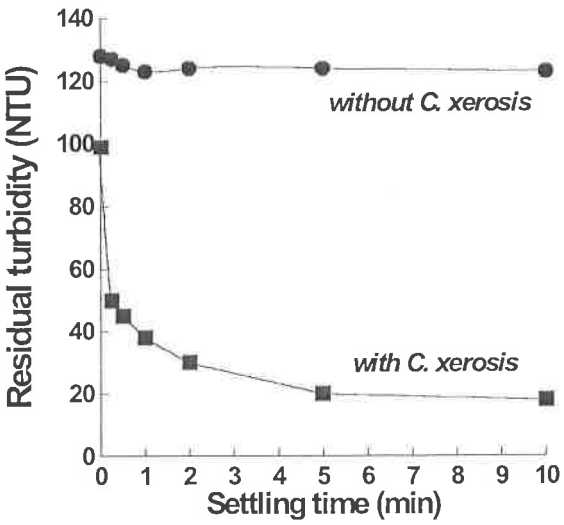

Figure 3. Residual turbidity of fluorite suspension as a function of settling time in the presence or absence of 40 mg/l of *Corynebacterium xerosis* (pH 7.0 +/- 0.2).

in a settling time of 1 min. Higher concentrations than 40 mg/l did not produce any improvement in floc quality.

Fig. 5 represents flocculation efficiency as a function of pH. Also shown are data for fluorite flocculation in the absence of microorganisms. At the bacterial cell concentration used, the suspensions were readily flocculated at pH values of 4.0 - 9.0, with an optimum pH of around 7.0.

Fig. 6 displays the zeta potential of *C. xerosis* cells and of fluorite particles as a function of pH. The *C. xerosis* cells had an isoelectric point at pH 2.0 while the isoelectric point of fluorite was near pH 10.0. Negatively charged organisms should ready adhere to the positively charged fluorite at neutral pH values. At pH 7.0, which gave best flocculation results, the *C. xerosis* cells have a zeta potential of -30 mV and fluorite particles of about + 60 mV.

DISCUSSION

The experimental data indicate that *C. xerosis* cells adhere to fluorite particles and promote flocculation of fine fluorite suspensions over a range of pH values. The quality of the flocs obtained are comparable to those produced with long chain polymers. The flocs are very "tight" and dense and contain little water. The flocculation occurs rapidly, within 1min., showing that these bacteria are more efficient than the *Arthrobacter* species used by Deshpande (5) for clearing manganese mining wastewaters, which required a minimum of 30 min settling time.

The mechanism of flocculation has been discussed elsewhere and primarily occurs by cell bridging (7,14). This mechanism is specially efficient in systems where the microorganism is smaller or of

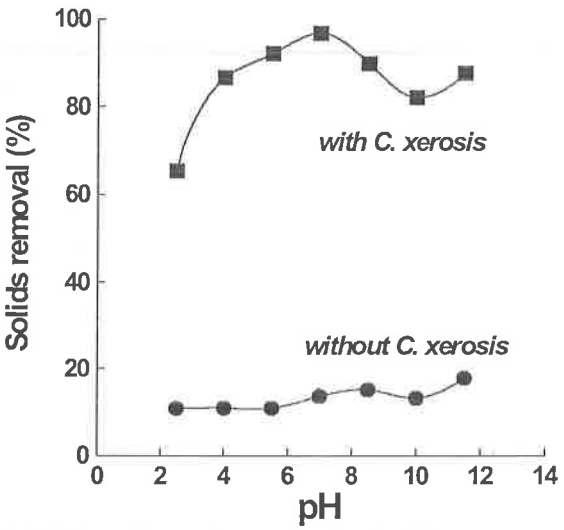

Figure 5. Solids removal efficiency of fluorite suspensions as a function of pH in the presence or absence of 40 mg/l of *Corynebacterium xerosis* cells (1 min settling time).

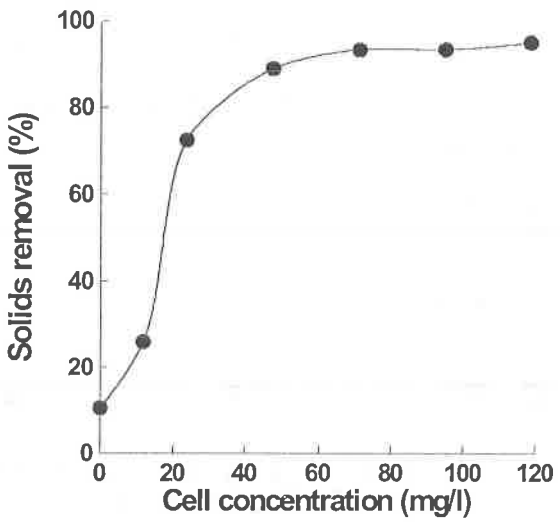

Figure 4. Solids removal efficiency of fluorite suspensions as a function of *Corynebacterium xerosis* cells (pH 7.0 +/- 0.2, 1 min settling time).

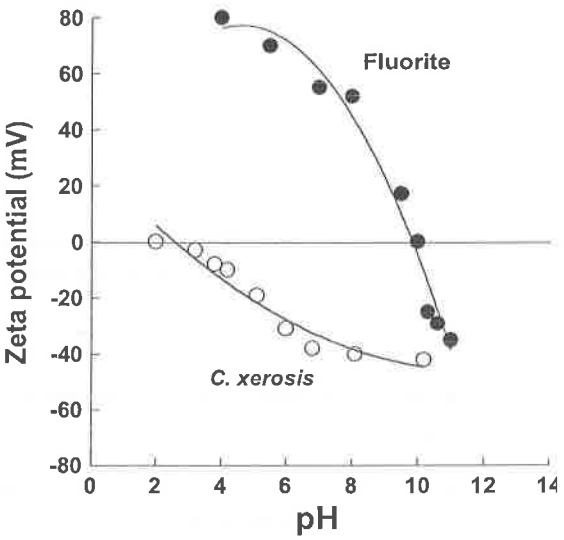

Figure 6. Zeta potential of *Corynebacterium xerosis* cells and fluorite particles as a function of pH. Ionic strength mantained by addition of $1x10^{-3}$ M $NaNO_3$.

a similar size to the particles (6). In the present case, the median size of the particles was 12.5 μm and only 2% of the fluorite has a particle size smaller than 1 μm (approximate size of *C. xerosis* cells).

In many cases, the adherence of a microorganism to a mineral surface can be predicted by a calculation based on the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (6,18,19). According to this theory, the total long range interaction (> 1nm) between surfaces is a summation of van der Waals and coulombic interactions (1,4). This theory explains the optimum flocculation behavior at neutral pH values in the fluorite - *C. xerosis* system. At pH 7.0 the fluorite has a high positive charge (about + 60 mV) and the bacteria a high negative charge (about - 30 mV).

However, a specific adsorption interaction should not be neglected. Bacteria having similar electrokinetic behavior were not able to promote the same degree of flocculation of fluorite (8). It has been shown that some Gram-negative bacteria have specific adsorption mechanisms to solid surfaces, associated with the surface lipopolysaccharides (3), and it is possible that a similar type of interaction may occur with this Gram-positive organism. Gram negative bacteria tested by us had no effect on settling (results not shown). However, the members of the *Acintomycetaceae* have previously been shown to promote flocculation of fine particles (5) and one possibility is that this is due to the distinctive nature of their cell wall material (11). In addition, the *Corynebacterium* group can produce short chains of cells because of incomplete separation after cell division (2) and this leads to a pseudo-polymeric structure, promoting cell bridging.

The concentration of *C. xerosis* required to flocculate fine fluorite suspensions is lower than that of other microorganisms reported in the literature. For instance, flocculation of hematite suspensions with the yeast *Candida parapsilosis* requires about 50 kg/t of bioflocculant (12), while flocculation of fluorite with *C. xerosis* is possible with only 4 kg/t. However, the dosage required is still higher than that used in flocculation with long synthetic polymers. A long chain polyacrylamide (average molecular weight $1x10^6$) flocculates some suspensions at concentrations below 0.1 kg/t.

Microorganisms are potential low cost flocculants for solid-liquid operations in mineral processing. Further studies are necessary to increase their competitiveness with common flocculants used in the mining industry. These will include research to identify more bacterial strains able to adhere to mineral surfaces, investigate the mechanisms involved, develop procedures to isolate the biochemical fraction responsible for flocculation, and to optimize the production of the bioflocculants on large scale.

ACKNOWLEDGMENTS

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RESUMO

Floculação de partículas finas de fluorita com a *Corynebacterium xerosis*

O tratamento de partículas finas dispersas em líquidos é comum em diversas indústrias e especialmente importante no processamento de minérios. A eficiência de operações de sedimentação pode ser substancialmente aumentada com a floculação. O objetivo deste trabalho foi estudar a floculação de partículas de fluorita com a bactéria *Corynebacterium xerosis*. A metodologia empregada para avaliar a floculação incluiu experimentos de floculação, microeletroforese e microscopia ótica. Os resultados demonstraram que as células da *Corynebacterium xerosis* se aderem na superfície da fluorita, promovendo a agregação das partículas. Flocos de alta qualidade podem ser obtidos rapidamente em pH 7,0 a uma concentração de microrganismos de 40 mg/l, dosagem esta consideravelmente menor do que aquelas previamente relatadas na literatura. Os resultados são discutidos em termos das propriedades superficiais do mineral e do microrganismo.

Palavras-chave: adesão, *Corynebacterium xerosis*, floculação, fluorita.

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PRODUCTION OF BIOSURFACTANT BY HYDROCARBON DEGRADING *RHODOCOCCUS RUBER* AND *RHODOCOCCUS ERYTHROPOLIS*

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ABSTRACT

There is world wide concern about the liberation of hydrocarbons in the environment, both from industrial activities and from accidental spills of oil and oil-related compounds. Biosurfactants, which are natural emulsifiers of hydrocarbons, are produced by some bacteria, fungi and yeast. They are polymers, totally or partially extracellular, with an amphipathyc structure, which allows them to form micelles that accumulate at the interface between liquids of different polarities such as water and oil. This process is based upon the ability of biosurfactants to reduce surface tension, blocking the formation of hydrogen bridges and certain hydrophilic and hydrophobic interactions. The ability of biosurfactant production by five strains of *Rhodococcus* isolated from oil prospecting sites was evaluated. Surface tension measurement and emulsifying index were used to quantify biosurfactant production. The influence of environmental conditions was also investigated – pH, temperature, medium composition, and type of carbon source – on cell growth and biosurfactant production. Strain AC 239 was shown to be a potential producer, attaining 63% of emulsifying index for a Diesel-water binary system. It could be used, either directly on oil spills in contained environments, or for the biotechnological production of biosurfactant.

Key words: biosurfactant, hydrocarbon degradation, *Rhodococcus sp*

INTRODUCTION

The annual release of crude oil in the oceans is estimated to be around 1.7 to 1.8 metric tons (11) and the impact of this pollution can be severe environmental imbalance. The microbial biodegradation of hydrocarbons appears to be a promising tool to control such pollution, and it is not surprising that much research is in under progress to study the biochemistry and the genetics involved in this activity (4). Biosurfactants are directly involved in the process of oil and oil-related product removal from the environment. These bio-molecules are produced by several strains of bacteria and fungi. Bushnell and Haas (1) were among the first to demonstrate bacterial production of biosurfactants by isolating *Corynebacterium simplex* and strains of *Pseudomonas* in a mineral media, containing either kerosene, mineral oil or paraffin.

The genus *Rhodococcus* – bacteria with a diverse and efficient metabolism – is also able to transform, biodegrade or utilize as carbon source several hydrophobic compounds such as hydrocarbons, chlorinated phenols, steroids, lignin, coal and crude

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oil. This capability could be of great commercial and industrial importance.

Rhodococcus are aerobic, Gram-positive, non-motile, nocardioform actinomycetes, with a life cycle alternating between cocci and small rods, sometimes showing small filamentous projections (7).

Diesel oil is an excellent model for studying hydrocarbon biodegradation, since it is constituted of a variety of these molecules, such as paraffin, olefins, naphtha and aromatic compounds. The molecular weight of the hydrocarbons present in Diesel is also variable, with molecules containing from 9 to 20 carbon atoms. The ASTM, through the standards issued in ASTM D 975, classifies three different types of Diesel, 1-D, 2-D and 4-D. In Brazil, however, there is only one classification, issued by standard 04 of The National Oil Council. Brazil is a large diesel producer and user, and there have been several reports on Diesel spills in the environment, besides other pollution problems related to the extensive use of this fuel (2).

The aims of this work are to identify suitable bacterial strains for biosurfactant production, and to study their metabolism in order to obtain these biomolecules on a commercial scale, to be safely used in pollution control.

MATERIALS AND METHODS

Bacterial strains. The following strains were obtained from the alkanotrophic bacteria collection of The Microbial Ecology and Genetics Institute of The Science Academy of Ural Division, Russia: AC 74 *Rhodococcus ruber*, AC 87 *Rhodococcus ruber*, AC 239 *Rhodococcus ruber*, AC 265 *Rhodococcus erythropolis*, AC 272 *Rhodococcus erythropolis*. As negative controls, two biosurfactant non producing strains of *Escherichia coli* and *Bacillus cereus* were used.

Media used and growth conditions. Cultures of bacteria were maintained on either nutrient agar, or Bushnell and Haas mineral agar, MMBH, (1) containing 1% (v/v) of Diesel, previously sterilized by filtration through a Millipore membrane with pore size of 0.22 μm. Experiments on growth optimisation and biosurfactant production were performed using three different mineral media: M1, containing $MgSO_4$ 0.20 g/l; $CaCl_2$ 0.02 g/l; KH_2PO_4 1.0 g/l; NH_4NO_3 1.0 g/l; K_2HPO_4 1.0 g/l; $NaNO_3$ 3.0 g/l; lactose 1.0 g/l; Diesel 1% (v/v); $ZnSO_4.7H_2O$ 0.525 g/l; Mn $SO_4.4H_2O$ 0.20 g/l; $CuSO_4.5H_2O$ 0.705 g/l; $NaMoO_4.2H_2O$ 0.015 g/l; $CoCl_2.6H_2O$ 0.20 g/l; H_3BO_3 0.015 g/l; $FeSO_4.7H_2O$ 0,01 g/l; M2, containing $NaNO_3$ 7.0 g/l; K_2HPO_4 1.0 g/l; KH_2PO_4 0.50 g/l; KCl 0.10 g/l; $MgSO_4.7H_2O$ 0.50 g/l; $CaCl_2$ 0.01 g/l; $FeSO_4.7H_2O$ 0.01 g/l; yeast extract 0.10 g/l; Diesel 1% (v/v); and M3, containing $MgSO_4$ 0.20 g/l; $CaCl_2$ 0.02 g/l; KH_2PO_4 2.0 g/l; NH_4NO_3 2.0 g/l; $FeCl_3.6H_2O$ 0.05 g/l; K_2HPO_4 2.0 g/l; glucose 10.0 g/l; and Diesel 1% (v/v). All chemicals were of analytical grade.

Diesel used in the experiments. Standard Diesel, without additives, was obtained directly from Petrobrás "Alberto Pasqualini" Oil Refinery, Canoas, RS, Brazil.

Culture conditions. Cultures (in flasks of 125 ml with 30 ml of medium) were inoculated with 5-10% volume aliquot of a overnight preculture grown for 48 hours, and incubated at 37°C, 200 rpm, in a rotatory shaker. Experiments were carried out in triplicate. Microbial growth was quantified by the technique of Miles and Misra (14).

Measurement of surface tension. The change in surface tension of cultures was evaluated by using a Leconde Du Nouy tensiometer. Samples were prepared by centrifuging at 10,000 g for 20 min, and using the supernatant for the assay.

Emulsification index (E24). E24 of culture samples was determined by adding 2 ml of hydrocarbon to the same amount of culture, mixing with a vortex for 2 min, and leaving to stand for 24 hours. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (10).

Carbohydrate assay. Carbohydrate concentration was quantified by the method of Dubois (6). A volume of 0.5 ml of cell supernatant was mixed with 0.5 ml of 5% phenol solution and 2.5 ml of sulfuric acid, and incubated for 15 min before measuring absorbance at 490 nm.

Haemolytic activity. Bacterial strains were tested for haemolytic activity by plating cells onto blood agar and incubated at 37°C for 48 hours.

Antibiotic resistance. Resistance to antibiotics (8) was determined by using Sensi-Discs (Cefan Diagnóstica Ltd.) and nutrient agar according to the instructions of the manufacturer. Isolates were tested for sensitivity to carbenicillin (100 μg), chloramphenicol (30 μg), penicillin (10 UI), tetracycline (30 μg), ampicillin (10 μg), streptomycin (10 μg), erythromycin (15 μg).

RESULTS AND DISCUSSION

Microbial growth on Diesel as sole carbon source. The first set of experiments was a simple growth test on solid medium to allow for a fast strain selection. The three *R. ruber* strains, AC 74, AC 87 and AC 239, showed much better and faster growth than *R. erythropolis* on solid MMBH incubated at 37°C for 96 hours and they were, therefore, selected to be used in the subsequent experiments. Control strains (*Escherichia coli* and *Bacillus cereus*), did not grow or produced any biosurfactant as measured by the methods of E24 and surface tension. All five strains were tested for haemolytic activity, which is regarded by some authors as indicative of biosurfactant production and used as a rapid method for bacterial screening (3, 10, 12). None of strains showed any haemolytic activity, indicating that the use of this rapid method could lead to reduced numbers of selected strains and potential losses of important active microorganisms. None of the *Rhodococcus* strains showed any resistance towards the antibiotics tested (ampicillin, tetracycline, penicillin, erythromycin, chloramphenicol, and streptomycin). The lack of haemolytic activity and antibiotic resistance suggests that these strains might be safely used in field experiments.

Microbial physiology. The three selected strains of *R. ruber* grown in Bushnell and Haas medium were evaluated for biosurfactants production and efficient Diesel emulsification. Strain AC 239 showed faster growth and higher E24 and surface tension reduction, although all strains were able to grow and produce biosurfactants (results not shown). AC 239 strain was selected for optimisation of experiments.

One important feature in scaling up a biotechnological process is the design of culture media. Three different, inexpensive growth inducers were tested: lactose 1.0 g/l (M1), yeast extract 0.10 g/l (M2), and glucose 10.00 g/l (M3). Figs. 1, 2 and 3 show experiments results. M2, containing yeast extract produced in laboratory (16), was more important for cell metabolism than the additional carbon sources tested. Cells growing in M2 also produced more biosurfactant, as indicated by E24 and surface tension when compared to the other media. These results are in accordance with the work of Iqbal *et al.* (10), who demonstrated an enhancement in biosurfactant production, when growing *Pseudomonas aeruginosa* in Bushnell and Hass medium with heptadecane as the sole carbon source.

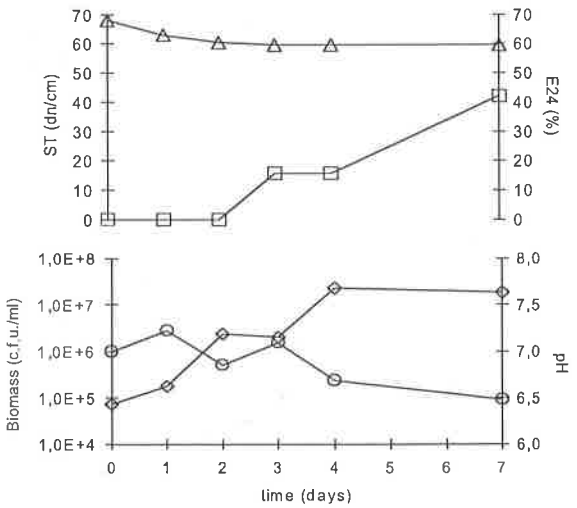

Figure 1 – Growth of *Rhodococcus* AC 239 in medium M1. Initial pH 7.0, 37 °C, 200 rpm, 1% Diesel (v/v). Δ – surface tension, ST, (dynes/cm), □ – E24, (%), ◊ – Biomass (cells/ml), O – pH.

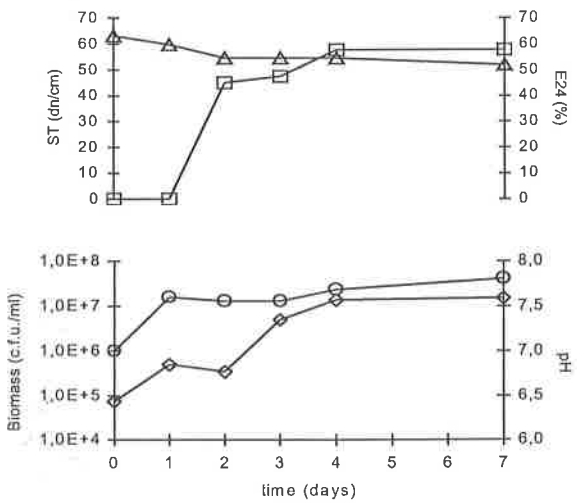

Figure 2 – Growth of *Rhodococcus* AC 239 in medium M2. Initial pH 7.0, 37 °C, 200 rpm, 1% Diesel (v/v). Δ – surface tension, ST, (dynes/cm), □ – E24, (%), ◊ – Biomass (cells/ml), O – pH.

The effects of temperature (30, 37, and 40 °C), pH (6.0, 7.0, and 8.0), and Diesel concentration (1 to 4% v/v), were determined in medium M2. Results presented in Fig. 4 show maximum values for biomass production and E24. The best environmental conditions for *R. ruber* AC 239 growing in M2 were 37°C, 200 rpm, initial pH 7.0, and 1% Diesel (v/v). These conditions can be used to further optimise biosurfactant production and make it economically

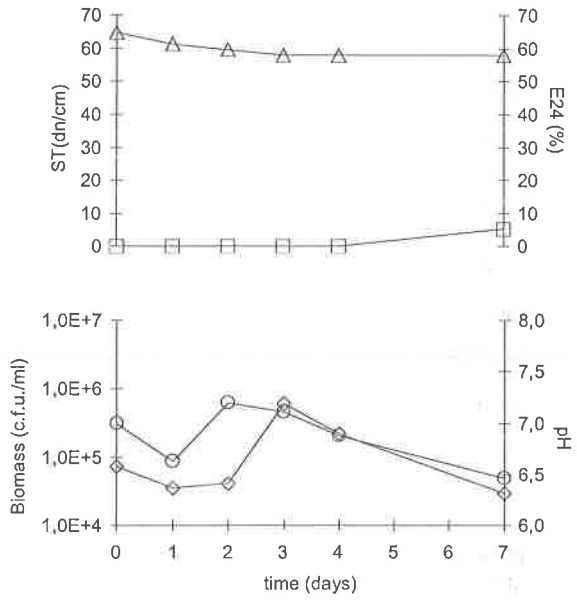

Figure 3 – Growth of *Rhodococcus* AC 239 in medium M3. Initial pH 7.0, 37 °C, 200 rpm, 1% Diesel (v/v). Δ – surface tension, ST, (dynes/cm), □ – E24, (%), ◊ – Biomass (cells/ml), O – pH.

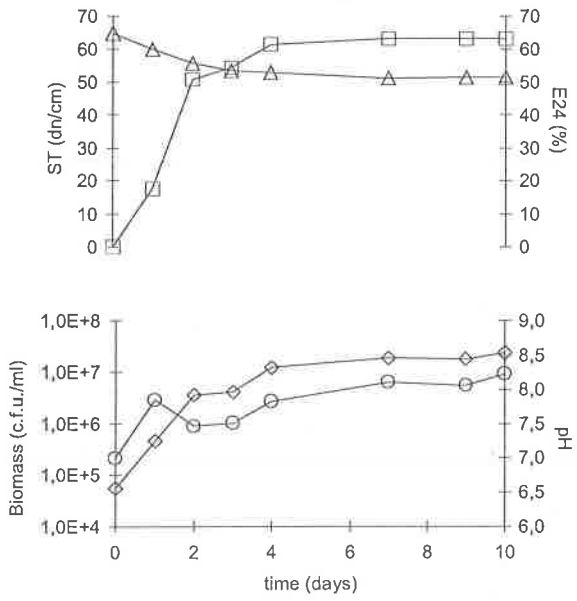

Figure 4 – Growth and biosurfactant production of *Rhodococcus* AC 239 in M2 at pH 7.0, 37 °C, 200 rpm, and 1% Diesel (v/v). Δ – surface tension, ST, (dynes/cm), □ – E24, (%), ◊ – Biomass (cells/ml), O – pH.

acceptable. Maximum values obtained for surface tension reduction were around 20 dynes/cm, comparable to findings by other authors (4, 15), but not economically competitive when compared to recommended values of at least 40 dynes/cm (13). Virtually all emulsifying activity of cultures was associated to cell fraction (Fig. 6). As was shown also by Hafesburg *et al.* (9), for *Rhodococcus* sp. growing on hexadecane. The yield of biosurfactant could almost certainly, therefore, be improved by lysing the cells prior to harvest.

Fig. 5 shows the increase in carbohydrate concentration in the culture medium when strain AC 239 was grown on Diesel. Since there was no carbohydrate in the medium, its appearance and increase along growth might be due to the nature of the biosurfactant released by *Rhodococcus ruber*, even in small amounts, since most of emulsifying activity was associated to cell. Previous research has shown that most of biosurfactant produced by *Rhodococcus* species is a glycolipid-type molecule (17), which further corroborates our findings.

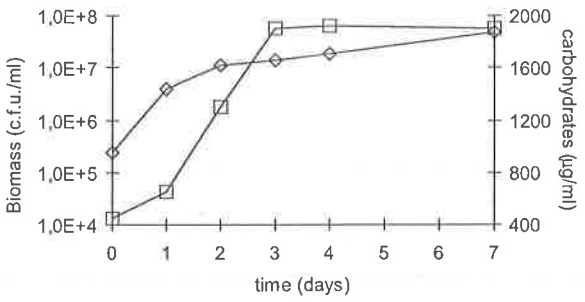

Figure 5 – Carbohydrates concentration in supernatants of *Rhodococcus* AC 239 cultures in M2. Initial pH 7.0, 37 °C, 200 rpm, 1% Diesel (v/v). □ – carbohydrate (µg/ml), ◊ – biomass (cells/ml).

Capability of AC 239 to emulsify other hydrocarbons. Table 1 summarises results obtained for *Rhodococcus* AC 239 growing on several hydrocarbon sources. The results demonstrated that this bacterium was able to grow on all oil related products as sole carbon source, showing variations in the morphological appearance of the cells. There was, apparently correlation between the molecular size of the C-hydrocarbon chain and emulsion formation. The results are particularly interesting with respect to combusted lubricant oil, which is a gas station waste, produced in large amounts. This oil residue is a potential pollution source, besides being an inexpensive potential substrate for industrial scale production of biosurfactant. Although cultures growing as clumps produced biosurfactants, as

demonstrated by results shown in Table 1 and Fig. 6, their E24 values were always 20 to 50% lower than values for cultures where only free cells were present. These observations suggest that this late form of cell growth should be promoted in an industrial process for surfactant bio-production.

Table 1 – Growth characteristics of *R. ruber* AC 239 on different hydrocarbon sources. Medium M2, 37 °C, 200 rpm, pH 7.0, and 7-days cultivation.

Hydrocarbon source	Growth in 7 days	Morphological appearance of colonies		Final pH
		Clumps	Secretion	
Petrol	+	–	+	7.05
Kerosene	+	+	–	7.18
Heptane	+	–	+	7.03
Pentane	+	–	+	7.04
Benzene	+	–	+	7.03
Diesel	+	+	+	8.30
Burned Lubricant oil	+	+	–	7.71
Lubricant oil	+	+	–	8.04

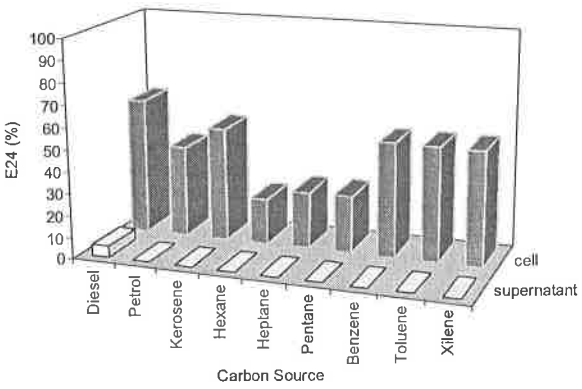

Figure 6 – Emulsifying index (E24) with different hydrocarbon for cell and supernatant fractions of *R. ruber* AC239 in medium M2 at 37 °C, 200 rpm, and 7-days cultivation.

CONCLUSIONS

We identified three strains of *Rhodococcus ruber* and two of *Rhodococcus erythropolis*, isolated from oil wells in Russia, which were able to grow on hydrocarbons as sole carbon sources and to produce biosurfactants. One strain of *R. ruber*, AC 239, was a good biosurfactant producer and was able to grow on several hydrocarbon sources, suggesting its possible exploitation in future biotechnological processes, either directly as a field-released microorganism, or as a biosurfactant producer under controlled conditions. We optimised medium composition and culture conditions for biosurfactant production by this strain, which appears to be associated to cell wall.

Further studies are under way to scale up growth conditions and biosurfactant production in bioreactors, and to identify genes involved in the synthesis of these biomolecules.

RESUMO

Produção de biossurfactantes por *Rhodococcus ruber* e *Rhodococcus erythropolis* degradadores de hidrocarbonetos

Há uma grande preocupação mundial relacionada à liberação de hidrocarbonetos no ambiente, decorrente da atividade industrial e do derrame acidental de óleo e seus componentes relacionados. Biossurfactantes, que são emulsificadores de hidrocarbonetos, são produzidos por algumas bactérias, bolores e leveduras. São polímeros, totalmente ou parcialmente extracelulares, com uma estrutura anfipática, que formam micelas que se acumulam na interface entre líquidos de diferentes polaridades tal como água e óleo. Este processo é baseado na habilidade dos biossurfactantes em reduzir a tensão superficial, impedindo a formação de pontes de hidrogênio e certas interações hidrofílicas e hidrofóbicas. Neste trabalho, foi avaliada a produção de biossurfactantes por 5 cepas de *Rhodococcus* isoladas de sítios de prospecção de óleo. A medição da tensão superficial e o índice de emulsificação foram usados para quantificar a produção de biossurfactante. Investigou-se também a influência de condições ambientais – pH, temperatura, composição do meio, e o tipo de fonte de carbono – no crescimento celular e na produção de biossurfactante. A cepa AC239 demonstrou ser uma potencial produtora de biossurfactantes, apresentando até 63% de índice de emulsificação para um sistema binário. Esta poderia ser usada diretamente em derrames de óleo no meio ambiente ou na produção biotecnológica de biossurfactante.

Palavras-chave: biossurfactante, degradação de hidrocarbonetos, *Rhodococcus sp.*

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DETECTION OF VIRAL PARTICLES IN FECES OF YOUNG DOGS AND THEIR RELATIONSHIP WITH CLINICAL SIGNS

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ABSTRACT

Gross and light microscopic studies of 100 stool specimens of young dogs were carried out. Viral particles were detected in 31% of the analized samples using negative contrast electron microscopic diagnostic technique. *Parvo-like* virus, *corona-like* virus and other non-identified particles were observed in 17%, 7% and 2% of the samples, respectively. *Parvo-like* and *corona-like* viruses were found together in 5% of the samples. More than half (58.82%) of the positive *parvo-like* virus specimens were from dogs aged between 6 weeks and 6 months. 42.85% of the *corona-like* virus positive samples were detected in dogs between 6 weeks and 6 months and a similar percentage was found in dogs older than six months of age. Dual infections with *parvo-like* and *corona-like* viruses were observed in 5% of the samples. Unidentified *virus-like* particles were found in two specimens. 80.63% of the samples containing viral particles were obtained from dogs with diarrhea.

Key words: *Parvo-like* virus, *corona-like* virus, diarrhea, dogs, electron microscopy

INTRODUCTION

Parvovirus (CPV) and coronavirus (CCV) have clearly been established as causes of enteritis and diarrhea in dogs and cats (5, 8, 11, 13, 15). Other viruses, like rotavirus, astrovirus, adenovirus, paramixovirus, calicivirus and picornavirus have been also observed in canine feces (4, 6, 9, 13, 17). Some of these viruses were found with equal frequency in normal feces and feces from animals with diarrhea, and have not been established as enteropathogens yet (11). Rotavirus has been recognized as one of the most important cause of neonatal diarrhea in humans, cattle, swine, sheep and mice (2, 11). In dogs rotavirus has been associated with a neonatal transitory diarrhea with lethal course in a few of them (9). An incidence of anti-rotavirus antibodies has been demonstrated in 79% to 86% of the animals (9, 11). These findings suggest that many animals present subclinical infections during their lives (9).

The incidence of these viruses in Argentina is not known. However, in a preliminary study carried out by the authors on 42 samples of canine feces, no rotavirus particle was detected (1).

The most frequent diagnostic methods for titration of viruses in feces are hemagglutination tests (HA) and enzime-linked immunosorbent assays (ELISA). CPV, CCV and other enteric viruses can be diagnosed

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by isolation from feces, detection by electron microscopy and identification by immune electron microscopy (3, 11).

Serologic tests to detect anti-rotavirus antibodies have been used in research but are not currently available for clinical diagnosis (11).

The objective of the present paper was to relate the clinical features of diarrhea of young dogs with the recognition of viral particles in the feces, through electron transmission microscopy. In order to establish other causes of diarrhea examination of feces through optical microscopy were also carried out.

MATERIALS AND METHODS

Fecal samples. The fecal samples (n: 100) were obtained from dogs submitted to the Small Animal Clinic of La Plata National University for clinical examination. Sixty two had diarrhea as the main problem.

The animals aged between 15 days and 12 months. Dogs were submitted to a complete clinical examination. These findings as well as vaccination status were registered in previously designed protocols.

Gross and microscopic examination of fecal samples. Gross examination of the feces was performed taking into account colour, consistency, presence of mucus and fresh blood. The light microscopy included the evaluation of fatty globules by Sudan III in 95% ethylic alcohol (16). A smear was stained with one drop of New Methylen Blue to count leukocytes, other cells and fibers. Direct coproparasitological examination was also included for identification of tapeworms, ascarids, hookworms, coccidia, oocysts and flagellated trophozoites of giardias.

Negative contrast electron microscopy (1). 1 ml of each fecal sample was homogeneized with 10 ml of phosphate buffered saline (PBS) pH 7.2, and centrifuged at 8,000 *g* during 15 minutes at 4°C. The sediment was discarded and a second centrifugation with a Beckman L7-65 (rotor 70.1 Ti) ultracentrifuge at 45,000 r.p.m. during two hours at 4°C was performed. The sediment was resuspended 1:1 with PBS, and one drop was placed on a cooper grid (carbon substrate), and allowed to stand one minute. The excess was removed with a piece of filter paper. Two grids were negatively stained with 2% phosphotungstic acid, pH 7 during 1 minute, and examined with a JEM 1200 EX II (Jeol) electron microscope. Each grid was scanned among 15 minutes before recorded as negative. The morphological identification was done according to the morphology and size of the viral particles (14).

RESULTS

The dogs were divided in three different groups according to their ages: Group 1: 1week to 6 weeks; Group 2: 6 weeks to 6 months and Group 3: 6 months to 12 months.

Viruses were detected in 31 fecal samples (31%). *CCV-like* viruses (Fig 1.) were found in 12 samples (12%), and *CPV-like* viruses (Fig. 2) were observed in 17 samples (17%).

The distribution of the viral findings according to the age of the animals is shown in Table 1.

The higgest frquency of viruses was observed in animals of Group 2: 10 positive samples (10%) for *CPV-like* viruses and 3 (3%) for *CCV-like* viruses.

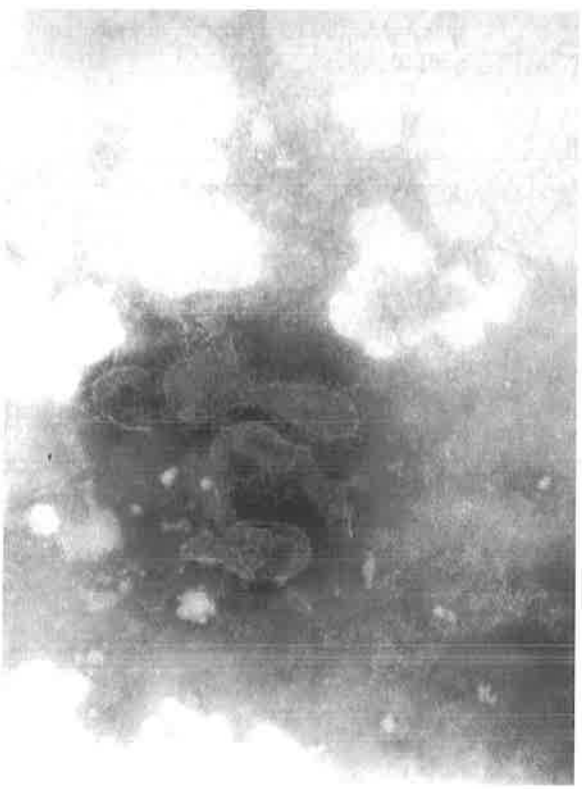

Figure 1 – Electron microscopy showing a *corona-like* virus, x 222K (negative strain).

Table 1 – *Parvo-virus* like (CPV) and *corona-virus* like (CCV) particles in fecal samples of dogs with and without diarrhea.

Virus detected	Ages of dogs												
	1w-6w				6w-6m				6m-12m				
	Normal		Diarrhea		Normal		Diarrhea		Normal		Diarrhea		Total
	n	%	n	%	n	%	n	%	n	%	n	%	%
CPV-L	1	1%	5	5%	1	1%	9	9%	–	–	1	1%	17%
CCV-L	1	1%	–	–	1	1%	2	2%	–	–	3	3%	7%
CPV/CCV-L	–	–	–	–	1	1%	4	4%	–	–	–	–	5%
Unidentified	–	–	–	–	1	1%	1	1%	–	–	–	–	2%
Total	2	2%	5	5%	4	4%	16	16%	–	–	4	4%	31%

W: week- m: month

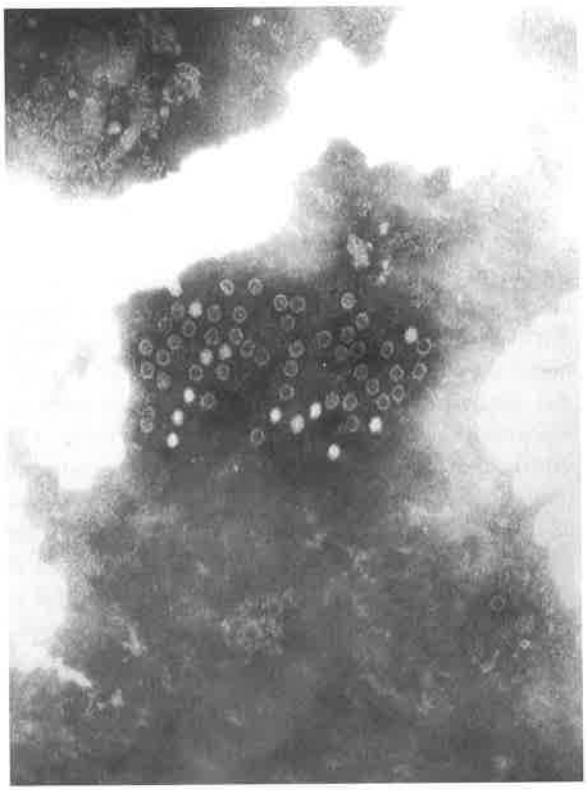

Figure 2 – Electron microscopy showing a group of *parvo-like* virus, x170 K (negative strain).

Five dual infections (5%) with CPV-*like* and *CCV-like* viruses were also observed. They were detected in animals aged between 6 weeks and 6 months. Unidentified virus-like particles were found in two specimens (2%).

Table 2 – Fecal consistency and body temperature of 31 dogs with CPV-like and CCV-like viral particles in their feces.

Consistency	*Parvo-like*	*Corona-like*	Parvo & *corona-like*	unidentified
Normal	2	2	1	1
Soft	4	3	–	1
Liquid	11	2	4	–
Body temperature				
Normal T°	15	5	2	1
Fever	2	2	3	1

The relationship between feces consistency, body temperature of the dogs and presence of viral particles is presented in Table 2.

The microscopic examination indicated that eleven positive fecal samples contained also a few nematod eggs (ascarids and hookworms). Only one sample had tapeworm eggs, and four presented giardias. Fatty globules and leucocytes counts were normal in all samples. Blood was found in 11 of the positive samples and 7 contained mucus.

None of the dogs had been immunized against canine parvovirus or corona-virus.

DISCUSSION

The identification of the CPV in feces can be carried out only during the elimination period of the viruses, which occurs between the 3nd and 9th day of infection (7). As serological or molecular studies were

not done, the low frquency observed in our study may be artificial.

Eighty point sixty three percent of the samples that presented viral particles belonged to dogs with soft (25.80%) and liquid (54.83%) feces. As fatty globules and leukocytes counts were normal, it can be assumed that diarrhea was not a consequence of maldigestion syndromes or bacterial infection (17).

Among 17 fecal samples with *CPV-like* particles, 2 presented normal consistency and 15 were liquid, six of them were also positive for ascarids, and 1 for flagellated trophozoites of giardias because they were in a few number, it was assured that they didn't produce disease.

Two normal, three soft and two liquid feces were found in the *CCV-like* positive fecal samples. This is in agreement with the findings by Pollock and Carmichael (9) who concluded that probably there is not a narrow association between CCV and severe gastroenteritis in dogs.

The results of the present study, showing a low incidence of *CCV-like* infection, may be artificial. This may be due to some problems associated with identification of this virus. The most important characteristic of this virus is the fragile envelope, whose integrity can be affected by the lenght of storage and by the handling of the sample. Since there was a limited control over the time of storage of the samples, a consequent loss in integrity of CCV could have interfered with the unequivocal identification of the virus (14).

Two dogs with *CPV-like* particles in their feces were febrile and 9 of them were depressed and anorexic. These findings are coincident with the results reported by Swango (12), who found that depression and fever in CPV or CCV infected animals are common.

Dual presence of *CPV-like* and *CCV-like* viruses ocurred in 5 dogs (16.12% of the positive samples). This result disagrees with those reported by Scherding (11), who said that the dual infection ocurrs in up to 25 per cent of cases of canine viral enteritis. The dogs with dual infection were more seriously ill than those infected with *CPV-like* or *CCV-like* viruses alone.

Fresh blood was found in 29.41% of the fecal samples containing *CPV-like* particles. This finding is not coincident with our preliminary report (18.60%) (1) and with Pollock and Carmichael (9), who found bloody diarrhea in 50% of the *CPV* infected dogs.

A high percentage of positive samples for *CPV-like* particles was found in puppies under 5 months of age (52.94%), confirming the age susceptibility to *CPV-like* infection described by Hammond and Timoney (6). Dehydration and acidosis develop more quickly in puppies than in older animals. The puppies of this study experienced a severe illness, were anorectic and depressed, and their feces were liquid.

In the present study, *CPV-like* particles were found to be more closely associated with canine gastroenteritis than *CCV-like* particles. This is coincident with the results of Roseto *et al.* (10), who found a prevalence of this virus associated with gastroenteritis.

In this study, rotavirus-like particles were not identified in the samples. Further surveys are needed to investigate the prevalence of this virus.

RESUMO

Detecção de partículas virais em fezes de cães jovens e sua relação com sinais clínicos

Foram analisadas macroscópica e microscopicamente 100 amostras de fezes de cães jovens colhidas em ambulatório clínico. Através de microscopia eletrônica de contraste negativo, foram detectadas partículas virais em 31% das amostras de fezes examinadas. Do total de amostras onde foram detectadas partículas virais, 17% continham partículas *parvo-like,* 7% continhan partículas *corona-like* virus e 2% continham partículas com morfologia não característica. Em 5% das amostras foi detectada a presença simultânea de partículas *parvo-like* e *corona-like*. Do total de amostras de fezes contendo partículas *parvo-like*, 58,82% eram de cães com 6 semanas a 6 meses de idade, o que também foi observado em 42,85% das amostras de fezes contendo partículas *corona-like* foram cães entre 6 semanas e 6 meses e um número semelhante foi encontrado em cães de mais de 6 semanas de idade. Uma dupla infecção de *parvo-like* virus e *corona-like* virus, foi confirmada em 5% dos especimens. Partículas "*virus-like*" não identificadas foram encontradas em 2 indivíduos Das amostras com identificação de partículas virais, 80,64% pertenceram a cães com diarréia.

Palavras-chave: parvo-like virus, corona-like virus, diarréia, cães, microscopia eletrônica

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ADHESIVE PROPERTIES OF AN OUTER STRUCTURE OF *CLOSTRIDIUM PERFRINGENS* TYPE A ISOLATED FROM PIGLETS WITH CATARRHAL ENTERITIS

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ABSTRACT

One strain (S32) of *Clostridium perfringens* type A was isolated from a case of catarrhal enteritis of piglets. This strain was able to adhere to HeLa cells showing an adherence index (AI) of 25.15 ± 1.26 (mean ± 1 standard error of the mean). Treatment of the bacterial cells with trypsin (0.25mg/ml) decreased in 70%-80% the AI and metaperiodate (10mg/ml) abolished completely the adherence, suggesting that the structure responsible for this phenomenon was probably a glycoprotein. Heating of bacterial suspensions (100°C/5 min) before carrying out the adhesion test decreased the AI rendering it equal to the negative controls. Rabbit homologous S32 antiserum inhibited the adherence up to dilutions of 1: 640, at least. The piglet ileal loop assay, carried out with strains S32 and Jab-1 (negative control) demonstrated that the strain S32 was able to adhere to the intestinal epithelial cells when examined after Gram staining. Transmission electron microcopy (TEM) demonstrated that S32 strain displayed a loose fibrillar material not seen with Jab-1. Stabilization of the bacterial cells with homologous antiserum of strain S32, followed by staining with rhuteniun red, revealed loose long fibrillar material on the outer surface of the cells, that sometimes could be seen spreading out from the cells and linking bacterial cells. The question whether this structure might be an adhesin for this strain of *Cl. perfringes* type A, perhaps playing a role in the pathogenesis of the catarrhal enteritis of piglets, is dependent on further studies.

Key words: *Cl. perfringens* type A, adherence, fibrillar structure, enteritis, piglets

INTRODUCTION

Clostridium perfringens type A (CpA), besides causing food poisoning in man, has also been reported as responsible for either necrotic or catarrhal enteritis in piglets, calves, chickens and other animals (7, 8, 9, 21). The exact mechanisms, which could be involved in the early steps of both diseases, however remain obscure. CpA intestinal infection poses a crucial question: how does the microorganism adhere to the

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intestinal epithelial cells of susceptible hosts? (4).

Whatever the disease to be considered it is feasible to assume that the vegetative bacterial cells must adhere to gut epithelial cells before going on with further mechanisms which are different for each enteropathogen (6, 12,13). So there are several reports in the literature on different structures that may act as colonization factors (CFs); putative colonization factors (PCFs); adhesin or adherence factors (AF) for other microorganisms (4, 10, 11).

As far as adhesins of CpA are concerned, no reports have been found in the literature. Airbuckle (1) and Sims *et al.* (18) based upon histological findings of pigs and foals, which died from necrotic enteritis, suggested that *Cl. perfringens* type C (CpC) was able to adhere to the intestinal cells of the diseased animals. More recently other workers have reported this adherence *in vivo* (2). Because CpA was isolated in pure culture from a piglet which died from catarrhal enteritis, the main purpose of this paper was to report the results of the experiments that have been undertaken in order to attempt the visualization of any external structure present on the surface of this bacterium, which could mediate the adherence of this isolate to the gut epithelial cells of its host, before triggering further mechanisms at the intestinal level.

MATERIALS AND METHODS

Bacterial strains and culture media. A pure culture of CpA, strain S32, was isolated in our laboratory at the University of Campinas, SP, Brazil, from a piglet, which died from acute catarrhal enteritis. Prof. T. Yamagishi (Department of Microbiology, University of Toyama, Japan) made typing of this strain (20). Another *Clostridium perfringens* strain (Jab-1) (not typed) which did not produce either toxin β or enterotoxin, isolated from the stools of a healthy piglet, was used as a negative control. The above bacterial strains were grown anaerobically (Gaspax System, BBL, Becton Dickinson, USA) on sulphide-polimyxin-sulphadiazine (SPS) agar and Columbia Agar Base (Difco Lab. Detroit, USA) to which gentamycin (40μg/ml) was added. Subcultures from isolated colonies were made in Brain Heart Infusion (BHI) and in Fluid Thioglycollate (FT) media at 37°C for 18h. BHI cultures were used for the experiments "in vitro" and "in vivo". Cultures were stored in FT medium at 8°C.

Adherence of *Clostridium perfringens* strains to HeLa cells. The adherence assays of *Cl. perfringens* strains S32 and Jab-1 to HeLa cells were carried out as described by Scaletsky *et al.* (17). For determination of the adhesion index, HeLa cells were first stained with Giemsa dye and thereafter examined under bright field microscopy using 320 magnification. Microscopic fields were chosen at random and the adhered bacteria were counted on 100 HeLa cells. The mean (x) ± SEM (standard error of the mean) was calculated (5).

Influence of trypsin, metaperiodate and heat treatments of *Cl. perfringens* S32 bacterial cells on their adherence to HeLa cells. Trypsin (0.25 mg/ml) diluted in 0.05M HEPES mixed with saline plus 1nM Calcium Chloride, pH 7.6 (stabilizer) was used. Briefly, 1.0 ml of standardized bacterial suspension (tube 10 of Mac Farland's scale) of S32 - BHI cultures was mixed with 1.0 ml of the enzyme and left at 37°C for 1h. Then, the trypsin-treated bacterial cells were washed 3x with 0.05M PBS, pH 7.4. Packed cells were resuspended in PBS to the original volume. Afterwards the adherence of this S32-trypsin treated bacteria were tested on monolayers of HeLa cells, as described above. In order to ascertain the effect of temperature on adherence of S32 strain of *Cl. perfringens*, standard bacterial suspensions were heated to 100°C for 5 min and to 56°C for 10 min and then submitted to the adherence test to HeLa cells as described by Scaletsky *et al.* (17).

In order to verify whether periodate had any influence on the adherence of strain S32 to HeLa cells a similar protocol was set up. Thus, 1ml of 20 nM metaperiodate was mixed with an equal volume of standardized suspension of S32 bacterial cells. The mixture was incubated at 37°C for 30 min and then centrifuged and washed 3x with PBS 0.05M, pH 7.4. Packed cells were resuspended in this buffer and the adherence test to HeLa cells was performed as mentioned in the previous item. Appropriate controls represented by untreated standardized cultures were included in these experiments.

Preparation of S32 total antiserum. For the preparation of *Cl. perfringens* S32 antiserum adult rabbits were immunized according to Batty and Walker (3), using for immunization or formalized bacterial whole cells treated bacterial whole cells grown in BHI medium.

Inhibition of S-32 adherence to HeLa cells by homologous antiserum. Equivalent quantities (0.5 ml) of packed bacterial cells from BHI S32 cultures, standardized to tube 10 of the Mac Farland's scale were mixed with the same amount of ten-fold dilutions of S32 antiserum. Mixtures were incubated for 30 minutes, at 37°C and thereafter centrifuged at 3,000 rpm for 10 min. Supernatants were discarded and the packed bacterial cells were harvested in 0.05M PBS, pH 7.4, to the original volume (1.0 ml). Afterwards, 0.1 ml aliquots of the bacterium suspension were dropped on HeLa cell monolayers which grew on coverslips placed in a 24-well tissue culture plates (Falcon, USA), containing Eagle's medium plus 2% of fetal bovine serum (FBS).

The readings of the adherence tests were then performed as described above. All experiments were carried out in triplicate taking care to include negative control represented by Jab-1 *Cl. perfringens* strain. Further assays were performed in the same day under equal conditions, without the addition of S32 antiserum. The inhibitory effect of serum treatment was evaluated by the comparison of the mean number (x) of S32 treated and non-treated adhered bacteria to HeLa cells ± 1 SEM (standard error of the mean).

Piglet ileal loop assay. Landrace newborn female piglets (weighting 1Kg?) were obtained from pregnant sows by Cesarean section carried out using all possible sterile precautions, so that the animals were considered " specific pathogen free" at least for up to 6h after birth. As soon as possible after parturition the piglets were maintained in clean cages and submitted to laparatomy after being anesthetized by ether inhalation. Ileal region was sutured at the proximal and distal positions using sterile common thread. The whole ileal segment was then intraluminally injected with sterile PBS and after gentle manipulation downwards, all PBS was aspirated with a 20-ml syringe and thereafter discarded. Fifteen 10 cm-intestinal loops were thus made with double stitches 1cm long, forming interloops separating each one from the adjacent loops.

Starting with the proximal loops, volumes of 0.5 ml of bacterial suspensions from strains S32 and Jab-1 subcultured in BHI medium at 37°C for 18h, were injected into them at different positions of the gut. Further negative controls consisting of sterile saline and BHI medium at 37°C for 18h, were similarly injected into the remaining loops. After inoculation the intestines were replaced into the abdominal cavity and peritoneum, muscles and skin levels were stitched with cotton thread. Three hours after surgery the animals were euthanized and the intestines removed from the abdomen. The loops were excised and small tissue fragments of the gut loops were cut, fixed with formalin and prepared as described by Yamagishi *et al.* (20). The fragments were then embedded into paraffin and processed for histological examination. Sections (5-6μm) stained with hematoxilin-eosin were examined, using bright-field microscopy at 400x and 1000x magnification. Some sections were stained by Gram's procedure adapted to tissues according to Moulry and McManus (15) and then examined as described above.

Transmission Electron Microscopy (TEM) studies. For TEM studies, equal volumes (100μl) of the S32 strain bacterial suspension from BHI cultures grown at 37°C for 18h and 1% phosphotungstic acid solution were mixed. Aliquots of 10μl were dropped on 300-mesh copper grids covered with a supporting parlodium film where a carbon film had been previously applied. The samples were then negatively stained for 2-3 min. TEM studies were also performed on section of bacterial cells of the S32 and Jab-1 strains, which were grown in BHI medium at 37°C for 24h. Four ml of these cultures were centrifuged and washed 3 times with 0.05M PBS, pH 7.4. Thus, 0.15 ml of undiluted S32 antiserum were mixed with either homologous or heterologous sedimented bacterial cells and left in contact at 37°C for 30min, with constant shaking. The mixtures were then centrifuged (3.000 rpm, for 15 min) and the bacteria washed with distilled water. The same strains with no addition of S32 antiserum were used as controls.

Both controls and serum-treated bacterial cells were shared into two aliquots. One of each was fixed with 5% glutaraldehyde buffered with 0.2ml of 0.2M sodium cacodylate, pH 7.4, for 30 min. To the other, 0.15% of ruthenium red solution was added to the fixative. After another centrifugation, the pellets were fixed with 5% buffered glutaraldehyde plus 0.05% ruthenium red, overnight. The bacterial cells were then fixed with 2.5% glutaraldehyde. After being washed with the buffer, the cells were dehydrated throughout an acetone series (30, 50, 70, 90, and 100%, 30 min each). Ruthenium red (0.05%) was also added to the bath with 30% and 50% acetone to

prevent remotion of the dye from the bacterial cells. The material was embedded with the Epon 812 resin (14) and cut with glass knives on a Porter Blum MT-2 ultramicrotome. The sections were double-stained with uranyl acetate and lead citrate (19) and observed on a Zeiss EM-9S2 transmission electron microscope, operated at 60KV.

RESULTS AND DISCUSSION

The results obtained with the strain S32 of *Cl. perfringens* submitted to the adherence test to HeLa cells showed that this strain had an adhesion index (AI) equal to 25.15 ± 1.26 (x ± 1SEM) while the control strain JAB-1 showed an AI as low as 0.52 ± 0.32 (data not shown). It was observed in this experiment that S32 strain was highly adherent to HeLa cells displaying themselves paralleled along the margins of the membranes of the HeLa cells (Fig. 1).

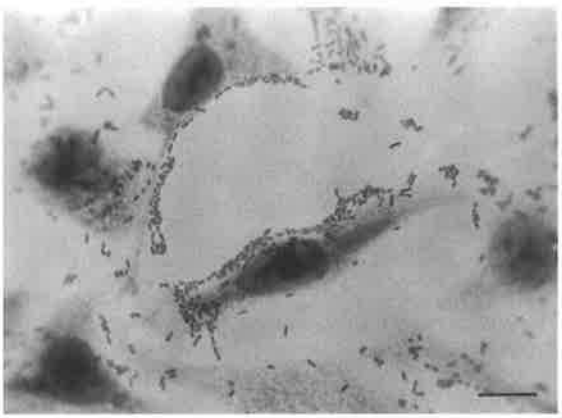

Figure1.Strain S32 of *Clostridium perfringens* adhered to HeLa cells. The parallel arrangement of the bacteria along the cellular membrane is easily observed. Magnification 1152 x. Bar (11mm) = 10μm

As shown in Table 1 when the bacterial cells were treated with different concentrations of trypsin before the adherence test to HeLa cells, there was a marked decrease of the AI, around 70%-80%. This finding suggested that at least part of the surface structure of S32 strain responsible for its adhesion to HeLa cells could be of protein nature.

In order to look for external structures of carbohydrate nature, strain S32 of *Cl.perfringens* was treated with different concentrations of metaperiodate which abolished significantly the adherence of this bacterium to HeLa cells (Table 1), suggesting that some type of glycoside was part of the adhesive structure. In other words, the surface structure of strain S32 responsible for HeLa adhesion, could be a glycoprotein.

With regard to the effect of temperature on adherence of strain S32 it was observed a decrease in the AI which was more pronounced with boiled bacterial cells (Table 1), similar to AI found for strain Jab-1. These data suggested that the biological activity of the structure responsible for the adhesion of strain S32 to HeLa cells was thermolabile, fitting with the hypothesis that this structure may be a glycoprotein.

Tests to look for the inhibition of the adherence of strain S32 to HeLa cells with homologous total antiserum (reciprocal tube agglutination titer equal to 320) were also performed. The results obtained are shown in Table 1 where an inhibition titer around 1:640 was observed. Higher dilutions of the antiserum did not interfere significantly in the process of adherence, though no adhesion index equal to the control (25.15±1.78) was obtained anymore.

Table1.Influence of heating, homologous antiserum, metaperiodate and trypsin on the adherence of *Cl. perfringens* S32 strain to HeLa cells.

Treatment	Adhesion index(AI) to HeLa cells (mean) ± (standard error of the mean) $\bar{X}$ ± (SEM)
None	25.15 ± 1.26
Heat 100C°/5min	1.50 ± 0.06
Heat 56°C/10min	4.92 ± 1.47
S32 antiserum (net)	6.00 ± 0.02
S32 antiserum (1/10)	9.93 ± 0.56
S32 antiserum (1/40)	12.35 ± 1.15
S32 antiserum (1/160)	15.65 ± 1.36
S32 antiserum (1/640)	18.10 ± 0.93
S32 antiserum (1/2560)	21.02 ± 1.12
Trypsin (0.25mg/ml)	4.32 ± 0.10
Metaperiodate (10mg/ml)	5.03 ± 0.10
Metaperiodate (5mg/ml)	6.48 ± 0.07
Metaperiodate (2.5mg/ml)	9.53 ± 0.31

The piglet ileal loop assay using newborn animals demonstrated that *Cl. perfringens* S32 strain was able to adhere to the intestinal epithelial cells. It is important to emphasize the fact that the short time (3h) used for the *in vivo* assays did not allow the production of several cytolytic toxins produced by this anaerobe which could affect the histological appearance of the gut epithelium. So, inoculation of

strain S32 into the loops of the proximal, intermediary and distal intestinal regions of the small intestine did not show any histological lesions other than those seen with the loops inoculated with sterile BHI or the negative control (strain Jab-1) (data not shown).

When the histological sections were stained by the Gram's method only gram-positive bacteria could be seen stuck to the intestinal epithelium (Fig. 2) suggesting that these clusters were formed by the injected bacteria and that this result was not due to the quick colonization of normal flora of the gut. This phenomenon was reinforced by the findings of the controls injected into the loops with sterile saline or BHI, which showed at the same time no visible bacteria, neither attached to the epithelium lining nor to the gut lumen.

Figure 2. Transverse section of the small intestine of the piglet, after the inoculation of crude culture of strain S32 of *Clostridium perfringens*, into the distal position of the gut. Several bundles of the bacteria are seen adhered to the epithelium lining. Staining by H.E. Magnification 1024 x. Bar (10 mm) = 10μm

The *in vivo* tests carried out in this work are in agreement with histological studies performed by Arbuckle (1) in natural cases of necrotic enteritis observed in piglets. That author showed very clearly that the strains *of Cl. perfringens* (type C) were adhered to the intestinal villi of some sick animals, which were killed before being allowed dyeing from the disease. The report of one case of necrotic enteritis among foals was also studied similarly (18). These authors were also able to show several gram-positive bacteria adhered to gut villi of one animal, which had its intestine studied histologically. The electron microscopy studies by negative staining method tried to identify any structure of proteic nature on the bacterial surface of strain S32. At least 5 strains of *Cl. perfringens* including Jab-1 were examined by this technique, as controls. All of them showed a "naked"appearance of their outer surface (data not shown). Strain S32, on the other hand, showed a very loose non-fimbrial structure (Fig. 3). Staining with ruthenium red, which is specific for the visualization of polysaccharide structures (glycocalix), was carried out with ultrathin sections. In this method we have also used the stabilization of possible outer surfaces, using for that purpose total S32 antiserum. Strain S32 so treated showed very clear fibrillar loose material extending in some cases from one bacterial cell to another and spreading out from the bacterial cells towards different directions (Fig. 4)

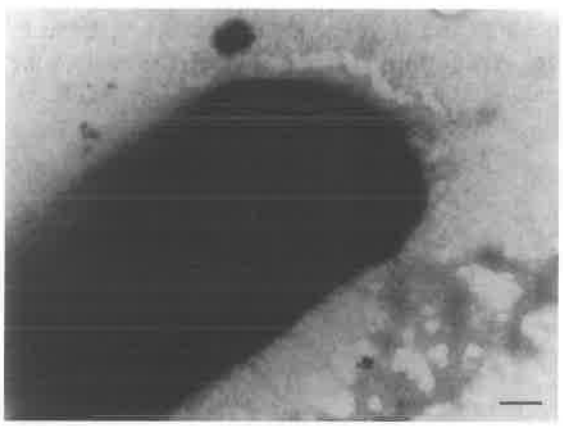

Figure 3. Strain S32 of *Clostridium perfringens* as seen by Transmission Electron Microscopy(TEM), negatively stained by 1% phosphotungstic acid. A loose structure is seen on the bacterial cell surface. Magnification 72,186 x. Bar (8mm) = 0.1μm.

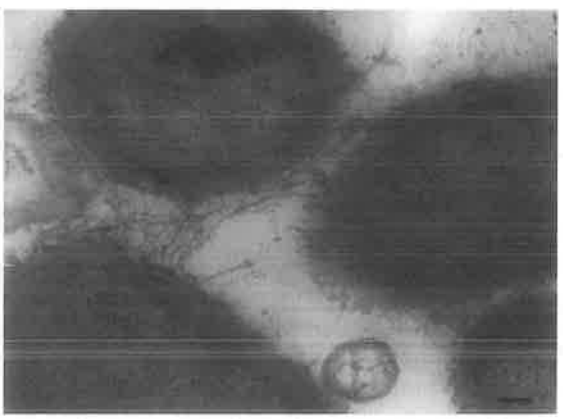

Figure 4. Ultrathin sections of the strain S32 of *Clostridium perfringens* after the stabilization of the outer structures by using homologous S32 antiserum and staining by ruthenium red which shows loose bundle around the bacteria, connecting some of them to each other. Magnification of 58,535x. Bar (6.5mm) =0.25μm.

We are not aware of any report on *Cl. perfringens* colonization factors. Our data suggest that with this bacterium, when the site of infection is the gut, an initial obligate stage represented by adhesion of bacterial cells to the villi does occur. Whether the structure is always similar to that observed in strain S32 with regard to other strains of *Cl. perfringens* remains to be further studied.

We were lucky to come across strain S32 of *Cl. perfringens*, which showed some kind of very tiny structure, even when examined by transmission electron microscopy, using the negative staining technique. This technique was efficient only with this strain and allowed *in vitro* and *in vivo* tests and has permitted us to go further with more detail in the adhesion tests to HeLa cells. We finished with the surface structure stabilization by total homologous antiserum complemented with staining with ruthenium red. The results obtained support the glycocongugates nature of the surface structure of strain S32 responsible for its adhesive properties. As far as we know, after the first papers on *in vivo* adherence by *Cl. perfringens* (1,16,18) this is so far the first report to demonstrate that *in vivo* and *in vitro* adherence occurs with this strain of *Cl. perfringens*, whose adhesive properties may imply that they could play a putative role in the catarrhal enteritis of piglets caused by this strains of *Cl. perfringens*.

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RESUMO

Propriedades adesivas de uma estrutura externa de *Clostridium perfringens* tipo A isolada de leitões com enterite catarral

Uma amostra (S32) de *Clostridium perfringens* tipo A foi isolada de um caso de enterite catarral em leitões. Esta amostra foi capaz de aderir a células HeLa mostrando um índice de adesão (AI) de 25,15 ± 1,26 (media ± 1 erro padrão da media). Tratamento das células bacterianas com tripsina (0,25mg/ml) diminuiu 70%-80% e metaperiodato (10mg/ml) aboliu significantemente a adesão, sugerindo que a estrutura responsável por esta adesão era provavelmente uma glicoproteína. O tratamento pelo calor das suspensões bacterianas (100°C/5min) diminuiu o AI ao nível dos controles negativos. Soro de coelho anti-S32 inibiu a aderência a células HeLa até a diluição de 1:640, pelo menos. O teste da alça ligada de leitão recem nascido demonstrou que a amostra S32 era capaz de aderir às células epiteliais intestinais, conforme demonstrado pela coloração de Gram de secções histológicas do intestino dos animais inoculados. O estudo em Microscópio Eletrônico de Transmissão demonstrou que a amostra S32 de *Cl. perfringens* mostrava um material de natureza fibrilar frouxa, ao contrário da amostra Jab-1 (controle negativo) que demonstrava uma aparência "nua ou lisa". A estabilização das células bacterianas com antissoro homólogo (S32), seguida de coloração com vermelho de rutenio, revelou de maneira mais nítida que longos materiais fibrilares, de aparência frouxa, se estendendo para longe da célula bacteriana, ligando por vezes estas células entre si. A possibilidade desta estrutura ser uma adesina para esta amostra de *Cl. perfringens tipo* A, talvez desempenhando um papel na patogenia da enterite catarral de leitões, depende de mais estudos.

Palavras-chave: *Cl. perfringens* tipo A, aderência, estrutura fibrilar, enterite, leitões.

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EQUINE LEUKOENCEPHALOMALACIA ASSOCIATED WITH INGESTION OF CORN CONTAMINATED WITH FUMONISIN B_1

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SHORT COMMUNICATION

ABSTRACT

This article describes clinical, etiologic and pathologic diagnosis of an outbreak of equine leukoencephalomalacia. Two samples of the corn consumed by the affected horses contained fumonisin B_1 at levels of 46 and 53 µg/g and *Fusarium moniliforme,* a good *in vitro* mycotoxin producer.

Key words: corn, leukoencephalomalacia, fumonisin, *Fusarium moniliforme*, chromatography

Fumonisins are a group of toxic metabolites produced by *Fusarium* spp. and *Alternaria* spp. fungi, especially *Fusarium moniliforme* (2,10,15), a species well adapted to tropical climates (8,17). These mycotoxins have been detected in oats and corn-based food, even after pelletization (5,19). Among the several types of fumonisin known, fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) have been isolated from naturally contaminated foods and feeds. FB_1, the most toxic of these compounds (13), is produced in large amounts and is responsible for various toxicoses in domestic animals, including equine leukoencephalomalacia (ELEM) (12,13), pulmonary edema and hydrothorax in pigs (6), diarrhea and reduced body weight in broiler chicks (8) and carcinogenity in rats (15). FB_1, FB_2 and FB_3 are generally found in feed at a ratio of 8:2:1 (18).

The clinical course of ELEM is directly related to the amount of toxin ingested and may be influenced by individual tolerance. Clinical disease may be observed from a few hours to several days after consumption of contaminated rations, but a correct diagnosis is normally possible after an average period of 72 hours. Feed containing more than 10 µg/g of FB_1 is considered toxic and of high risk if administered to animals. Previous studies of ELEM outbreaks have shown that feed contaminated with 1 to 126 µg/g of FB_1 produces clinical signs of the disease in horses (12,19).

The aim of this short communication was to describe an outbreak of ELEM in horses and correlate the disease with the presence of both *Fusarium moniliforme* and FB_1 in corn ingested by the affected animals.

The episode occurred in a farm located in southern Brazil, during the spring of 1996, when 3

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to 4 years old thoroughbred horses (3 males and 1 female; approximate body weight: 400 Kg) were affected. The animals were being fed native grass supplemented with 2 kg of corn/horse/day. Three horses presented clinical signs of ELEM seven days after ingestion of contaminated ration and the forth horse showed similar signs three days later. All horses died within 18 to 30 hours from the start of clinical disease and were necropsied immediately after death. Brain tissue was macroscopically examined and selected areas were sampled for routine histopathology.

Two samples of corn fed to the affected horses were analysed for the presence of FB_1. All the procedures used for extraction, purification, and quantification of FB_1 by high-performance liquid chromatography (HPLC) (GBC Scientific Equipment Pty Ltd. - Victoria, Australia) were adapted from previous studies (3,12). 10 g of ground corn were mixed with 50 ml acetonitrile/water (50:50) and blended for 5 min. This suspension was then filtered through Whatmann IV filter paper and 2 ml of the filtrate was mixed with 6 ml of deionized water. The diluted filtrate was then applied to a silica C_{18} cartridge (500 mg) previously conditioned with 2 ml of acetonitrile and 2 ml of water. FB_1 was eluted with 2 ml of acetonitrile/water (70:30) and dried in liquid nitrogen and diluted in 200 µl of acetonitrile/water (70:30). 100 µl of FB_1 solution was added to an equal volume of 0.1 M borate buffer and to 100 µl of o-phthaldialdehyde (OPA) solution (30 mg of OPA dissolved in 9.5 ml of acetonitrile containing 0.5 ml of 2-mercaptoetanol and stored in dark at 7°C for less than 1 week). After 10 minutes, 200 µl of 0.01 M boric acid were added to the mixture. 20 µl of chromatographic solution were analysed by a reverse-phase isocratic system with fluorescence detection. FB_1 concentrations were determined under an excitation wavelength of 365 nm and emission wavelength of 460 nm.

The mobile phase was prepared using a buffer of 0.1 M sodium phosphate and acetonitrile (60:40). A 5 µm C_{18} (250 x 4.6 mm) column was used at a flow rate of 1 ml/min, and quantification was determined by peak area.

Corn samples obtained from the warehouse, and suspected to be responsible for this outbreak, were grounded and cultured. 10 grams of corn kernels from each sample were added to 90 ml of sterile distilled water to obtain a 10^{-1} stock dilution, from which ten fold serial dilutions up to 10^{-6} were made using the same diluent. Duplicate 1 ml volumes of each dilution were added to Petri dishes containing 10 to 15 ml of Potato Dextrose Agar (16). The plates were then incubated at 27°C for 5 days and observed daily. The fungal colonies recovered were identified according to Nelson (10). Colonies of *Fusarium moniliforme* isolated from the suspected corn were inoculated into Erlenmeyer flasks with 50 g of sterile corn at two different humidity levels (34 and 45%), in triplicates. The cultures were homogenized and incubated at 27°C for 17 days, after which they were autoclaved, placed on trays and dried at 40°C for 15 hours, to facilitate FB_1 extraction and quantification.

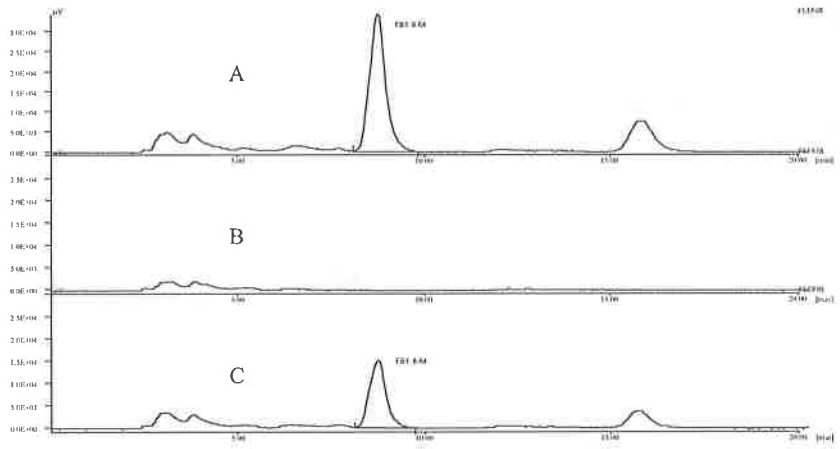
Contamination of corn by fumonisin and the consequent onset of ELEM are known to occur worldwide (5,7,12). A seasonal occurrence of ELEM has also been described, associated with the ingestion of mouldy corn and the isolation of *Fusarium moniliforme* from feeds (9). In Brazil, the highest frequencies of this toxicosis were recorded from the end of fall until the beginning of spring (1,4,11).

Seasonality of ELEM outbreaks may be linked with the humidity levels required for growth of *Fusarium moniliforme* and production of FB_1 in significant amounts, or, additionally, with the need to supplement horse diets with corn-based rations during shortage of native pasture. In this report, each horse received 2 kg/day of corn, and two samples of this feed were found to be contaminated with FB_1 at levels of 46 and 53 µg/g (Fig. 1).

The concentrations of fumonisin B_1 detected in our study were at least five times higher than the known toxic concentration for horses (12,20). Contamination with FB_3 and FB_2 at levels of 7 and 13 µg/g, respectively, has been reported for corn administered to horses that also developed ELEM (20).

In this outbreak, the delay in the onset of clinical signs was probably due to the relatively low amounts of FB_1 present in the feed. Although the signs consisted of blindness, uncoordination, anterior limb crossing, circling, aggressiveness, recumbency and eventual death, and were thus indicative of ELEM. The definitive diagnosis was based on determination of FB_1 levels in the corn feed as well as gross and histological findings. Histopathology revealed areas of necrosis in the white matter of the CNS yet these lesions were not extensive. According to Kellerman *et al.* (7), who studied the effects of chronic exposure to FB_1, a reduced spread of necrosis could be related to short time of exposure to the toxin. Gross changes

Figure 1. HPLC chromatograms of fumonisin B_1.



A - Chromatogram of FB_1 standard (10 µg/ml).
B - Chromatogram of negative sample.
C - Chromatogram of corn sample which caused the outbreak of ELEM (53 µg/g de FB_1).

observed at necropsy in our study were restricted to the nervous system and consisted of cerebral hemisphere asymmetry, mild edema, and increased tissue softness. Focal areas of haemorrhage were randomly distributed over the subcortical white matter, the basal nuclei, and the mid brain. Microscopically, CNS lesions were observed in the white matter and consisted of spongiform degeneration, punctate haemorrhage and perivascular haemorrhages.

The isolation of *Fusarium moniliforme* from rations administered to horses affected by ELEM has been reported in several outbreaks (1,11). The isolated strain (LAMIC 2999/96) was shown to be a good producer of FB_1. Cultures of this isolate yielded up to 440 and 670 µg/g when cultured *in vitro*, at humidity levels of 34 and 45%, respectively. It was also observed that the concentration of FB_1 was directly related to relative humidity, where levels between 34% and 45% were more efficient at inducing a high production of this mycotoxin at room temperature. Similar findings have also been reported by other authors (14).

The etiologic diagnosis of ELEM constitutes an important tool for the prevention of this toxicosis. The frequency of ELEM appears to be significantly higher in regions where weather conditions promote the growth of fungi (i.e., rainy seasons with moderate temperatures). A conclusive clinical diagnosis of ELEM is often very difficult due to the fact that the neurological signs are similar to those consequent to the neurological alterations that occur due to encephalitis and rabies. Therefore, a conclusive diagnosis must be based on gross and histopathologic alterations of the CNS together with the detection of fumonisin in contaminated rations.

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RESUMO

Leucoencefalomalácia eqüina associada à ingestão de milho contaminado por fumonisina B_1

Descreve-se o diagnóstico clínico, etiológico e patológico de um surto de leucoencefalomalacia eqüina. Duas amostras do milho consumido pelos eqüinos afetados apresentaram 46 e 53 µg/g de fumonisina B_1 e uma linhagem de *Fusarium moniliforme*, boa produtora da toxina em cultivos *in vitro*.

Palavras-chave: milho, leucoencefalomalácia, fumonisina, *Fusarium moniliforme*, cromatografia.

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KILLER TOXIN OF *SACCHAROMYCES CEREVISIAE* Y500-4L ACTIVE AGAINST FLEISCHMANN AND ITAIQUARA COMMERCIAL BRANDS OF YEAST

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ABSTRACT

The strain *Saccharomyces cerevisiae* Y500-4L, previously selected from the must of alcohol producing plants and showing high fermentative and killer capacities, was characterized according to the interactions between the yeasts and examined for curing and detection of dsRNA plasmids, which code for the killer character. The killer yeast *S. cerevisiae* Y500-4L showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast and also against the standard killer yeasts K2 (*S. diastaticus* NCYC 713), K4 (*Candida glabrata* NCYC 388) and K11 (*Torulopsis glabrata* ATCC 15126). However *S. cerevisiae* Y500-4L showed sensitivity to the killer toxin produced by the standard killer yeasts K8 (*Hansenula anomala* NCYC 435), K9 (*Hansenula mrakii* NCYC 500), K10 (*Kluyveromyces drosophilarum* NCYC 575) and K11 (*Torulopsis glabrata* ATCC 15126). No M-dsRNA plasmid was detected in the *S. cerevisiae* Y500-4L strain and these results suggest that the genetic basis for toxin production is encoded by chromosomal DNA. The strain *S. cerevisiae* Y500-4L was more resistant to the loss of the phenotype killer with cycloheximide and incubation at elevated temperatures (40°C) than the standard killer yeast *S. cerevisiae* K1.

Key words: Killer yeast, *Saccharomyces cerevisiae*, killer toxin

INTRODUCTION

The killer system in yeasts has been extensively investigated since it was first described in *Saccharomyces cerevisiae* by Bevan and Makower (1). Killer strains secrete a protein toxin which is lethal to sensitive strains of the same genus and, less frequently, strains of different genera (10). Among the yeasts, killer, sensitive, and neutral strains have been described. Eleven distinct patterns of the range of killer activity against killer yeast have been found (K1-K11) according to the interaction between the killer yeasts (12, 15).

Genetic studies have shown that the killer phenotype of *S. cerevisiae* is inherited cytoplasmically and has been linked to the presence of a double stranded RNA (dsRNA) associated with virus-like particles within the cytoplasm of the killer cells. However, dsRNA need not always be the determinant. In other genera, like *Kluyveromyces lactis*, the information for the killer phenotype is carried by linear dsDNA (6, 11). The killer character

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of *Candida* sp. (13, 14) and *Hansenula anomala* (7) is encoded by chromosomal genes, not by extrachromosomal ones.

In *Saccharomyces cerevisiae*, two distinct dsRNA species exist: L-dsRNA (4.6-4.8 Kb) and M-dsRNA (1.0-1.8 Kb) (12). It has been established that L-dsRNA encodes the major virus-like particle capsid protein, and M-dsRNA encodes the killer toxin synthesis (2, 3). L-dsRNA encodes a protein for encapsidation of M-dsRNA and plays an essential role in maintenance or expression of the killer phenotype (3). Kitano *et al.* (8) furthermore observed two new killer types belonging to *Saccharomyces*. Using tetrad analysis, their killer genes were found to be encoded on chromosomal DNA. One gene, designated KHR, was on chromosome IX and another, designated as KHS, was on chromosome V (4).

The purpose of this investigation was to characterize the previously isolated killer strain *S. cerevisiae* Y500-4L, which shows high fermentative capacity.

MATERIALS AND METHODS

Yeast strains. *Saccharomyces cerevisiae* Y500-4L and *Hansenula* sp. Y66-1 killer yeasts, previously isolated from the must of alcohol producing plants (9). Two commercial brands of yeast (Fleischmann e Itaiquara) were used as sensitive strains. The standard killer yeasts are listed in Table 1.

Table 1- Standard killer yeast strains used

Strains	Killer type
Saccharomyces cerevisiae KL88	K1
Saccharomyces diastaticus NCYC 713	K2
Saccharomyces capensis NCYC 761	K3
Candida glabrata NCYC 388	K4
Debaryomyces vanrij NCYC 577	K5
Kluyveromyces marxianus NCYC 587	K6
Pichia membranaefaciens NCYC 333	K7
Hansenula anomala NCYC 435	K8
Hansenula mrakii NCYC 500	K9
Kluyveromyces drosophilarum NCYC 575	K10
Torulopsis glabrata ATCC 15126	K11

Media. YEPD medium (1.0% yeast extract, 2.0% peptone, 2.0% glucose) and YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose) were used for yeast culturing. YEPD-MB agar (YEPD containing 0.1M citrate-phosphate buffer pH 4.5, 0.01% methylene blue and 2.0% agar) was used for the determination of killer phenotype.

Interaction between killer yeasts. Killing ability and resistance were determined by inoculating killer strains onto YEPD-MB agar and after incubation for 2 days at 25°C the sensitive yeast suspension, previously incubated on YEPD medium for 2 days at 25°C, was sprayed onto YEPD-MB agar. The plates were incubated for 2 more days at 25°C and the strains with killer activity appeared surrounded by a clear zone.

Extraction and electrophoresis of double-stranded RNA plasmids. Extraction was performed by a modification of the method of Goto *et al.* (5). Yeast cells grown in YEPD medium were harvested and washed with 50 mM EDTA (pH 8.0). After centrifugation at 5000 rpm for 10 minutes, the cells were incubated for 1 hour at 60°C in 600µl of a solution containing 25 mM EDTA, 200 mM Tris-HCl (pH 8.0), 25 mM NaCl and 1.0% sodium dodecyl sulfate. The cell suspension was then centrifuged and the aqueous phase treated twice with an equal volume of phenol, phenol-chloroform (1:1) and chloroform for extraction of the plasmids. The upper phase was transferred and precipitated with isopropanol (1:1). The precipitate was dissolved in buffer and analyzed by 1% agarose gel electrophoresis (75V, 0.7A for 2 hours).

Curing test. Killer yeast cells grown in YM medium were suspended in sterilized water (10^6 cells/ml) and 10 µl streaked onto YM agar with or without 0.2 ppm cycloheximide. The plates were incubated at temperatures of 25°C (control), 37°C, 38.5°C or 40°C for 3 days. Colonies of the yeast which grew in each treatment were inoculated onto YM agar, and after incubation at 25°C for 36 hours, were replica-plated on YEPD-MB agar plates and then the sensitive yeast sprayed. After 2 days, the cured strains, which had lost their killer activity, were detected.

RESULTS AND DISCUSSION

Interaction between yeasts. Killing ability and resistance of *S. cerevisiae* Y500-4L, previously selected (9) as being a strain showing high fermentative capacity, were determined by interaction between yeasts. The results are shown in Table 2. The

killer yeast *S. cerevisiae* Y500-4L showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast, suggesting that this strain could be a potential competitor in alcoholic fermentations using these commercial brands as starter cultures. It also showed killer activity against the standard killer yeasts K2, K4 and K11. This killer character was similar to that of the K1 type killer. However, the strain Y500-4L showed sensitivity to the killer toxin produced by the standard killer yeasts K8, K9, K10 and K11 and was immune to the action of the K2, K3 and K5 killer toxins.

Extraction and electrophoresis of double-stranded RNA plasmids. Double-stranded RNA plasmids of killer strains were analyzed by agarose gel electrophoresis (Fig. 1). Standard killer type strains K1, K2 and K3 contain the two species of plasmid previously reported (15). The larger species (L-dsRNA) had a molecular weight of 5.0 Kb; the killer type strain K1 showed the presence of M1-dsRNA with 1.8 Kb and the strains K2 and K3 showed M2 and M3-dsRNA with 1.4 Kb. The molecular weights of each species of dsRNA were similar to those described by Wickner (12).

The killer strain *S. cerevisiae* Y500-4L showed the presence of L-dsRNA with 5.0 Kb but no M-dsRNA plasmid was detected. This result was the same as that obtained for another strain of *S. cerevisiae*, Y-9, described by Kitano (8), suggesting that the genetic basis for toxin is encoded by chromosomal DNA in these strains.

Fig. 1 also shows the result of the electrophoretic analysis of plasmid samples from *Hansenula* sp. Y66-1. No plasmids were detected. According to Kagiyama (7), the killer character of this genus is encoded by chromosomal genes.

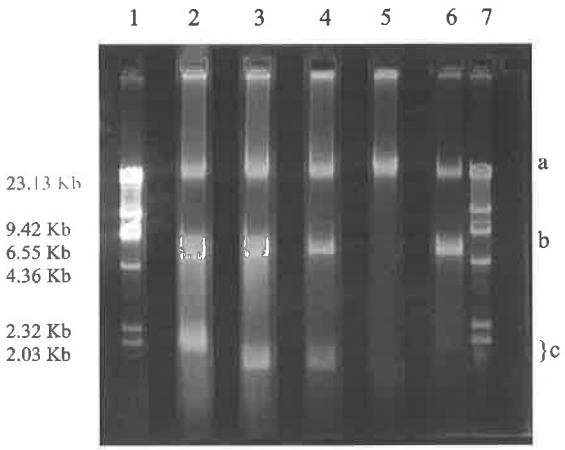

Figure 1-Agarose gel electrophoresis of dsRNA from killer yeasts. **a**- DNA; **b**- L-dsRNA; **c**- M-dsRNA;
1 and **7**- λ HindIII (molecular weight marker); **2**- standard killer yeast type K1; **3**- standard killer yeast type K2; **4**- standard killer yeast type K3; **5**- strain Y66-1; **6**- strain Y500-4L.

Table 2- Killing reaction of killer yeast against various yeasts

Seeded strains / Killer strains	K1	K2	K4	K6	K7	K8	K9	K10	K11	Y66-1	Y500-4L	Fleisch.	Itaiquara
K1	-	+	+	-	-	-	-	-	+	-	-	+	+
K2	+	-	-	-	-	-	-	-	-	-	-	-	+
K3	+	-	+	-	-	-	-	-	-	-	-	-	++
K4	-	+	-	-	-	-	-	-	-	+	-	ND	ND
K5	+	-	-	-	-	-	-	-	+	-	-	ND	ND
K6	-	-	-	-	-	-	-	-	-	-	-	ND	ND
K7	-	-	+	-	-	-	-	-	+	+	-	ND	ND
K8	+	+	++	-	-	-	-	-	++	+	++	ND	ND
K9	++	++	++	-	-	-	-	-	++	+	++	ND	ND
K10	+	+	++	-	-	-	-	-	+	+	+	ND	ND
K11	+	++	-	-	-	-	-	-	-	++	+	++	++
Y66-1	-	-	++	-	-	-	-	-	++	-	-	-	-
Y500-4L	-	++	+	-	-	-	-	-	+	-	-	+++	++++

- = No killing activity
+ = Killing activity. Each + = 3 mm f (clear zone)
ND indicates that the test was not carried out.

Curing test. The results are shown in Table 3. The curing of the phenotype means the loss of killer toxin production by cycloheximide treatment or temperature.

Table 3- Curing of killer yeasts

	Methods of curing			
Strains	Cycloheximide	Temp. 37°C	Temp. 38.5°C	Temp. 40°C
K1	-	75 %	95 %	-
Y500-4L	55 %	0 %	0 %	40 %
Y66-1	0 %	-	-	-

The standard killer yeast K1 *S. cerevisiae* was cured easily by incubation at elevated temperature. At 37°C and 38.5°C, 75% and 95% respectively of curing were obtained. It did not grow in 0.2 ppm cycloheximide or at 40°C.

The strain *S. cerevisiae* Y500-4L was shown to be more resistant to curing than killer standard K1. Only 40% of curing was obtained when incubated at 40°C or 55% when grown in 0.2 ppm cycloheximide. These results indicated that this strain is more resistant to the loss of killer capacity.

The strain *Hansenula* sp. Y66-1 was not cured by cycloheximide treatment and did not grow at elevated temperature (37-40°C). These results were expected because the killer character in this genus is encoded by chromosomal genes.

ACKNOWLEDGMENTS

The authors thank CNPq for their financial support.

CONCLUSIONS

The killer strain *S. cerevisiae* Y500-4L, which have high fermentative capacity, was characterized. This yeast showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast and also against other genera like *Candida*, conferring a selective advantage over sensitive strains competing to grow in the same environment.

No M-dsRNA plasmid was detected in the strain Y500-4L, suggesting that the genetic basis for toxin production is encoded by chromosomal DNA. This result was in accordance with the greater resistance to treatments for the loss of the capacity to produce killer toxin. These results showed a potential use of this competitive yeast in alcoholic fermentation.

RESUMO

Toxina "killer" de *Saccharomyces cerevisiae* Y500-4L ativa contra leveduras comerciais Fleischmann e Itaiquara

A linhagem de *Saccharomyces cerevisiae* Y500-4L com alta capacidade fermentativa e atividade "killer", previamente selecionada de mosto de fermentação de usina de álcool, foi caracterizada quanto ao espectro de atividade e quanto à perda do caracter "killer". A linhagem "killer" de *S. cerevisiae* Y500-4L, mostrou alta atividade "killer" contra as leveduras comerciais Fleischmann e Itaiquara, e também contra as linhagens "killer" padrões K2 (*S. diastaticus* NCYC 713), K4 (*Candida glabrata* NCYC 388) e K11 (*Torulopsis glabrata* ATCC 15126) e mostrou ser sensível às toxinas produzidas pelas leveduras padrões "killer" K8 (*Hansenula anomala* NCYC 435), K9 (*Hansenula mrakii* NCYC 500), K10 (*Kluyveromyces drosophilarum* NCYC 575) e K11 (*Torulopsis glabrata* ATCC 15126). A linhagem de *S. cerevisiae* Y500-4L não apresentou plasmídio M-dsRNA e, provavelmente, o caracter genético responsável pelo fenótipo "killer" é codificado por genes cromossomais. Em ensaios para a perda do fenótipo, a linhagem *S. cerevisiae* Y500-4L apresentou maior resistência ao tratamento com cicloheximida e a temperatura elevada (40°C) do que a levedura *S. cerevisiae* padrão "killer" K1.

Palavras-chave: Levedura "killer", *Saccharomyces cerevisiae*, toxina "killer"

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FERMENTATION AND RECOVERY OF L-GLUTAMIC ACID FROM CASSAVA STARCH HYDROLYSATE BY ION-EXCHANGE RESIN COLUMN

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ABSTRACT

Investigations were carried out with the aim of producing L-glutamic acid from *Brevibacterium* sp. by utilizing a locally available starchy substrate, cassava (*Manihot esculenta* Crantz). Initial studies were carried out in shake flasks, which showed that even though the yield was high with 85-90 DE (Dextrose Equivalent value), the maximum conversion yield (~34%) was obtained by using only partially digested starch hydrolysate, i.e. 45-50 DE. Fermentations were carried out in batch mode in a 5 L fermenter, using suitably diluted cassava starch hydrolysate, using a 85-90 DE value hydrolysate. Media supplemented with nutrients resulted in an accumulation of 21 g/L glutamic acid with a fairly high (66.3%) conversation yield of glucose to glutamic acid (based on glucose consumed and on 81.74% theoretical conversion rate). The bioreactor conditions most conducive for maximum production were pH 7.5, temperature 30°C and an agitation of 180 rpm. When fermentation was conducted in fed-batch mode by keeping the residual reducing sugar concentration at 5% w/v, 25.0 g/L of glutamate was obtained after 40 h fermentation (16% more the batch mode). Chromatographic separation by ion-exchange resin was used for the recovery and purification of glutamic acid. It was further crystallized and separated by making use of its low solubility at the isoelectric point (pH 3.2).

Key words: *Brevibacterium* sp., L-glutamic acid, cassava hydrolysate, batch and fed-batch process, ion-exchange resin, purification

INTRODUCTION

L-amino acids have a wide spectrum of commercial use as food additives, feed supplements, infusion compounds, therapeutic agents and precursors for the synthesis of peptides or agrochemicals (14). Monosodium glutamate (MSG), the sodium salt of glutamic acid is used commercially as a flavour enhancer, usually in combination with nucleotides inosinate to provide an expansion and extension of taste in processed food such as soups, biscuits, noodles, Chines foods, meat and vegetable processing etc. (12). Glutamic acid mother liquor in MSG production is being used in the manufacture of Sauce and as soil conditioner, fertilizer etc. Several strains of *Corynebacterium* and *Brevibacterium* are used as cost effective bioconverters, which have been exploited by the fermentation industry to provide various amino acids, including L-glutamic acid (1).

Owing to the importance of the particular industrial fermentation, much efforts were still going

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on to improve the glutamic acid fermentation process especially from the standpoint of savings in production cost (3, 10). Cassava *(Manihot esculenta* Crantz*)* popularly known as tapioca, is one of the major tuber crops of the world, being cultivated extensively in tropical countries and obviously provides a major source of calories to millions of the people in the world. On dry weight basis, tapioca contains about 80-82% starch (fresh roots have about 22-30% starch) of which 55-60% is recoverable as starch. It is an excellent substrate for the reducing sugars such as (maltose, glucose etc), especially because of its ease of liquefaction.

The present study was undertaken with an aim to test the efficiency of cassava starch to be used as the substrate for L-glutamic acid production in batch- and fed-batch processes. The optimized parameters in the fermentation using pure glucose (11) were applied to the cassava starch hydrolysate for the production of glutamic acid. Attempts were also made to isolate and purify the L-glutamic acid using chromatographic techniques with ion-exchange resin.

MATERIALS AND METHODS

Microorganism and cultivation

A strain of *Brevibacterium* sp. (DSM 20411) was used in the present study. The growth medium, culture preservation conditions and inoculum preparation were same as mentioned elsewhere (8).

Preparation of cassava starch

Fresh cassava tubers obtained locally were washed and peeled. The process of starch extraction from these tubers consisted of wet milling the washed cassava roots followed by washing the starch from the fibrous mass. Starch was sedimented in settling containers and air-dried.

Hydrolysis of cassava starch

Liquefaction of the starch slurries (5%, w/v) was carried out using a thermostable α-amylase (Termamyl 120, Novo Industries, Bagsvared, Denmark) produced from a strain of Bacillus licheniformis. The enzyme was having 120 KNU/g activity (one Novo α-amyl ase unit (NU) is defined as the amount of enzyme that hydrolyses 5.26 g starch/h under Novo's standard conditions). The pH of the slurry was adjusted to 6.0 and reaction was carried out in a stirred reactor with enzyme at 85°C for 2 h. The resulting solution was cooled to 60°C and after adjusting the pH 4.8-5.0 (with 1 N HCl), saccharification was carried out with a fungal glucoamylase (AMG 300, Novo industries, which has an activity of 300 AGU/ml) (one Novo amyloglucosidase unit is defined as the amount of enzyme that splits one micromole of maltose per minute at 25°C). Reaction was carried out for 18-24 h at 60°C and was then stopped by heating to 90°C for 10 minutes. The hydrolysate was double filtered using a nylon cloth and later by using Whatman N° 1 filter paper so as to get the clear hydrolysate. Hydrolysate of different dextrose equivalent (DE) values containing different concentrations of sugars were prepared by the analysis of hydrolysate at different time intervals for the reducing sugars.

Fermentation

Batch process in flasks. Initial studies were carried out by taking 50 ml media in 250 ml Erlenmeyer flasks to study the effect of starch hydrolysate with different DE values on the growth and activity of *Brevibacterium* sp. Media were prepared by taking hydrolysate of different DE values and supplemented with $NaNO_3$ 0.7 g, KH_2PO_4 0.12 g, 1 ml mineral solution ($FeSO_4.7H_2O$, $MnSO_4$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCI, each 1 mg), 100 µl corn steep liquor and one drop of Tween 80 in 100 ml starch hydrolysate (pH 7.2). After autoclaving, the media were inoculated with 5% v/v suspension (10^8 cells/ml) of 20 h old *Brevibacterium* sp. (9). The optimum parameters obtained in earlier studies were maintained throughout the period of fermentation such as pH 7.5, temperature 30°C, and agitation speed 180 rpm (8-10). Samples were withdrawn as whole flask at desired time intervals for analysis. The results reported are the average of three sets of experiments.

Batch process in fermenter. Cassava starch hydrolysate (85-90 DE) was diluted to 5% initial sugar concentration and was supplemented with $NaNO_3$ 0.7 g, KH_2PO_4 0.12 g, 1 ml mineral solution ($FeSO_4.7H_2O$, $MnSO_4$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCI, each 1 mg), 100 µl corn steep liquor and one drop of Tween 80 in 100 ml starch hydrolysate (pH 7.2). Fermentation was carried out with a

working volume of 2.5 L in a 5 L fermenter (BIOFLO III, New Brunswick Scientific, Edison, N, J., USA). Dissolved oxygen was maintained at 60% of air saturated medium.

Fed-batch process in fermenter. Fed-batch process was also carried out in the fermenter. The initial concentration of reducing sugars in the medium was 5%, and at the stages, where the concentration fell to 2% (as determined by the analysis of fermentation medium), starch hydrolyzate solution containing 10% reducing sugars, was added to bring the sugar concentration of fermenting medium as 5%. Fermentation conditions were the same as for batch process.

Filtration and Centrifugation of Broth

Two batches, each consisting of 2.5 L fermented broth (obtained from batch fermentation in fermenter) was filtered using a microfiltration unit (Millipore, USA), fitted with a 50 mm membrane diameter with a pore size of 45 μm under vacuum using a pump. Both the filtrates were combined and then centrifuged at 10,000 rpm for 10 minutes to get the supernatant, which was then used for the recovery of the product.

Preparation of Resin

Spherical particles of cation exchange resin, Amberlite IR 120 (Hi-media) was used. Prior to use, the resin was pre-conditioned according to the method of Moore and Stein (6). The resin (100 g) was washed thoroughly two times with 4 N HCl. After two washes with distilled water, the resin was then washed with 2 N NaOH until the filtrate was alkaline. The resulting material (sodium salt of the resin) was suspended in 3-times its volume of 1 N NaOH and heated over a steam bath for 2 h with occasional mixing. The supernatant fluid was decanted after 30 minutes of settling and replaced with fresh hot 1 N NaOH. The procedure was repeated two times. The resin was filtered and washed with 2 L of distilled water to make it free of alkali. The resin was finally stored as the moist sodium salt.

Packing the Column

Resin (as above) was placed in a column (3 cm × 50 cm) containing distilled water and filled upto 25 cm^3. The excess water was removed using a siphon.

Separation Process by Ion-Exchange Column

The chromatographic conditions were selected to minimize the inhibitory effect of co-existing inorganic ions on the adsorption of amino acids by ion-exchange resins, following the method of Samejima (13). Removal of impurities from the broth was done by filtering and centrifuging the broth. The pH of the broth dropped from 7.5 to 4 with 1 N HCl, which was the most important factor affecting the adsorption of glutamic acid on the resin because the ionic forms vary with the pH.

The processes involved in the column were adsorption and elution. In the adsorption process, the broth used was adjusted to a suitable pH of 1.8-2.0, using 1 N HCl to charge the glutamic acid so that the ion exchange between the glutamic acid and the resin could occur. The broth was continuously recycled at a flow rate of 20 ml/minute (retention time 50 minutes) until glutamic acid was fully adsorbed in the column, leaving other ions. In the elution process, the pH was increased to 3.8-4.0 by treating the broth with urea and sodium hydroxide. This was done to release the glutamic acid bound on the resin by changing the glutamic acid charge.

Crystallization

After adsorption and elution, the eluent containing a high amount of glutamic acid was acidified to pH 3.2, the isoelectric point of glutamic acid with 1 N HCl. Storage at 20°C for 48 h resulted in the formation of crystals of glutamic acid. After evaporation of the eluent, the dry solid crystals were obtained.

Analytical Methods

Bacterial growth was determined by measuring the optical density (OD) of the culture broth at 610 nm using an UV-visible spectrophotometer (UV-160 A Shimadzu, Japan). Total carbohydrates in the samples were detected by phenol-sulphuric-acid method (4). Starch content was determined by hydrolyzing the substrate with 10% HCl and estimating the glucose content by DNS reagent (5). pH measurements were made by a standard pH meter (model 361 μ, Systronics, Ahmedabad, India). Thin layer chromatography (TLC) was used (Silica gel G, Solvent mixture-n butanol/glacial acetic acid/water 4:1:1, v/v) for the qualitative detection of L-glutamic acid (2). The TLC plate with ninhydrin showed only

one spot, which was identical with authentic sample of L-glutamic acid, hence ninhydrin colour reaction method was used for quantitative estimation of L-glutamic acid (15). Product purity was reconfirmed by IR-spectrum (Perkin Elmer Model 882) using pure L-glutamic acid as a standard (3).

RESULTS AND DISCUSSION

Growth and glutamic acid production based on the hydrolysate having different DE values. Fig. 1 shows the growth pattern of *Brevibacterium* sp. on cassava starch hydrolysate. In general, higher DE hydrolysate supported better growth of the culture, which was maximum with the 85-90 DE hydrolysate (OD 1.92 at 18 h). Apparently, the rate of cell growth was directly related with the DE values of the hydrolysate, as higher the DE value, lesser was the time to achieve maximum cell growth in hydrolysate of different DE values. In case of 15-20 DE hydrolysate, the growth was slowest and it took 30 h for the cells to achive the maximum growth.

Fig. 2 shows the consumption of reducing sugars by *Brevibacterium* sp. at different DE values. The pattern was similar in all cases with a consumption of more than 85% sugars (a maximum of 94% with DE 85-90). Fig. 3 shows the glutamate production at different time intervals. A maximum of 8.8 mg/ml

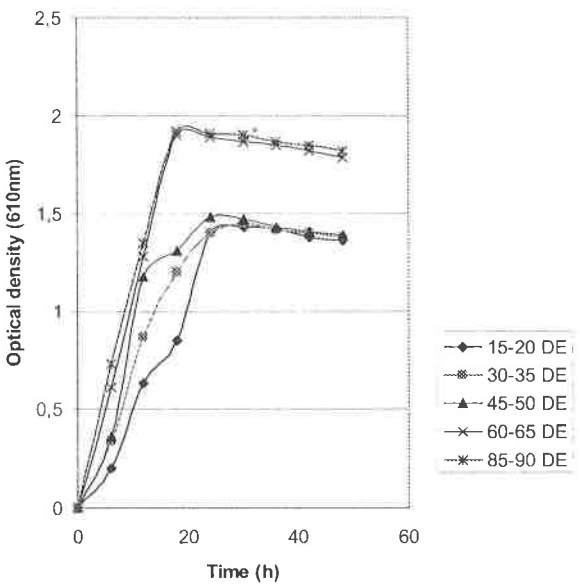

Figure 1 – Growth pattern of *Brevibacterium* sp. in cassava starch hydrolysate medium of different DE values.

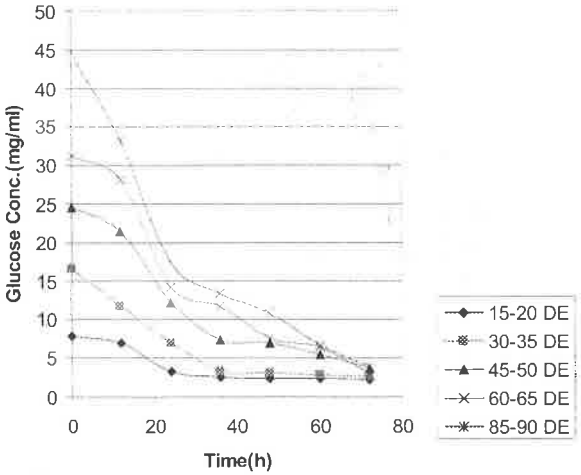

Figure 2 – Consumption of sugars by *Brevibacterium* sp. in different DE starch hydrolysate.

L-glutamic acid was obtained after 60 h fermentation with the medium having 85-90 DE. While considering the percentage conversion of sugars to L-glutamic acid (based on glucose consumed and 81.74% as the theoretical conversion rate) (8); it was maximum (34%) with the hydrolysate having DE value 45-50 as shown in Fig. 4. On the other hand, with 85-90 DE hydrolysate, the conversion was lowest (~27%). Thus, if conversion factor has to be considered as a major criterion, a low DE value hydrolysate, i.e. 45-50 DE would be sufficient for L-glutamic acid production. There are reports in the literature where a wide variety of applications have been mentioned for low DE starch hydrolysate (7).

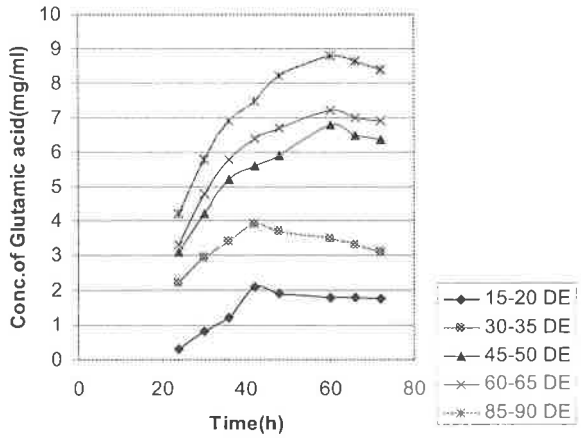

Figure 3 – Yields of L-glutamic acid by *Brevibacterium* s. in different DE value starch hydrolysate.

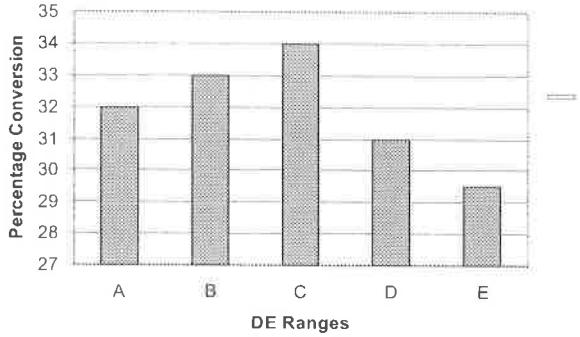

Figure 4 Percentage conversion of cassava starch hydrolysate of L-glutamic acid.

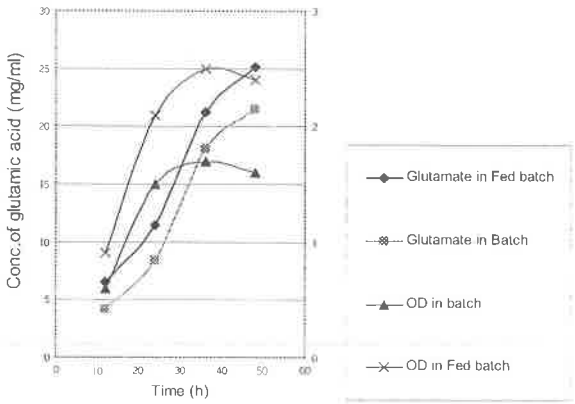

Figure 5 – Comparison of the growth profile and glutamic acid production in batch and fed batch processes.

In this context, our findings are significant. Yet, another advantage of partially hydrolyzed starch was that the glucose could be made available in a kind of controlled release process, which avoided the kind of repression, which normally one faces with fermentation feed stocks.

Studies in fermenter. Table 1 shows the summary of the fermentation process (batch mode) such as cell growth, substrate consumption and the corresponding product fermentation. From the data it was evident that more than 95% of the reducing sugars were consumed and within 40h fermentation the accumulation of glutamic acid was nearly 21 g/L, which was approximately two and half fold more than what was obtained in shake flask studies. Based on the glucose consumed and also by assuming 81.7% as the theoretical conversion, we got conversion of about 66.3%.

Fig. 5 shows a comparison of bacterial growth and L-glutamic acid production in batch and fed-batch process. A maximum of 25 g/L of glutamate was obtained in fed-batch process, which was 16% more than the batch mode. Hence, the maintenance of an active biomass constantly for a long period could enhance the accumulation of glutamate to a certain extent only.

Recovery of glutamic acid. The fermented broth contained various impurities such as bacterial cells, macromolecules, pigments, inorganic substances, organic substances etc., which were removed by filtration and centrifugation. Glutamic acid was purified from cation exchange resin. The elution profiles of the glutamic acid concentration from the column are shows in Fig. 6. Symmetrical peaks of glutamic acid concentration and absorbance were obtained. Glutamic acid was recovered for the highest

Table 1 – The overall changes during L-glutamic acid fermentation (in 5 L fermenter) using *Brevibacterium* sp.

Time	Optical density (610 nm)	Conc. Of glutamic acid (mg/ml)	Glucose consumption (%)
12	1.65	4.15	21.84
24	2.12	8.35	42.64
36	2.07	4.70	89.78
48	1.98	25.20	92.73
60	1.95	25.14	93.10

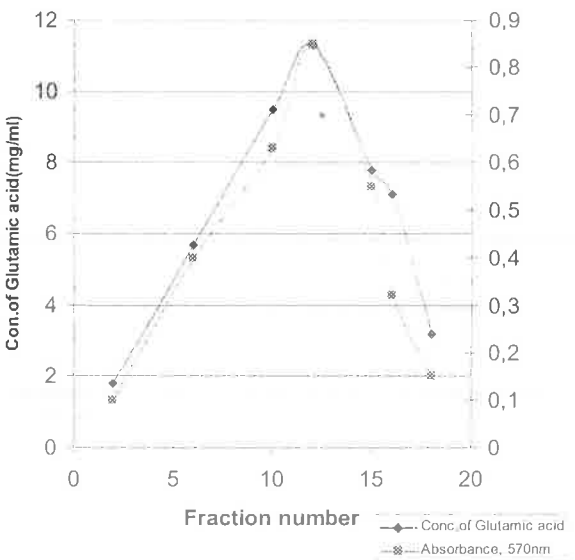

Figure 6 – Glutamic acid recovered at different elution volumes through ion-exchange resin column.

yield, taking the fractions of 8 to 17 (total elution volume of 200 ml) through the ion-exchange column. By changing the pH to the isoelectric point (3.2) and by the subsequent cooling of the eluent, glutamic acid was crystallized out. The purity of the final product, which was reconfirmed using IR spectrum, showed similar peak at the same frequencies. All functional groups of the product (NH_2 COOH, and CH_2) showed a frequency similar to standard glutamic acid as shown in Fig. 7.

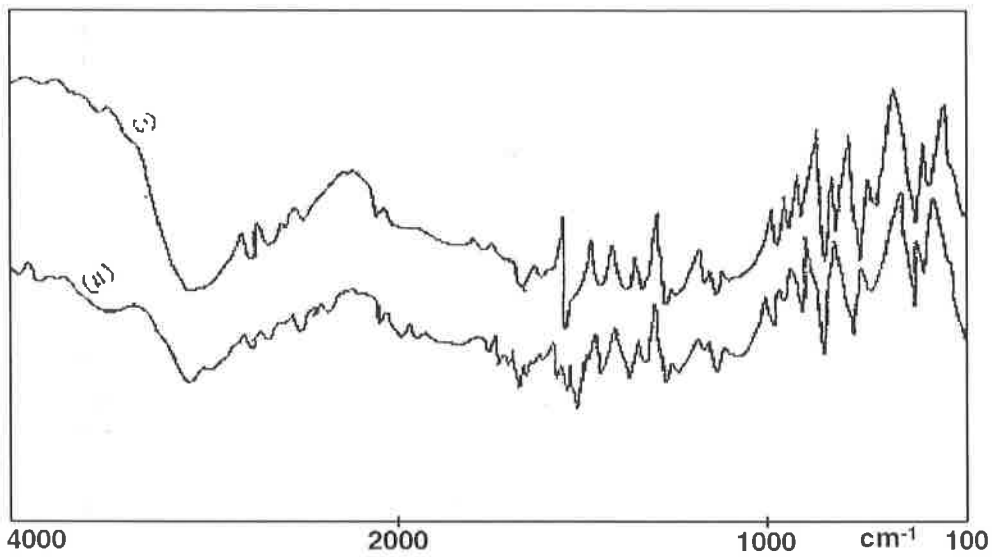

Figure 7 IR-spectra of standard (I) and purified (II) samples of L-glutamic acid.

CONCLUSIONS

The results of the present study have indicated the possibility of utilizing starchy tubers as a raw material for L-glutamic acid production. It further indicated that as a soluble non-sweet, easily digestible carbohydrates, the low DE starch hydrolysate could find a potent industrial application (as a low cost raw material for amino acid fermentation). By making use of various combinations of environmental factors, ion-exchange resins could effectively be utilized for concentration and separation of glutamic acid.

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RESUMO

Produção de ácido L-glutâmico a partir de um hidrolisado de amido de mandioca usando resina de troca iônica

Pesquisas foram realizadas com o objetivo de produzir ácido glutâmico a partir de *Brevibacterium* sp. utilizando um substrato disponível na região, a mandioca (*Manihot esculenta* Crantz). Estudos iniciais, desenvolvidos em shaker, demonstraram que mesmo obtendo elevado rendimento com 85-90 DE (Dextrose Equivalent value), a taxa de conversão máxima (~34%) foi obtida usando um hidrolisado de amido parcialmente digerido, i.e. 45-50 DE. As fermentações foram realizadas em um fermentador de 5 L, usando um hidrolisado de amido de mandioca adequadamente diluído, preparado à partir de um valor DE de 85-90. O meio enriquecido com nutrientes resultou em um acúmulo de 21 g/L de ácido glutâmico, com uma elevada (66,3%) taxa de

conversão da glicose em ácido glutâmico (baseada em glicose consumida e em uma taxa de conversão teórica de 81,74%). As condições mais favoráveis, levando à uma máxima produção, foram pH 7.5, temperatura 30°C e agitação de 180 rpm. Quando a fermentação foi conduzida em um reator do tipo descontinuo alimentado, onde a concentração de açúcares redutores era mantida em 5% w/v, foram obtidos 25.0 g/L de glutamato após 40 h (16% a mais do que no modo descontinuo). Para a recuperação e purificação do ácido glutâmico, foi utilizada a separação por cromatografia com resina de troca inônica. O ácido foi posteriormente cristalizado e separado, levando-se em consideração a sua baixa solubilidade no ponto isoelétrico (pH 3.2).

Palavras-chave: *Brevibacterium* sp, L-ácido glutâmico, hidrolisado de mandioca, processo batch e fed-batch, resina de troca iônica, purificação

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PROPERTIES OF A NEW FUNGAL β-GALACTOSIDASE WITH POTENTIAL APPLICATION IN THE DAIRY INDUSTRY

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ABSTRACT

β-Galactosidase or β-D-galactoside-galactohydrolase (EC. 3.2.1.23) is an important enzyme industrially used for the hydrolysis of lactose from milk and milk whey for several applications. Lately, the importance of this enzyme was enhanced by its galactosyltransferase activity, which is responsible for the synthesis of transgalactosylated oligosaccharides (TOS) that act as functional foods, with several beneficial effects on consumers. *Penicillium simplicissimum*, a strain isolated from soil, when grown in semi-solid medium showed good productivity of β-galactosidase with galactosyltransferase activity. The optimum pH for hydrolysis was in the 4.0-4.6 range and the optimum pH for galactosyltransferase activity was in the 6.0-7.0 range. The optimum temperature for hydrolysis and transferase activity was 55-60°C and 50°C, respectively, and the enzyme showed high thermostability for the hydrolytic activity. The enzyme showed a potential for several industrial applications such as removal of 67% of the lactose from milk and 84% of the lactose from milk whey when incubated at their original pH (4.5 and 6.34, respectively) under optimum temperature conditions. When incubated with a 40% lactose solution in 150 mM McIlvaine buffer, pH 4.5, at 55°C the enzyme converted 86.5% of the lactose to its component monosaccharides. When incubated with a 60% lactose solution in the same buffer but at pH 6.5 and 50°C, the enzyme can synthetize up to 30.5% TOS, with 39.5% lactose and 30% monosaccharides remaining in the preparation.

Key words: β-Galactosidase, galactosyltransferase, galactooligosaccharides, lactose, prebiotic effect

INTRODUCTION

β-galactosidase or β-D-galactoside-galactohydrolase (EC. 3.2.1.23) is used industrially to obtain the hydrolyzates of lactose from milk and milk whey for utilization in bakery products, ice creams, animal feed and as a sugar source for several fermentation products (23). Furthermore, economic aspects of transport and storage require the concentration of milk whey by evaporation or ultrafiltration. The easy crystallization of lactose, however, represents a strong limitation in such

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processes. Monosaccharides derived from hydrolysis are highly soluble and usually prevent the crystallization of the remaining lactose (23). Enzymatic hydrolysis of lactose from milk and milk whey is also desirable for lactose-intolerant individuals (10).

Enzymologists have recently attributed great importance to the galactosyltransferase activity of β-galactosidases to obtain the synthesis of oligosaccharides with 2 or more galactose units starting from lactose. Solutions with high lactose concentrations, such as evaporated milk whey, submitted to the action of β-galactosidase from some microorganisms suffer a transgalactosylation reaction, producing a galactooligosaccharide mixture or transgalactosylated oligosaccharides (TOS) which act as functional foods, with several beneficial effects for their consumers. Diets enriched with TOS significantly increase the population of *Bifidobacterium, Lactobacillus* and some species of *Streptococcus*, with a consequent decrease in the concentration of putrefactive bacteria in the gut of humans and other animals (prebiotic properties). This change in the intestinal flora (bifido effect) composition has been proposed to be responsible for the decrease of putrefactive products in the feces, for a lower blood cholesterol content (1,13), higher Ca^{2+} absorption, a smaller loss of bone tissue in ovariectomized rats (2), and a lower incidence of colon cancer (21). Besides, TOS are non-cariogenic sugars and can be widely used as additives in several infant formulations, and in the manufacture of candy, pastry, bread and jams because of their heat stability (17).

This work describes the hydrolytic and transgalactosylase activities of a semi-purified β-galactosidase produced by a strain of *Penicillium simplicissimum* isolated from soil. The enzyme was applied to milk, milk whey and to a highly concentrated lactose solution, showing several desirable properties for industrial application.

MATERIALS AND METHODS

Enzyme. β-galactosidase from *Penicillium simplicissimum* was produced by semi-solid fermentation, semi-purified by precipitations with $(NH_4)_2SO_4$ and cold acetone followed by exchange chromatography on DEAE-Sephadex A-50 and CM-cellulose columns. These procedure allowed to obtain a fraction with 27.66 U/mg of protein and a purification of 9.7 times (3).

Enzyme assay. The hydrolytic activity of β-galactosidase was determined using two substrates, i.e. *O*-nitrophenyl-β-D-galactopyranoside (ONPG) and lactose. In the first case, properly diluted aliquots of 0.1 ml of enzyme solution were incubated with 1.0 ml of 2.0 mM ONPG solution in 75 mM McIlvaine buffer, pH 5.0, at 50°C. After 5 min the reaction was stopped by the addition of 3.0 ml of 0.1 M NaOH and the *O*-nitrophenol (ONP) liberated was estimated at 420 nm with a Pharmacia spectrophotometer (Molar Absorptivity = 5,200 $cm^{-1}.M^{-1}$). When lactose was utilized as substrate, the reaction medium consisted of 1.0 ml of appropriately diluted enzyme solution and 2.0 ml of 3.0% lactose solution in 75 mM McIlvaine buffer, pH 5.0, at 50°C. After 30 min the reaction was stopped by deproteinization with 0.1 ml of 0.33 N $Ba(OH)_2$ plus 0.1 ml of 5.0% (w:v) $ZnSO_4$, followed by centrifugation at 1,600 x g for 10 min. The released glucose was determined by the glucose-oxidase method, as cited by Cruz *et al* (4). In both cases an enzyme unit (U) was defined as the amount of enzyme necessary to liberate 1 μmol of the products per min under the assay conditions. Protein content was determined by the method of Lowry *et al* (16), and bovine serum albumin was utilized as standard.

Effect of pH and temperature on hydrolytic activity. The pH effect of the purified enzyme on the hydrolysis of both substrates, was estimated after incubation of the reaction systems in 75 mM McIlvaine buffer in the 2.6 to 7.6 pH range, at 50°C. The optimum temperature for ONPG and lactose was determined in the 35 to 70°C range in 75 mM McIlvaine buffer, pH 5.0. Heat denaturation whitout the presence of substrates was studied by treating the enzyme samples in the same buffer and at the same molarity at 55°, 60°, 65° and 70°C for 2 h. Aliquots of 0.2 ml of each treatment were removed every 5 minutes, cooled immediately in an ice bath and kept at 4°C for 24 h before the determination of residual activity using ONPG as substrate.

Optimal pH and temperature for galactosyltransferase action. To study the effects of pH and temperature on galactosyltransferase action it was utilized reaction systems consisting of 50 ml of 20% (w:v) lactose solution in 75 mM McIlvaine buffer and 18 U of hydrolytic activity of the enzyme. Optimum pH was determined in the 3.0 to 7.6 range

at 45°C and optimum temperature in the 40° to 60°C range, at pH 6.5. The reactions were stopped after 8 h by treatment with boiling water for 5 min and the reaction products were analyzed by high performance liquid chromatography (HPLC).

Preparation and enzymatic treatment of milk and milk whey. Milk was prepared by rehydration of 100 g of defatted powdered milk in distilled water at the 1:10 ratio (w:v). The fractions to be used for milk whey production were supplemented with 2 N HCl until the pH dropped to 4.5, for isoelectric precipitation of their proteins. After centrifugation at 1,600 x g for 5 min, the total sugar content of 50 ml aliquots of the milk and milk whey thus obtained were adjusted to 5.1% (w:v) according to the method of Dubois *et al.* (6), and the samples were incubated with 10.2 U at their original pH (6.34 and 4.5, respectively), at 50°C for 12 h. Samples were removed at predetermined time intervals, the reactions were stopped by treatment with boiling water for 5 min and the sugars were analyzed by HPLC.

Hydrolysis of highly concentrated lactose solutions. To verify the hydrolytic action of the enzyme on concentrated solutions, 100 ml of a 40% (w:v) lactose solution in 150 mM McIlvaine buffer, pH 4.5, was incubated with 100 U of β-galactosidase at 50°C. Samples of 2.0 ml were collected every 6 h and the reaction was stopped by deproteinization with the $Ba(OH)_2$-$ZnSO_4$ system as described above. The sugars in the reaction medium were quantified by HPLC.

TOS production. A 50 ml aliquot of the reaction medium was prepared with 26.6 U of β-galactosidase and lactose solution in 150 mM McIlvaine buffer, pH 6.5, at a final concentration of 60% (w:v), which was the maximal lactose concentration permitting total solubilization in that medium. The reaction system was maintained at 50°C and 2 ml samples were removed at predetermined time intervals for sugar analysis by HPLC.

Carbohydrate analysis. The carbohydrates in the reaction medium were analyzed with a Shimadzu LC-10A chromatography apparatus, model RID 6-A, equipped with a refraction index detector and a 250 x 4.6 mm Supelcosil LC-NH_2 column in a room at 20°C. A system consisting of acetonitrile-water (4:1) was used as solvent and the flow rate was 2.0 $ml.min^{-1}$. Glucose, galactose, lactose, (Merck) and the galactooligosaccharides raffinose and stachyose (Sigma) were used as standards. Calculations were performed using the external standardization techniques. All experiments were conducted in triplicate and the relative standard deviation was less than 5%.

RESULTS AND DISCUSSION

Effect of pH. As shown in Fig. 1, the enzyme acted in a similar way on both substrates tested, but showed a broader range of optimum pH for the hydrolysis of lactose. Its maximum activity in pH 4.0 to 4.6 coincided with the behavior of other fungal β-galactosidases such as those produced by *A. oryzae* (20) *A. niger* (9), *Alternaria alternata* (14) and *Sterigmatomyces elviae* (19). Fig. 1 also shows that, under the assay conditions used, the optimum pH for galactosyltransferase action was in the neutral range, with a peak at pH 6.5, in contrast to the hydrolytic activity which peaked within the acid range. Higher transferase action at higher pH than the optimum pH

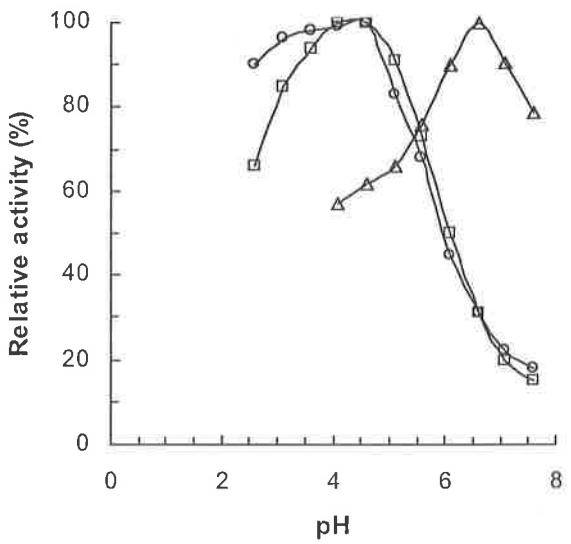

Figure 1 – pH effect on hydrolysis of lactose (o), ONPG (□) and galactosyltransferase activity (Δ) of β-galactosidase from *Penicillium simplicissimum*.

for hydrolytic activity has been previously described by Kikushi and Ishiwata (14), using β-galactosidase from *Alternaria alternate* and by Cruz *et al* (5) using fructosyltransferase from *Aspergillus japonicus* for fructooligosaccharides (FOS) synthesis from concentrated solutions of sucrose. According to authors, these findings may be explained by inhibition

of transferase activity by the high monosaccharides concentrations (specially glucose) in the reaction medium at optimum hydrolytic pH.

Effect of temperature. As shown in Fig. 2, β-galactosidase from *Penicillium simplicissimum* showed higher activity on lactose at temperatures below 60°C and lower activity at temperatures above 55 and 60°C compared to its action on ONPG. These differences may be possibly explained by thermal denaturation, since the enzyme was exposed to these temperatures for a longer time (30 minutes) when lactose was used as substrate, as opposed to only 5 min when ONPG was used as substrate. Fig. 3 shows that hydrolytic activity of the enzyme was reduced by only 14% during exposure to 60°C for 120 min. It is also possible that the transferase activity of the enzyme is more sensitive to heat than the hydrolytic activity, a fact that would explain the maximum galactosyltransferase activity observed at

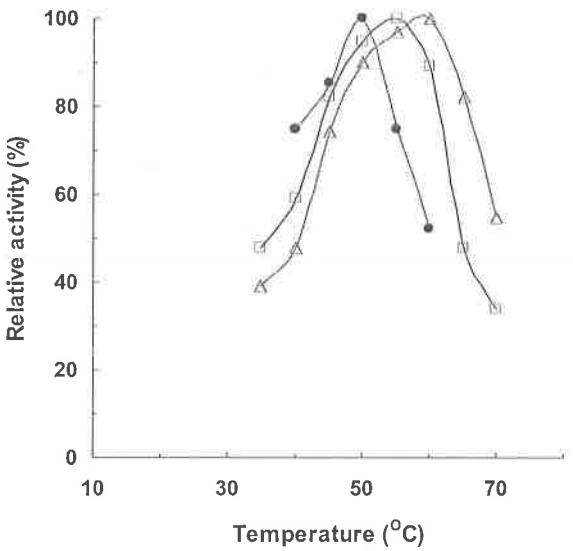

Figure 2 – Temperature effect on hydrolysis of lactose (□), ONPG (△) and galactosyltransferase (●) activity of the enzyme.

50°C (Fig. 2). In any case, the thermostability of β-galactosidase from *Penicillium simplicissimum* is higher than that of the enzymes produced by *A. niger* (9), *A orizae* (20), *Alternaria alternata* (14) and some yeasts (19) already proposed for milk lactose removal and/or TOS synthesis. Thermostable enzymes are desirable in industrial processes because they can accelerate the reactions and prevent microbial contamination (8).

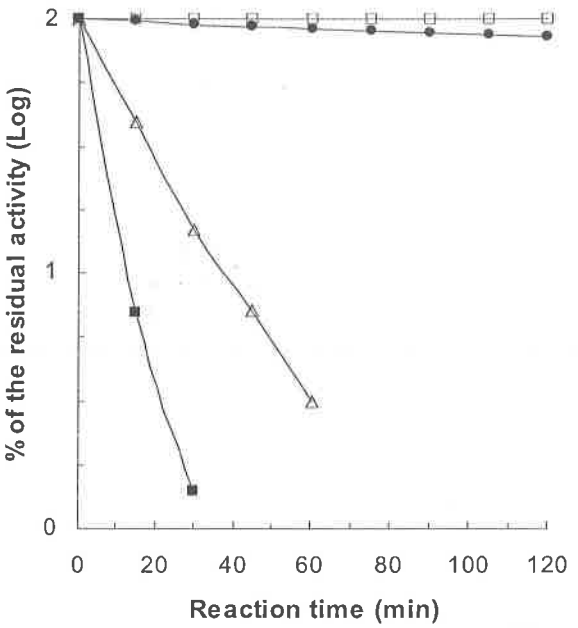

Figura 3 – Thermal denaturation of β-galactosidase from *Penicillium simplicissimum*. (□55°C; ●60°C; △65°C; ■70°C)

Lactose removal from milk and milk whey. As shown in Fig. 4, after 10 h of the proposed treatment only 33% and 16% lactose remained in milk and in milk whey, respectively. The higher efficiency of the enzyme in the hydrolysis of milk whey compared to milk lactose is explained by the differences in pH between the tests, i.e., pH 4.5 for whey and pH 6.34 for milk lactose. Despite the reduced hydrolytic activity shown by the enzyme at pH above 6.0, under the assay conditions (Fig. 1) the rate of milk lactose hydrolysis (67%) can be considered highly satisfactory. According to Gikas and Lopes-Leiva review (8), some traditional enzymatic processes for milk lactose removal available on the market present a final hydrolytic value of 50 to 70% at reaction times longer than 18 h. The high rate of lactose hydrolysis obtained here perhaps may be explained by the high Ca^{2+} content of milk. It should be pointed out that, under the present assay conditions, divalent cations stimulated the performance of the enzyme, while chelating agents (EDTA, for example) inhibited the action of the enzyme (3).

In the enzymatic treatment of milk and milk whey the higher TOS synthesis was verified at 3 h of reaction. However it is difficult to explain the smaller TOS production in milk treatment that in milk whey treatment, especially because its reaction was

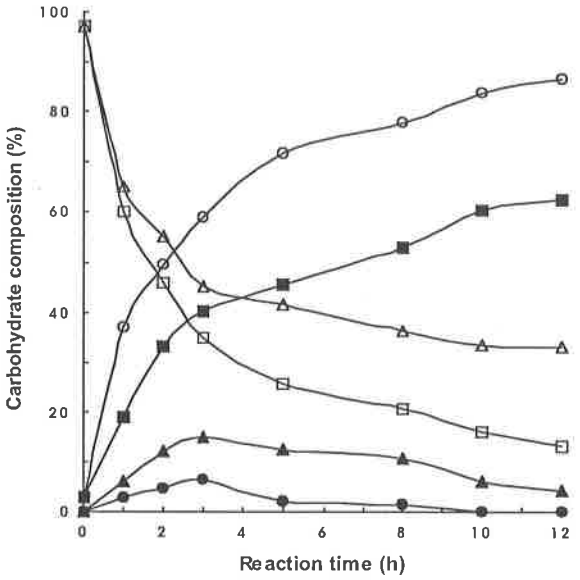

Figure 4 – Hiydolysis of lactose from milk and milk whey by β-galactosidase from *Penicillium simplicissimum*. (M – milk; MW – milk whey) – △ M lactose; □ MW lactose; ■ M monosacch.; ○ MW monosacch; ▲ M TOS; ● MW TOS.

performed at higher pH. Fig. 4 also shows that TOS synthetized in the milk treatment decreases quickly after the 3 h of reaction, simultaneously with the monosaccharides increase. This could suggest that the same higher content of Ca^{+2} that stimulates the hydrolitic activity, inhibits the transferase activity. Besides, could the highest protein content in milk reduce the galactosyltransferase activity? Unfortunately the specialized literature has not discussed those questions. No change in milk protein solubility was observed during the course of the experiment, suggesting the absence of proteases in the enzymatic mixture used.

Hydrolysis of highly concentrated lactose solutions. As shown in Table 1, highly concentrated lactose solutions such as those present in milk whey concentrates can be hydrolyzed by β-galactosidase from *Penicillium simplicissimum* as long as the enzyme is applied to the solution at high enzyme:substrate ratios and under conditions of optimum pH and temperature. Under these conditions, when 100 U of β-galactosidase was added to 100 ml of a 40% lactose solution the reaction was time-dependent up to 24 h, when the residual lactose content dropped to only 13.5%, with 83% monosaccharides being obtained. When the reaction time reached 48 h the process reached equilibrium with a production of

Table 1 – Hydrolysis of lactose by β-galactosidase from *Penicillium simplicissimum*.

Time (h)	Galac + Gluc (%)	Residual lactose (%)	TOS* (%)
6	77.2	17.2	5.6
12	81.7	13.8	4.5
24	86.0	13.5	0.5
36	79.1	16.5	4,4
48	76.2	19.5	4.3
60	77.2	17.4	5.4
72	77.8	17.4	4.8

Lactose 40%; pH 4.5; Temperature 50°C; Enzyme/Substrate ratio = 2.5 U/g
* Transgalactosylated oligosaccharides

about 77% monosaccharides, 17.5% residual lactose and the rest as TOS. The increase of the remaining lactose from 13.5 to 17.4% in the interval time between 24 and 48 h is inside of admitted variation coefficient. However it could also be explained by the phenomenon of the lactose re-synthesis, suggesting that in high concentration, glucose can also act as acceptor molecule of galactose.

Industrially, the concentration of milk whey is an extremely desirable process because of the economy in the cost of storage and transport processes, which are limited by the easy crystallization of lactose. Thus, the enzymatic hydrolysis of lactose in these concentrates also represents a suitable process since its constituent monosaccharides are highly soluble and prevent the crystallization of the remaining lactose.

TOS production. As shown in Fig. 5, the maximum percentage of TOS obtained was 30.5%, reached at the 16 h of reaction, with 39.5% of remaining lactose and 30% of monosaccharides, mainly glucose. Among the TOS, only tri- and tetrasaccharides were synthesized and longer chain oligosaccharides were not found at any time during the course of the reaction. This observation confirms the results described by Smart *et al.* (22), according to which high lactose concentrations favor the synthesis of short- chain oligosaccharides. It was also observed that complete inhibition of oligosaccharide synthesis occurred when the monosaccharides content of the reaction medium reached the total concentration of TOS. From this reaction time (16 h) on, oligosaccharide hydrolysis, rather than synthesis, occurred, which was more marked than lactose hydrolysis. This was also observed for the lowest

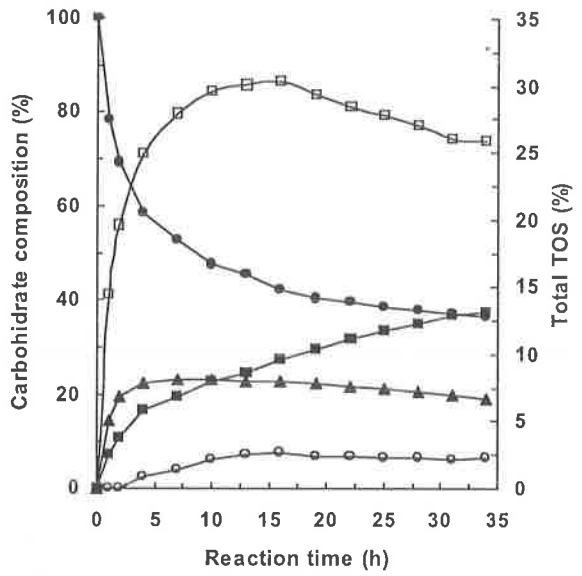

Figure 5 – Reaction course for transgalactosylated oligassacharides (TOS) synthesis in the best conditions of pH (6.5), temperature (50°C) and lactose concentration (60% w:v) – ● lactose, ■ monosacchides, ▲ trissacharides; ○ tetrassacharides; □ total TOS.

lactose concentrations tested, which presented a lower rate of lactose conversion to TOS.

The maximal percentage of lactose conversion (30.5%) to TOS obtained in this work can be considered quite satisfactory when compared to the values reported by others. "Oligomate 50", a trade mark registered for a product marketed in Japan, contains 50% to 52% TOS (17). However, this product is obtained by two successive enzymatic treatments, with a consequent increase in cost. The values reported here are slightly higher than those reported by Dumortier *et al* (7) – 29% conversion – working with β-galactosidase from a strain of *Bifidobacterium bifidum* and with an initial lactose content of 60%. The value obtained here corresponds to an oligosaccharide content of 183 mg/ml and is 60% higher than the 114 mg/ml value obtained with β-galactosidase from *Aspergillus oryzae* by Iwasaki *et al.* (11). It is also much higher than the content of 80.7 mg/ml, 111.5 mg/ml and 81.5 mg/ml obtained by Ji *et al.* (12) with β-galactosidases from *Aspergillus oryzae*, *Kluyveromyces fragilis*, and a mixture of both, respectively.

The prebiotic properties of several oligosaccharides have been demonstrated with the administration of reduced amounts of the products to animals (13, 15, 18). Thus, it is possible that the product obtained here, with 183 mg/ml or 30.5% of TOS, is quite acceptable for use in the formulation of diets with a prebiotic effect. Furthermore, according to Matsumoto *et al.* (17), it is possible to separate the oligosaccharides from the residual lactose and glucose using cation-exchange resins, thus obtaining a final product with 85-99% TOS. Studies on this topic are currently underway in our laboratory.

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RESUMO

Propriedades de uma nova β-galactosidade fúngica com potencialidade para emprego na indústria de latícinios

A β-galactosidase ou β-D-galactosídeo-galactohidrolase (EC. 3.2.1.23) é uma importante enzima utilizada industrialmente na hidrólise da lactose do leite e soro de leite para diversas aplicações. Ultimamente, sua importância foi realçada por sua atividade de galactosiltransferase responsável pela síntese de oligossacárides transgalactosilados (TOS) que atuam como alimentos funcionais, trazendo diversos efeitos benéficos para seus consumidores. *Penicillium simplicissimum*, uma linhagem isolada do solo, mostrou grande produtividade de β-galactosidase com atividade de galactosiltransferase quando crescida em meio semi-sólido. Seu pH ótimo para hidrólise está na faixa de 4,0-4,6 e para a atividade de galactosiltransferase na faixa de 6,0-7,0. A temperatura ótima para hidrólise e transferase foram encontradas a 55-60°C e 50°C, respectivamente e a enzima mostrou maior termoestabilidade para a atividade de hidrolase. A enzima mostrou potencialidade para diversas aplicações industriais como a remoção de 67% da lactose do leite e 84% da lactose do soro de leite quando incubada nos respectivos pHs oroginais (4,5 e 6,34, respectivamente) em condições ideais de temperatura. Quando incubada com uma solução a 40% em tampão

de McIlvaine 150 mM, pH 4,5 a 55°C a enzima converteu 86,5% da lactose em seus monossacarídeos componentes. Quando aplicada a uma solução com 60% de lactose no mesmo tampão mas em pH 6,5 e 50°C, a enzima pode sintetizar até 30,5% de TOS, deixando 39,5% de lactose residual e 30% de monossacárides.

Palavras-chave: β-galactosidase, galactosiltransferase, galactooligossacárides, lactose, efeito prebiótico

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INFLUENCE OF RAW MEAT NATURAL BACKGROUND FLORA ON GROWTH OF *ESCHERICHIA COLI* O157:H7 IN GROUND BEEF

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ABSTRACT

Escherichia coli O157:H7 is a foodborne pathogen of increasing importance. It has been involved in several threatening outbreaks, most of them associated with meat products. In this study, the influence of some bacteria from the natural background flora of raw meat over *E.coli* O157:H7 in ground beef stored under refrigeration and at room temperature was evaluated. Different levels of *E.coli* O157:H7 (10^1-10^2, 10^3-10^4 and 10^6-10^7 CFU/g), inoculated in ground beef samples, were challenged with strains of non-pathogenic *E.coli*, *Pseudomonas putida* or *Leuconostoc* sp. Growth of the pathogen was monitored using standard cultural methods and an ELISA-type rapid method. Non-pathogenic *E.coli*, *Pseudomonas putida* and *Leuconostoc* sp. did not affect growth of *E.coli* O157:H7 in ground beef, both under refrigeration and at room temperature. Based on these findings, the low occurrence of *E.coli* O157:H7 in raw meat may not be attributed to antagonistic effects of bacteria from the natural background flora.

Key words: *Escherichia coli* O157:H7, antagonism, ground beef

INTRODUCTION

Enterohemorrhagic *Escherichia coli* is a foodborne pathogen of increasing importance. It was identified as a human pathogen in 1982, when *E. coli* serotype O157:H7 was associated with two outbreaks of hemorrhagic colitis (20). Since then, many outbreaks have been reported, culminating in 1996 in Japan with a foodborne outbreak that affected at least 6,309 children from 62 Sakai schools (9, 23).

Most confirmed *E. coli* O157:H7 outbreaks have been associated with the consumption of undercooked ground beef and less frequently, other types of foods like unpasteurized milk and apple cider (6).

Geographically, the focus of attention on *E. coli* O157:H7 has been largely on the North American continent. However, recent reports reveal that *E. coli* O157:H7 and other serotypes of enterohemorrhagic *E. coli* are responsible for human disease in other parts of the world as well. The apparent geographic clustering of *E.coli* O157:H7 may be due to awareness by physicians and testing laboratories (13, 24). Some reports have addressed on infections caused by Shiga-toxin producing *E. coli* and its presence in food in developing countries like Argentina, Chile and Thailand (5, 15, 22). Moreover, in Argentina *E.coli* serotype O157:H7 has been associated with 2 to 18% hemolytic uremic syndrome

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(HUS) patients and with 4.5 to 7% of children with bloody diarrhea (14).

So far, there is no report on occurrence of foodborne outbreaks due to Shiga-toxin producing *E.coli* in Brazil. Even the involvement of this pathogen in human cases of hemorrhagic colitis in this country is not known. Gomes *et al.*, 1994, observed that Shiga-toxin producing *E.coli* was present in only 0.4% of children diarrheic stool samples, but none of the isolated strains was O157:H7 (10). The occurrence of *E.coli* O157:H7 or of other Shiga-toxin producing *E.coli* in cattle has also been very low (3).

Many explanations for the low occurrence of *E.coli* O157:H7 in raw meat may be considered. Studies showed that the carriage of *E.coli* O157:H7 in cattle is transient and seasonal and the prevalence of this pathogen in animals is low (1, 2). Besides this, interactions between microorganisms in raw meat are believed to be important in the selection of the microflora (7). Antibacterial activity of lactic acid bacteria (LAB) and *Pseudomonas* spp. over other microorganisms is well known (4, 17).

This study was conducted to observe possible antagonism between bacteria that are part of the background flora of raw meat and *E. coli* O157:H7 in ground beef samples kept under refrigeration and at room temperature. The study was carried out through challenge tests done with strains of non-pathogenic *E.coli*, *Leuconostoc* sp. and *Pseudomonas* sp. isolated from Brazilian raw meat.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli O157:H7 strain EDL 933 was isolated from a hamburger outbreak (17). Non-pathogenic *Escherichia coli*, *Pseudomonas putida* and *Leuconostoc* sp. were isolated from Brazilian fresh raw meat products, purchased in local supermarkets of the city of São Paulo, Brazil.

The *E.coli* strain selected for this study was isolated using methods recommended by APHA (12) and characterized as non-pathogenic using DNA probes (16). The *Pseudomonas putida* strain was isolated using cetrimide-fucidin-cephaloridine agar (CFC agar – pseudomonads agar base type CM 559 with selective suplement SR 103; Oxoid), incubated at 30°C for 48 hours, and identified as *Ps. putida* using the VITEK system (bio-Meriéux). The *Leuconostoc* sp. strain was isolated using MRS agar (MRS broth plus 1.5% agar) for lactic acid bacteria, with incubation at 30°C for 48 hours, and identified as *Leuconostoc* sp. according to Schillinger and Lücke, 1987 (21).

Preparation of meat

Samples of bovine *semitendinosus* muscle were purchased in local supermarkets of the city of São Paulo, Brazil. Under aseptic conditions, the external layer (approximately 0.5 cm thick) of the muscle was removed and internal portions were grounded in a sterile meat grinder. The ground meat was divided into portions of 25 g in sterile plastic bags and kept frozen until used.

Preparation of cultures

The *E. coli* O157:H7 and the non-pathogenic *E. coli* strains were cultivated in TSB at 35°-37°C for the time needed to reach 10^8-10^9 CFU/ml, determined through a spectrophotometric calibration curve. The *Ps. putida* and the *Leuconostoc* sp. strains were grown in TSB at 25°C and in MRS broth at 30°C, respectively, for the time needed to reach 10^8 CFU/ml, also established through a spectrophotometric calibration curve. The bacterial cultures were serially diluted in 0.1% peptone water and 0.1 ml of each dilution was plated onto TSA or MRS agar plates (MRS broth plus 1.5% agar), for determination of the exact number of CFU/ml.

Challenge tests

Portions of 25 g of ground beef were inoculated with 2.5 ml of the *E. coli* O157:H7 and the challenge cultures, using proper dilutions in order to get the following combinations:

- *E. coli* O157:H7 (0, 10^1-10^2, 10^3-10^4 or 10^6-10^7 CFU/g) and non-pathogenic *E. coli* (0, 10^1-10^2, 10^3-10^4 or 10^6-10^7 CFU/g);
- *E. coli* O157:H7 (0, 10^1-10^2, 10^3-10^4 or 10^6-10^7 CFU/g) and *Pseudomonas putida* (0, 10^3-10^4 or 10^6-10^7 CFU/g);
- *E. coli* O157:H7 (0, 10^3-10^4 or 10^6-10^7 CFU/g) and *Leuconostoc* sp. (0 or 10^6-10^7 CFU/g).

Six equal samples were prepared for each inoculation level and combination. After

homogenization of the inoculated meat samples by hand massaging of the plastic bags, four samples were kept under refrigeration (8,5°C) and analyzed after 24, 48, 72 and 96 hours. The two remaining samples were kept at room temperature (25°C) and analyzed after 24 and 48 hours. Negative controls, consisting of non-inoculated meat portions and of meat portions inoculated with only one of the microorganisms at each inoculation level, were also included.

Analysis of inoculated meat samples

Each ground beef sample was homogenized with 225 ml of 0.1% peptone water in a Stomacher (Seward Medical Ltd.) and subsequent decimal dilutions were made using the same diluent. Portions of 0.1 ml of each dilution were plated onto MacConkey-sorbitol agar (MCS, Difco) for enumeration of *E. coli* O157:H7 (sorbitol negative colonies) and non pathogenic *E. coli* (sorbitol positive colonies), onto cetrimide-fucidin-cephaloridine agar (CFC agar, Oxoid) for enumeration of *Pseudomonas* sp., or onto MRS agar (MRS broth plus 1.5% agar) for enumeration of lactic acid bacteria. The temperature and time of incubation for MCS agar was 35-37°C for 18-24 hours, for CFC agar, 25°C for 48 hours and for MRS agar, 30°C for 48 hours. Colonies of *E. coli* O157:H7 on MacConkey-sorbitol agar were identified using suitable biochemical (glucose, lactose and sorbitol fermentation, production of gas, H_2S, indol, urease and lysine descarboxilase and motility) and serological tests, according to Ewing, 1986 (7) and Toledo *et al.*, 1982a,b (25, 26).

Enumeration of *E. coli* (non-pathogenic) was also performed on Petrifilm™EC plates (3M Microbiology, St. Paul, MN), with incubation for 18-24 hours at 35°-37°C. For the enumeration of *E. coli* O157:H7, the Petrifilm™ kit HEC (3M Microbiology, St. Paul, MN) was used. This kit is based in a ELISA-type test, carried out with colonies grown on Petrifilm EC plates. The colonies are transfered from the plate to a reactive disc and O157:H7 antigens, if present, are used to capture enzyme-labeled anti-O157 antibodies (i.e., conjugate) in the first development step. The antibody location is detected in the second development step when the bound enzyme converts an identifying substrate to a permanent black spot on the disc. Each spot indicates an O157:H7 presumptive-positive colony.

RESULTS AND DISCUSSION

Figs. 1a and 1b illustrate the growth of *E. coli* O157:H7 in the samples kept under refrigeration and at room temperature, respectively, when the inoculation level of both *E. coli* O157:H7 and the challenging microorganisms was 10^6-10^7 CFU/g. When *E. coli* O157:H7 was inoculated individually in the meat samples and kept under refrigeration (Fig. 1a), the counts remained relatively constant throughout the 96 hours of experiment. A similar observation occurred when the other competing microorganisms were also present. At room temperature (Fig. 1b), the counts of all microorganisms increased similarly and were almost identical to that of control treatments in which *E. coli* O157:H7 was alone.

The curves in Fig. 1a and 1b are almost coincident, presenting counts that didn't change significantly during the experiment.

Figure 1a

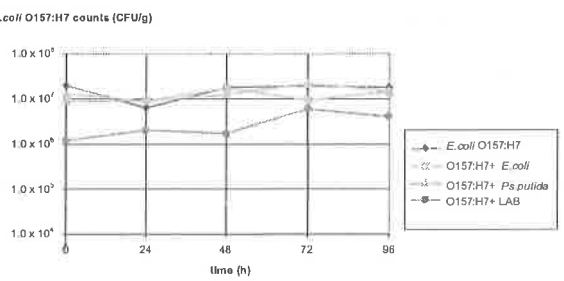
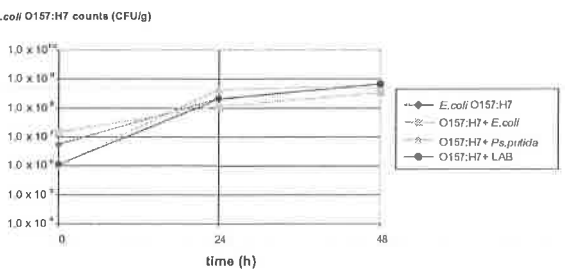


Figure 1b



Figures 1a and **1b** – Counts of *E. coli* O157:H7 in ground meat samples inoculated with *E. coli* O157:H7 and non-pathogenic *E. coli*, *Pseudomonas* spp. (*Ps. putida*) or lactic acid bacteria (LAB – *Leuconostoc* sp.), using an inoculation level of 10^6-10^7 CFU/g. **1a** = meat samples kept under refrigeration; **1b** = meat samples kept at room temperature.

When the intermediate inoculation levels of 10^3-10^4 CFU/g for *E. coli* O157:H7 and 10^6-10^7 CFU/g for the challenging microorganism was considered (Figs. 2a and 2b) some differences in the growth curves were noted. In Fig. 2a, they were due to variations in the number of CFU/g of *E. coli* O157:H7 in the inoculum. However, the counts after 96 hours were very similar to the initial ones. In Fig. 2b, a lower count of *E. coli* O157:H7 in the presence of non-pathogenic *E. coli* was observed at 24 hours, probably caused by difficulties to enumerate low numbers of colonies of the pathogen in the presence of high number of colonies of non-pathogenic *E. coli*. These difficulties increased when the lowest inoculation level (10^1-10^2 CFU/g) was assayed and results were not considered.

These results suggest that the presence of non-pathogenic *E. coli*, *Pseudomonas putida* or *Leuconostoc* sp. did not interfere with the growth or survival of *E. coli* O157:H7 in ground beef samples kept under refrigeration or at room temperature, regardless of the level of contamination. Santos *et al.*, 1995, also observed that *E. coli* O157:H7 counts remained approximately constant in ground meat kept for 12 days at 9.5°C (20). These were less than one log cycle changes in *E. coli* O157:H7 numbers, whereas indigenous Gram negative bacteria increased their counts from the fourth up to the twelfth day at this temperature.

Greer and Dilts, 1995, observed that spoilage bacteria grew on both fat and lean tissue whereas pathogens grew on fat tissue only (11). Therefore, differences in the affinity for different portions of meat by the microorganisms tested in this study may be the cause for the absence of interference over the multiplication of each other.

The correlation between results of enumeration of *E. coli* O157:H7 using the standard cultural method and the ELISA-type rapid method was high (97.2%).

Results of the current study suggest that the growth of *E. coli* O157:H7 in artificially contaminated ground beef was not influenced by the presence of different concentrations of non-pathogenic *E. coli*, *Pseudomonas putida* or *Leuconostoc* sp. at refrigeration temperature or at room temperature, indicating that this pathogen is a good competitor. Thus, the low occurrence of *E. coli* O157:H7 in ground beef may not be attributed to competition by other microorganisms.

Figure 2a

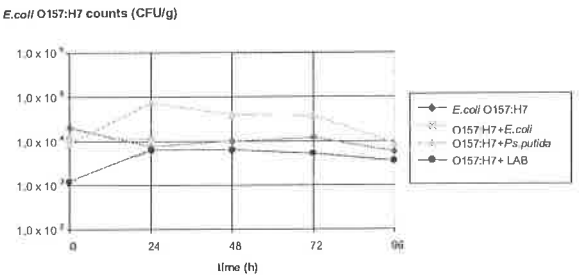
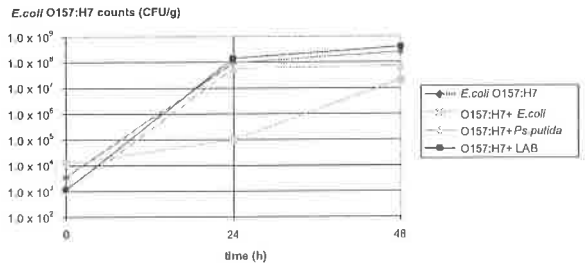


Figure 2b



Figures 2a and **2b** – Counts of *E. coli* O157:H7 in ground beef samples inoculated with *E. coli* O157:H7 and non-pathogenic *E. coli*, *Pseudomonas* spp. (*Ps. putida*) or lactic acid bacteria (LAB – *Leuconostoc* sp.), using an inoculation level of 10^3-10^4 CFU/g for *E. coli* O157:H7 and of 10^6-10^7 CFU/g for the other bacteria. **2a** = meat samples kept under refrigeration; **2b** = meat samples kept at room temperature.

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RESUMO

Influência da microbiota natural da carne na multiplicação de *Escherichia coli* O157:H7 em carne bovina moída

Escherichia coli O157:H7 é um patógeno de origem alimentar de importância crescente, tendo

sido envolvido em diversos surtos ameaçadores, a maioria deles associada ao consumo de produtos cárneos. Neste estudo foi avaliada a influência de algumas bactérias da microbiota natural da carne crua sobre *E. coli* O157:H7 em amostras de carne bovina moída armazenadas em refrigeração e à temperatura ambiente. As amostras foram inoculadas com diferentes níveis de *E. coli* O157:H7 (10^1, 10^3 e 10^6 UFC/g) e de *E. coli* não patogênica, *Pseudomonas putida* ou *Leuconostoc* sp. A multiplicação do patógeno foi monitorada através de metodologia convencional e através de método rápido do tipo ELISA. *E. coli* não patogênica, *Pseudomonas putida* e *Leuconostoc* sp. não exerceram influência sobre a multiplicação de *E. coli* O157:H7 em carne moída, tanto em refrigeração como à temperatura ambiente. Assim sendo, a baixa ocorrência de *E. coli* O157:H7 em carne crua não pode ser atribuída a efeitos antagônicos de bactérias de sua microbiota natural.

Palavras-chave: *Escherichia coli* O157:H7, antagonismo, carne moída

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HEPATOSPLENOMEGALY CAUSED BY AN EXTRACT OF CYANOBACTERIUM *MICROCYSTIS AERUGINOSA* BLOOM COLLECTED IN THE MANGUABA LAGOON, ALAGOAS -BRAZIL

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ABSTRACT

Cyanobacteria (*Microcystis aeruginosa*), which produce powerful hepatotoxic cyclopeptides, were collected and submitted to the determination of toxicity through intraperitoneal injections made in 30 and 90 days-old Swiss albino mice. The liver and the spleen were histopathologically analyzed and the weight and vital signs development were monitored. Test of toxicity resulted in a LD_{50} of 154.28 mg.Kg^{-1}. *M. aeruginosa* represented 95% of the analyzed biomass. The ratios between liver weight and body weight in the animal inoculated with a single dose were 6.0% and 7.2%, with multi doses 7.0% and 7.5% and in the control animals 4.0% and 5.0%, for adult and young animals, respectively. There was an accentuated increase in the volume and weight of the spleen, and the animals inoculated with a single dose showed a ratio between spleen weight and body weight of 0.67% and 0.37%, with multidoses 1.22% and 1.05% and the control animals the ratio was 0.12% and 0.15%, for adult and young animals, respectively. The young animals inoculated with single and multi doses had an increase of 150% and 407% in the spleen size while the adults increased, 607% and 845%, respectively, in relation to the control. The histopathological analysis showed strong differences in the structure of the hepatic parenchyme in control animals and in those exposed to the *M. aeruginosa* extract. The main alterations were the congestive aspect, including the sinusoid, and intrahepatic haemorrhagia. The histopathological analysis showed considerable increase in the number of multinuclear giant cells in the spleen of the animals intoxicated by *M. aeruginosa*.

Key words: *Microcystis aeruginosa*, cyanobacteria, hepatomegaly, splenomegaly

INTRODUCTION

The blooms of toxin-producing cyanobacteria frequently observed in estuary and lagoon environments have been registered in various parts of the world and have caused the intoxication and death of domestic and wild animals (4, 5, 12, 16, 17, 23, 26, 27).

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According to Pearson (18), the toxins produced by cyanobacterium may be accommodated into three categories: neurotoxins, hepatotoxins and lipopolisaccharides. The hepatotoxins, such as the microcystins produced by *Microcystis*, *Oscillatoria*, *Anabaena* and *Nostoc,* are the most studied and have been involved in the majority of the environmental incidents. They are cyclic peptides formed by seven amino acids. Two positions at the structural chain of these microcystins may be occupied by different amino acids which form a series of distinct molecules and the most common and toxic is the microcystin-LR that present in the variable positions the amino acids leucine and arginine (4, 20). *Aphanizomenon* is another species, less cited in the literature, though it may cause numbness in fishes and may be transmitted to man through consumption (3). The predominant species in the plankton of fresh and brackish water belong to the genera *Anabaena*, *Nodularia*, *Coelosphaerium, Microcystis*, *Aphanizomenon*, *Gloeotrichia* and *Oscillatoria*, all involved in incidents connected with poisoning of animals (8).

The presence of cyanobacterial toxins in rivers and water reservoirs, used as drinking water sources, has been of great concern to health investigators and authorities around the world. Recently, in Caruaru/ Brazil, 1996, there was an accident in a hemodialysis center, and the death of 56 patients was attributed to a hepatic intoxication caused by toxins present in the water. The toxic effects and the risks to the population due to the presence of cyanobacteria in water sources are very big and the critical examples are diarrhea, nausea, muscle weakness, cutaneous paleness and liver tumors (4, 6, 13). Another aggravating point is the persistence of cyanobacterial toxins in water when they are not removed or destroyed by the conventional treatment system exposing the population to the consequences of these toxins (1, 14).

In the Estuary-Mundaú/Manguaba Lagoon Complex (CELMM), in Alagoas/Brazil (9° 35' 00'' - 9° 45' 00'' S. Lat. and 35° 42' 30'' - 35° 57' 30'' W. Long.), the blooms of cyanobacterium are popularly known as "verdete" due to their green color on the water surface. The CELMM is the major food and income source for the people living at the lagoon neighborhoods.

In this study, we studied the toxicity to mice of *M. aeruginosa* collected at the estuary of the Manguaba lagoon. LD_{50} valves were determined and physical and histopathological evaluations of the liver and the spleen of the animals were also done.

MATERIALS AND METHODS

Sampling

The samples of cyanobacterium and water were collected at the surface of water sources in the "Camurupim Station" of the Manguaba Lagoon, Maceió-AL, Brazil. A plankton collecting net (mesh 45 μm) was used to determine the concentration of plankton plant species. The water samples were placed in snap cap glass recipients with a capacity of 250 ml and fixed with 4% formaldehyde. The plankton samples for the bioassay were placed in plastic bottles and taken to the laboratory in a box with ice.

Concentration of the plankton sample

The plankton plant sample was concentrated through filtration in a plankton net (mesh 45μm) at the collecting site and then frozen at -20°C. The sample was freeze-dried in the laboratory using a micromodular lyophilizer (Edwards, Crawley, England). Every algae extract was frozen -20°C until assayed.

Identification of plankton plant species

0.1 ml of the sample maintained in formaldehyde (4%) was examined in an optical microscope for the determination of plankton plant species, according to Desikachary (10), Sournia (24), Bourdley (2), Eskinazi-Leça (11), Tregouboff and Rose (25), Silva (22) and Chamixaes (7).

Inoculum preparation and LD_{50} determination

The toxicity and LD_{50} of the sample were determined through intraperitoneal injection of the algae extract in male Swiss albino mice. Increasing concentrations (77.14; 154.28; 308.57; 462.85 and 617.14 mg/Kg of animal weight) of the algae extract were prepared in 0.9% salt solution and applied in the peritonium (0.1 ml of sample per 10g of body weight). A control, containing only 0.9% salt solution, was also included. The experiment was carried out in quadruplicates, using mice weighing 35.15 ± 2.43 g. After applications, animals were observed during a period of 48 hours, kept in an aerated place with food and water.

Bioassay conducted to develop mice hepato and splenomegaly

Animals were selected according to their weight. Young and adult animals, respectively 30 and 90 days old, were used.

The animals were inoculated with sublethal doses. Each animal of the groups of young and adult animals received a single application of 38.57 $mg.Kg^{-1}$ of *M. aeruginosa*. To another group of adult animals, 5 doses of 30.85 $mg.Kg^{-1}$ of *M. aeruginosa* were applied at every 72-hours. LD_{50} was determined as described above.

All experiments were done in quadruplicates using young and adult animals. After 14 days, animals were weighed and anesthetized (see procedures below) for the extraction of liver and spleen. The weight of the liver and spleen was determined.

Biopsy

At the end of the experiments, the animals were submitted to a biopsy for morphological analysis of their organs and fixed in 10% formaldehyde for histopathological analysis, according to the following steps:

1. Anesthesy - The animals were placed in closed containers with sulfuric ether wetted cotton. After loosing conscience and living signals, they were taken to the surgery room.

2. Surgical Preparation and Animal Biopsy - Nippers, scissors and scalpels were used for the surgical opening of the animals. After being fixed to the surgical table, skin and muscles of the abdominal wall were opened, maintaining the peritoneal integrity in order to evaluate the extension of the organs and the wounds provoked by local infection. Afterwards, the peritoneum was opened for morphological analysis and posterior removal of the liver and spleen.

Statistical Analysis

The statistical analysis of the experimental data (maximum and minimum, standard deviation e standard error) was done using GraphPad Prism version 1.03 software.

RESULTS

In the samples of water collected at the Manguaba Lagoon, 95% of the analyzed biomass was represented by *M. aeruginosa*. The minimum lethal dose which killed more than 50% of the animals (LD_{50}) was 154.28 $mg.Kg^{-1}$. Figs. 1 and 2 show that there is a visible disproportion of the sizes of liver and spleen in control and in the inoculated animals. Fig. 1 shows animals with the total peritoneum and Fig. 2 shows animals with the peritoneal muscles cut.

As shown in Fig. 3, there was a reduction of 13.14% to 10.00% in the weight of young and adult animals, respectively, inoculated with a sublethal dose of *M. aeruginosa*. The inoculated young animals gained the same weight as the control animals (Fig. 3). In the bioassay with adult animals, the loss of weight was not recovered during the experiment, even in animals inoculated with a single dose (Fig. 3).

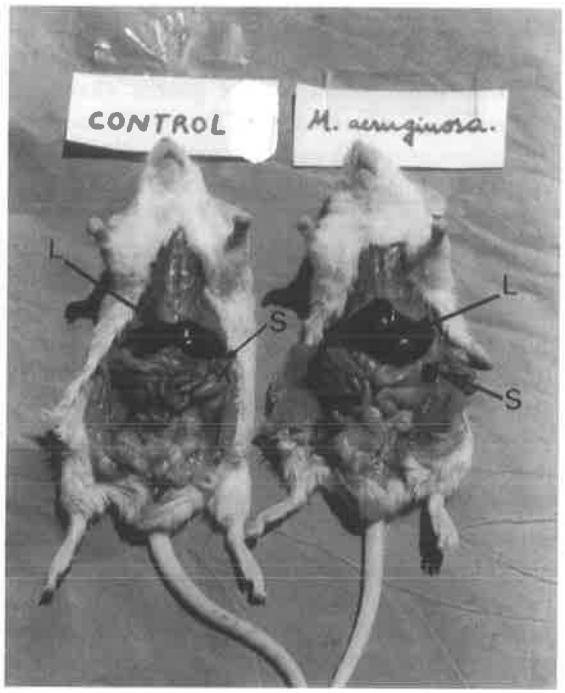

Figure 1. Photography of mice (control and inoculated with sublethal doses of *M. aeruginosa).* Showing an increase of the liver (L) and spleen (S) sizes in the inoculated animal. I is the inoculation point and X indicates the location of spleen in the non-inoculated animal.

These data are inaccordance with the results indicated in Fig. 4, which shows that the weight of the liver of young animals inoculated with a single dose of *M. aeruginosa* increased between 12% and 16%, while in the adult animals the increase was between 48% and 72%, in relation to the control. The weight proportion of the liver in the control animals was around 4% and 5%, respectively, in young and

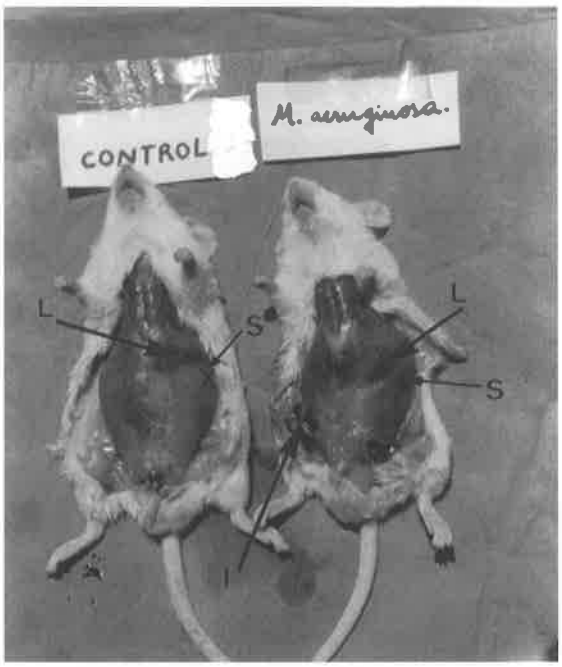

Figure 2. Photography of mice (control and inoculated with sublethal doses of *M. aeruginosa)* Showing the accentuated increase of the liver (L) and principally spleen (S) sizes in the inoculated animal.

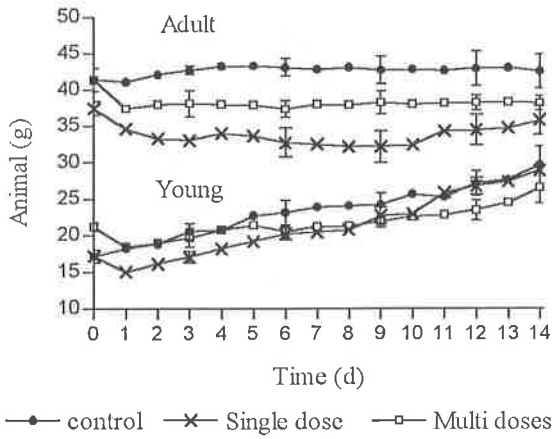

Figure 3. Variation of weight in young and adult mice (30 and 90 days old, respectively) inoculated with *M. aeruginosa* and the control group.

adult animals, while in animals inoculated with a single dose the proportion was of 6.0% and 7.2% and in animals with multi doses, 7.0% and 7.5%, in young and adult animals, respectively (Fig. 5).

According to Fig. 6, the accentuated increase in the volume (Figs. 1 and 2) and weight of the spleen (Fig. 6) indicates the high degree of toxicity of *M. aeruginosa.* The young animals inoculated with a single dose and multi doses had an increase of 150% and 407% in the spleen while the adult animals had an increase of 607% and 845%, respectively, in relation to the control. The relation between spleen weight and body weight in the control animals was around 0.12% and 0.15%, respectively, for young animals and adult animals. For the animals inoculated with a single dose this relation was 0.67% and 0.37% and for those with multi doses the relation was 1.22% and 1.05%, for adult and young animals, respectively (Fig. 7).

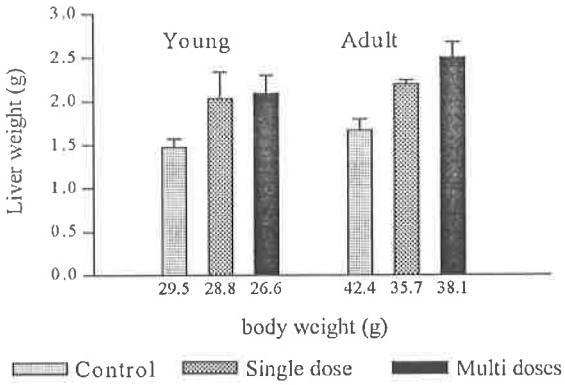

Figure 4. Hepatomegaly in young and adult mice (30 and 90 days old, respectively) inoculated with sublethal doses of *M. aeruginosa* and the control group. The horizontal numbers represent the average weight in the last day of the bioassay.

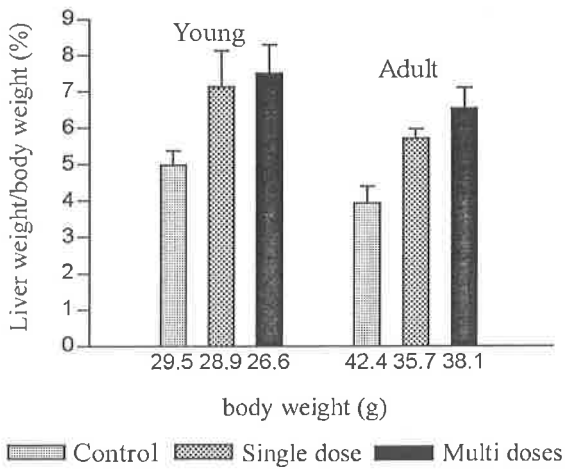

Figure 5. Proportion between liver weight and body weight of young and adult animals (respectively, 30 and 90 days old), inoculated with sublethal doses of *M. aeruginosa* and the control group. The numbers represent the average weight in the last day of the bioassay.

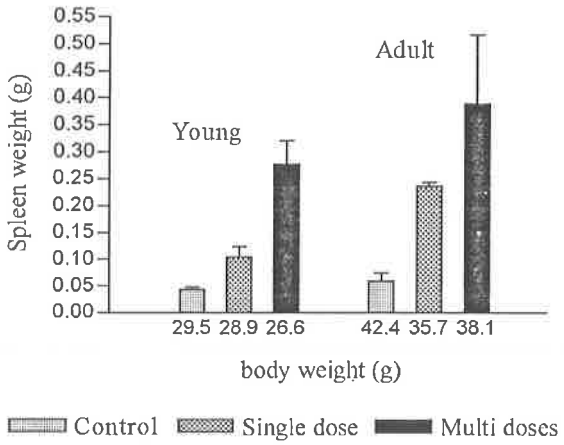

Figure 6. Splenomegaly in young and adult mice (30 and 90 days old, respectively) inoculated with sublethal doses of *M. aeruginosa* and the control group. The numbers represent the average of the weight in the last day of the bioassay.

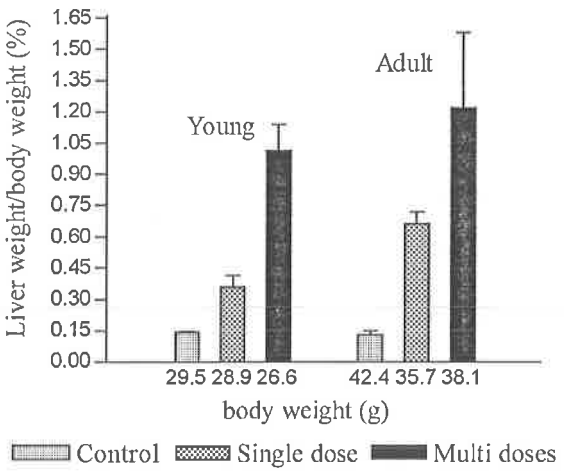

Figure 7. Proportion between spleen weight and body weight of young and adult animals (30 and 90 days, respectively old) inoculated with sublethal doses of *M. aeruginosa* and the control group. The numbers represent the average of the weight in the last day of the bioassay.

The histological cuts of the livers of the control animals and of those exposed to *M. aeruginosa* (Figs. 8 and 9) showed an outstanding difference in the structure of the hepatic parenchyma. In the intoxicated animals, a congestive and hemorrhagic aspect predominated and the light of the vases, including the sinusoid, was full of bloody globules. Another well evident aspect was a vesiculation in the cytoplasm of the hepatocyte. For this reason, the inoculated animals developed an accentuated hepatomegaly.

The Figs. 10 and 11 show a considerable increase in the number of giant multinuclear cells in the spleens of the animals inoculated with *M. aeruginosa*.

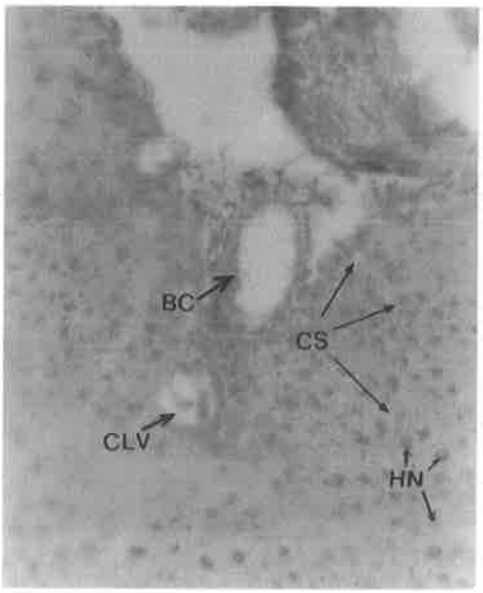

Figure 8. Microphotograph of the liver (H.E. 10X) of a control Swiss albino mice. The arrows show the biliary canaliculi (BC), the central lobular vein (CLV), the hepatocyte nucleus (HN) and the capillary sinusoid (CS).

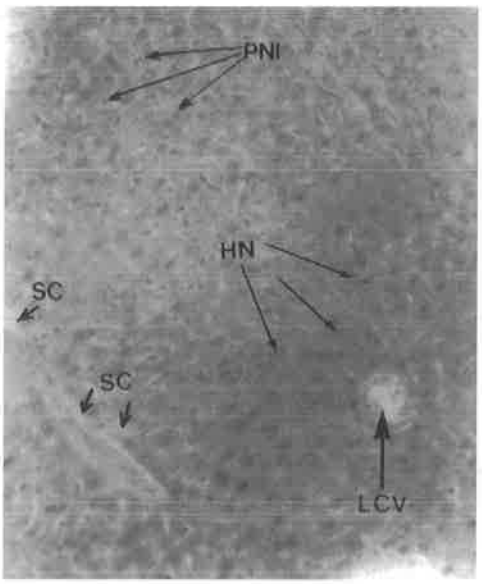

Figure 9. Microphotograph of the liver (H.E. 10X) of a Swiss albino mice inoculated with sublethal doses de *M. aeruginosa*. The arrows show capillary sinusoid (SC), lobular central vein (LCV), hepatocyte nucleus (HN) and polymorphus nuclear infiltrates (PNI).

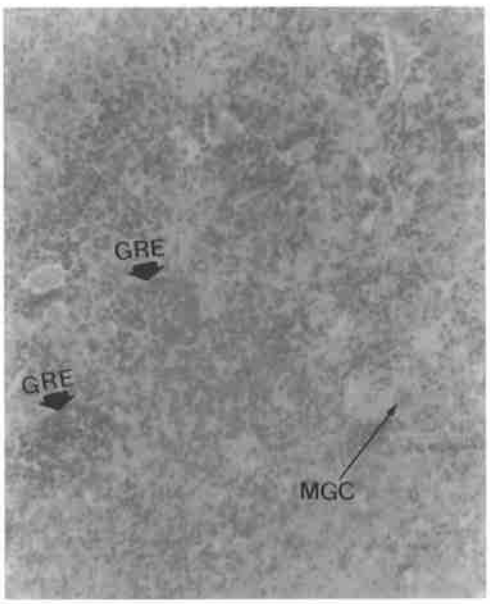

Figure 10. Microphotograph of the spllen (H.E. 10X) of a control Swiss albino mice. The arrows show groups of endothelial reticule (GRE) and some multinuclear giant cells (MGC).

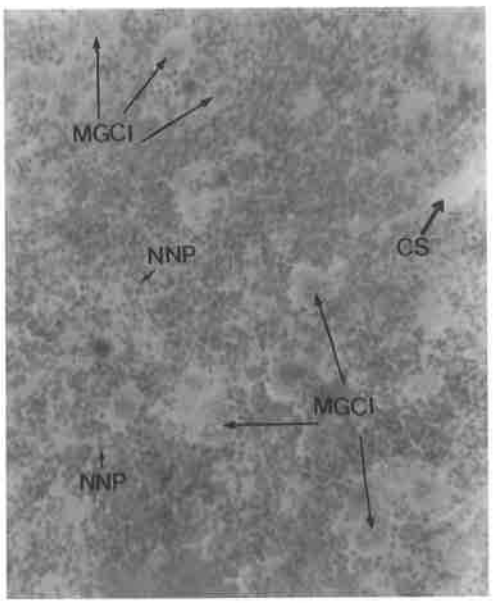

Figure 11. Microphotograph of a spleen (H.E. 10X) of a Swiss albino mice inoculated with sublethal doses of *M. aeruginosa*. The cut shows reactive alterations and the desegregation of the splenic parenchyme, with numerous nuclear polymorphus (NNP), congest sinusoid (CS) and multinuclear giant cell infiltrates (MGCI).

DISCUSSION

The microcystin-LR produced by the cyanobacterium is the best characterized toxin in the study of the hepatic toxicity in laboratory animals (4, 6). The hepatotoxins (mainly microcystins and nodularine) are, respectively, heptapeptides and pentapeptides that produce necrosis in the liver and are responsible for the majority of intoxication cases (4). The DL_{50} value found in this study (154.28mg.Kg^{-1}) indicates that the material collected in the CELMM presents a high toxicity compared to other samples of cyanobacterium species collected in other localities (19, 21, 26, 27). The symptoms observed in the animals after injection of the extracts were: diarrhea, convulsions, muscle weakness, tachycardia, fluffing, etc. These symptoms are compatible with the effects described for the microcystin-LR toxin (4).

The liver is one of the most important sites of lesions caused by toxic substances due to its intense participation in the reactions of biotransformation of chemical substances of the organism. Frequently, it is affected by infectious processes and intoxications, leading to hepatomegaly (6). It's known that splenomegaly occurs as a consequence of hepatic insufficiency, which causes blood congestion in the hepatic vessel due to decrease in activity and increase in the hydrostatic pressure in this vessel. This increase causes a blood reflow through the splenic vein toward the spleen, causing its swelling. This is also attributed to the intense production of lymphaticus and to the retention of particles in the blood.

The mice bioassays is showed an increase in the weight and volume of the liver and spleen in relation to the body weight of the intoxicated animals, mainly in animals with the cumulative dose of 154.28 mg.Kg^{-1} (Figs. 2, 4 and 6). The pathological examination of the liver (Figs. 8 and 9) showed an intrahepatic haemorrhage which increased the volume of blood in the organ and caused hepatomegaly, a fact also verified by Salomon (21). The pathological examination of the spleen (Figs. 10 and 11) showed an increase in the giant multinuclear cells, generating an accentuated splenomegaly.

Another important fact is that when the animal undergoes successive treatments, the liver increases its volume in an attempt to recover its homeostasy

causing the increase in volume and weight observed in the experiments. However, the exposure to the drug for a longer time provokes the fibrosis of the liver causing the decrease in the volume and weight of the organ. For this reason, the liver weight/body weight ratio was increased in the test animals, with 6.0% and 7.0% (adults) and 7.2% and 7.5% (young), respectively, inoculated with a single dose and multi doses, while the ration for the control animals was of 4% and 5%, respectively for adult and young animals. It is necessary to remember that young animals present larger livers (occupying a great part of the left hypochondrium) than adult animals and consequently they were more resistant to the *M. aeruginosa* toxins. Adult and young animals were, however, highly affected by the drugs, mainly the animals inoculated with multi-doses. The toxins act in the liver causing an imbalance in the maintenance of the cell cytoskeleton, by the inhibition of the phosphatase proteins that are responsible for the production of the microfilaments responsible for the form and the holding of the cell. The most aggravating and important episode caused by *M. aeruginosa* was, however, demonstrated in the spleen (Figs. 6 and 7), where the organ weight/body weight ratios were 1.22% and 0.67% (adults) and 1.05% and 0.37% (young) for animals inoculated, respectively, with multi-doses and single dose. The control groups presented ratios of 0.12% and 0.15%, respectively, for adult and young animals. The increase in the relation of the liver and spleen are justified by the decrease in the animal weight in the last day of the experiment (Fig. 3) and by the increase in the volume and the weight of the organs. In the studies with multi-doses, there was an increase of 16.6% and 72% (liver), 407% and 845% (spleen) and with a single dosage the increase was of 12% and 48.8% (liver) and 150% and 607% (spleen), respectively, in young and adult animals.

The vesiculation observed in the hepatocyte cytoplasm (Fig. 9) is also a characteristic of the microcystin effects on the cells of the liver, which have been observed as an extensive vesiculation of the granular endoplasmatic reticula (9). The *M. aeruginosa* bloom samples isolated in the estuary region of the Manguaba Lagoon presented a great hepatotoxic and splenotoxic risk for the animals that necessitate the estuary water as well as for the human population which uses such water for consumption. The chances of intoxication increases mainly when *M. aeruginosa* accumulates next to the margins by the action of the wind, where the contact and ingestion are much greater. The toxins in solution are hardly eliminated by the normal processes in the water treatment stations or by boiling (15). This is worrying because the experiments showed that sublethal single rate and multi-doses of the algae extract, when applied to swiss albino mice, were capable of causing hepatopathies due to the toxic action of the microcystin-LR or another type of toxin. This produces a panorama of hepatic-splenic insufficiency that leads to an increase in the volume and weight of the organs, turning impossible the biotransformation of chemical substances in the organism of the animal, causing its death.

ACKNOWLEDGEMENTS

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RESUMO

Hepatoesplenomegalia causada por um extrato de cianobactéria *Microcystis aeruginosa* coletada na Lagoa Manguaba, Alagoas -Brasil

Cianobactérias (*Microcystis aeruginosa*), produtoras de potentes ciclopeptídeos hepatotóxicos conhecidos como microcistinas, foram coletadas e submetidas à determinação de toxicidade em camundongos "Swiss Albino" com 30 e 90 dias de idade, através de injeção intraperitoneal. O fígado e baço foram submetidos à analise histopatológica e o desenvolvimento de peso e os sinais vitais foram monitorados. A DL_{50} no teste de toxicidade foi de 154,28 $mg.Kg^{-1}$. Da biomassa analisada, 95% das espécies eram de *Microcystis aeruginosa*. A relação entre peso do fígado e peso corpóreo nos animais testes inoculados com dose única foi de 6,0% e 7,2%, com multidose 7,0% e 7,5% e nos controles, foi de 4% e 5% para animais adultos e jovens, respectivamente. Houve um aumento acentuado no volume e peso do baço, em relação aos dos animais controle, onde os animais inoculados com dose única tiveram uma relação entre o peso do baço e peso corpóreo de 0,67% e 0,37%, com multidose de 1,22% e 1,05% e nos controles foi de 0,12% e 0,15% para

animais adultos e jovens, respectivamente. Os animais jovens inoculados com dose única e multidose tiveram aumento do peso e do volume do baço em 150% e 407%, enquanto os adultos em 607% e 845%, respectivamente, em relação ao controle. A análise histopatológica mostrou uma diferença marcante na estrutura do parênquima hepático, entre animais controles e expostos aos extratos de *M. aeruginosa*. As principais alterações observadas foram o aspecto congestivo, inclusive dos sinusóides, e hemorragia intra-hepática. Enquanto que, a análise histopatológica do baço mostrou um aumento considerado do número de células gigantes multinucleares nos baços dos animais intoxicados com a *M. aeruginosa*.

Palavras-chave: *Microcystis aeruginosa*, Cianobactéria, hepatomegalia, esplenomegalia

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Manuscripts are accepted for publication only after they are critically reviewed. Papers are reviewed by referees indicated by the Section Editors. After review, the manuscript will be returned to the nominated author for revision according to suggestions made by the reviewers. The author should return the reviewed manuscript to the Editors.
The author is notified when a manuscript is received and also when it is accepted or rejected for publication.

On acceptance of the paper, the nominated author will be requested to send the text on a computer diskette. Galley proofs will be sent to the author for correction. They should be checked carefully and handled promptly (5 days) according to instructions which are attached.
Membership in Brazilian Society for Microbiology is not a prerequisite for acceptance of a manuscript for publication. Nonmembers scientists from Brazil and other countries are invited to submit papers for consideration for publication.
Submission of a manuscript implies that all authors and their institutions have agreed to its publication.

Revista de Microbiologia assumes no responsability for errors made by the authors. Furthermore, Revista de Microbiologia assumes no responsability for conclusions reached by the authors.

Types of papers

The following types of papers are acceptable for publication in Revista de Microbiologia (Journal of the Brazilian Society for Microbiology):

Research paper: the research paper reports results of original research which have not been published elsewhere. It consists of 12 to 15 double-space typewritten or computer-written pages plus appropriate references, Tables and Figures. An abstract with title (Resumo) and three to five key-words (palavras-chave) in Portuguese must also be included.

Short Communication: a Short Communication is a concise account of new and significant findings. It should be written according to the guidelines given for research papers (see below) but without the heading divisions. It's abstract and resumo (in Portuguese) should not exceed 50 words. Figures and Tables should be restricted to a maximum of two Figures or two Tables, or one Table and one Figure. The designation "short communication" will appear above the title of this type of paper. The author should specify that his manuscript is a short communication so that it can be properly evaluated during the review process.

Mini-review: Review articles should deal with microbiological subjects of broad interest. Specialists will be called upon to write them. In addition to an abstract in English and in Portuguese (resumo), they may contain a list of contents.

Preparation of Manuscripts

General

1 - All manuscripts should be typed double-spaced with wide margins and the pages should be numbered sequentially. Research papers should be restricted to 15 printed pages, including Figures and Tables. Short Communications should be restricted to 6 printed pages.
2 - All manuscripts should be submitted written in English. The Editor recommends that a manuscript should be read critically by someone fluent in English before it is submitted. Manuscripts in poor English will not be accepted.
3 - The paper should be organized in topics, as described in the next paragraph. The name of the topics should be typed in capital letters (e.g. ABSTRACT, INTRODUCTION, etc.).
4 - Abbreviations of therms and symbols should follow the recommendations of the IUPAC-IUB Commission and the Metric System is to be used throughout.
5 - As a rule, the references in the text should be cited by their numbers. Exceptionally, when authors are mentioned in the text, the mention should be done according to the following examples: Bergdoll (number) reported that..., Bailey and Cox (number) observed that..., or Smith *et al.* (number) mentioned that... Do not use capital letters.
6 - Authors of accepted papers will be requested to send a 3 1/2" diskette containing the text prepared in a P.C. based word processor.

Organization

TITLE PAGE: A separate page should be used to give the title of the paper, complete name (including first name and middle initial) and affiliation of each author. An asterisk should be placed after the name of the author to whom correspondence about the paper should be sent. The telephone and fax numbers of this author should be given on the bottom of the page. No text of the manuscript should appear on the title page.
The title should be as brief as possible, contain no abbreviations and be truly indicative of the subject of the paper. Expressions such as "Effects of", "Influence of", "Studies on", etc., should be avoided. Care should be exercised in preparing the title since it is used in literature retrieval systems.

ABSTRACT: The abstract should be typed in a separate page and should not exceed 250 words. It should summarize the basic contents of the paper. The abstract should be meaningful without having to read the remainder of the paper. An abstract should not contain references, tables or unusual abbreviations. Abstracts are reprinted by abstracting journals and hence will be read by persons who do not have access to the entire paper. Hence the abstract must be prepared with great care. Three to five key words should also be included.

RESUMO: *Resumo* is the abstract written in Portuguese. Its preparation should follow the same recommendations for the abstract in English. The *resumo* should also contain a title in Portuguese. The rules for the title in

Portuguese are the same for the title in English (see above). Three to five *palavras-chave* (key words) have also to be included. The *resumo* and the title in Portuguese should also be typed in a separate page.

INTRODUCTION: The introduction should begin on a new page and provide the reader with sufficient information so that results reported in the paper can be properly evaluated without refering to the literature. However, the introduction should not be an extensive review of the literature. The introduction should also give the rationale for and objectives of the study that is being reported.

MATERIALS AND METHODS: This section should provide enough information for other investigators to repeat the work. Repetition of details of procedures which have already been published elsewhere should be avoided. If a published method is modified, such modification(s) must be described in the paper. Sources of reagents, culture media and equipment (company, city, state, country) should be mentioned in the text. Names that are registered trade marks should be so indicated. Subheading often make this section easier to read and understand.

RESULTS: This section should, by means of text, tables and/or figures, give the results of the experiments. If a *Discussion* section is to be included, avoid extensive interpretation of results but do so in the *Discussion* section. If *Results* and *Discussion* are combined. then results should be discussed where, in the text, it is most appropriate. Tables should be numbered independently of the figures using Arabic numerals. All tables and figures must be mentioned in the text. The approximate location of tables and figures in the text should be indicated.

DISCUSSION: The discussion should provide an interpretation of the results in relation to known information.

ACKNOWLEDGMENTS: This section is optional and follows the *Discussion*. It aknowledges financial and personal assistance.

REFERENCES: Arrange the references in alphabetical order, by last name of the author. All authors must be cited. Number the references consecutively. Cite each reference in the text by its number. Journal names should be abbreviated according to the style of *Biological Abstracts* or *Chemical Abstracts*. All references given in the list should be cited in the text and all references mentioned in the text must be included in the list. List references according to the style shown in the following examples.

a. Paper in a journal
Campos, L.C.; Whittam, T.S.; Gomes, T. A.T.; Andrade, J.R.C.; Trabulsi, L.R. *Escherichia coli* serogroup O111 includes several clones of diarrheagenic strains with different virulence properties. *Infect. Immun.*, 62: 3282-3288, 1994.

b. Paper or chapter in a book
Nelson, E.B. Current limits to biological control of fungal phytopathogens. *In*: Arora, D.K.; Rai, B.; Mukerji, K.G.; Knudsen, G. (eds). *Handbook of applied mycology; soils and plants*. Marcel Dekker, New York, 1991, p.327-355.

c. Book by author(s)
Salyers, A.A.; Whitt, D.D. *Bacterial pathogenesis. A molecular approach*. ASM, Washington, 1994, 418p.

d . Patent
Hussong, R.V.; Marth, E.H.; Vakaleris, D.G. Manufacture of cottage cheese. *U.S. Pat. 3,117,870*. Jan. 14, 1964.

e. Thesis
Calzada, C.T. Campylobacter jejuni *e* Campylobacter coli - *caracterização em sorogrupos e biotipos das cepas isoladas no município de São Paulo, no período de 1983-1989.* São Paulo, 1991, 131p. (Ph.D. Thesis. Instituto de Ciências Biomédicas. USP).

f. Publication with no identifiable author or editor
Anonymous. The economy of by-products. *Álcool Alcoolquim.*, 2;33-40, 1985.

g. Communications in events (Symposia, conferences, etc)
Simão, G.S.; Silva, J.; Toledo, A.S.; Gontijo Filho, P.P. *Micobactérias não tuberculosas isoladas de pacientes com a síndrome de imunodeficiência adquirida.* XVII Congresso Brasileiro de Microbiologia, Santos, 1993, p.41.

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

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

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

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

Reprints

Fifteen reprints of each paper will be mailed to the nominated author, free of charge Additional reprints will be billed at cost price if requested upon returning the corrected galley proofs.

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