ISSN 0001-3714

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Volume 29 Número 4 Out. - Dez. 1998

FICHA CATALOGRÁFICA

Preparada pela Biblioteca do
Instituto de Ciências Biomédicas da Universidade de São Paulo

Revista de Microbiologia/Sociedade Brasileira de Microbiologia.
Journal of the Brazilian Society for Microbiology
— Vol. 29, nº 4 (out/dez 1998)
— São Paulo: SBM, [1970] -
v.:il; 27 cm

Trimestral
1970 - 1998, **4-29**
ISSN 0001-3714

1. Microbiologia I. Sociedade Brasileira de Microbiologia

NLM-QW4

Revista de Microbiologia

Journal of the Brazilian Society for Microbiology

Publication of the Brazilian Society for Microbiology - São Paulo - Brazil

Papers published in Revista de Microbiologia (Journal of the Brazilian Society for Microbiology) are indexed in:

Current Contents (USA); CNRS - Centre de la Recherche Scientifique (France); Chemical Abstracts Service (USA); Cambridge Scientific Abstract (USA); Commonwealth Mycological Institute (England); Hamdard National Foundation (Pakistan); IMLA - Index Medicus Latino Americano (Brasil); Institut Nauchtoi Informatsii (ex-URSS); Periodica (Mexico); Sumários Correntes Brasileiros (Brasil); UMI - University Microfilms International (USA).

Financial support: FINEP, FAPESP and CNPq.

Printed by WINNER GRAPH (phone: (011) 5584.5753)

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05508-900 - São Paulo - SP
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REVISTA DE MICROBIOLOGIA

Volume 29 number 4 October - December 1998

Revista de Microbiologia (1998) 29:237-245
ISSN 0001-3714

MOLECULAR EVOLUTION IN BACTERIA: CELL DIVISION

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Submitted: November 03, 1998; Approved: January 18, 1999.

REVIEW

ABSTRACT

Molecular evolution in bacteria is examined with an emphasis on the self-assembly of cells capable of primitive division and growth during early molecular evolution. Also, the possibility that some type of encapsulation structure preceeded biochemical pathways and the assembly of genetic material is examined. These aspects will be considered from an evolutionary perspective.

Key words: cell division, molecular evolution, bacteria, self-assembly

INTRODUCTION

Once the earliest microorganisms appeared on the Earth, the planet was changed in an irreversible manner by biological cycles, the presence of molecular oxygen from photolytic cleavage of water (27) and biological diversification of species. Since about 80% of the evolutionary history of life on Earth is restricted to microorganisms, an understanding of their molecular evolution is central to understanding the origin(s) of life and molecular evolution.

It is generally agreed that early life on Earth was capable of replicating and evolving by means of some type of primitive genetic material (4). This does not solve the mystery of the origin of the first genetic material nor how it became enclosed inside a primitive evolving, functional membrane that eventually became capable of septum formation and cell division. Moreover, evolution experimented with genetic codes and the result is diverse cellular designs, genetic coding and mechanisms of energy production and conversion that are efficient for life on Earth (18).

The physical-chemical environment in which self-assembly of primitive evolving prokaryotic cell(s) occurred was small, likely in the order of nms to μms. This makes the physical-chemical environment difficult to conceptualize when dealing with molecular evolution of bacteria. This also means parameters (e.g., temperature, pH, redox, pressure) used to describe microbial environments have no meaning on the scale of individual microbial cells (27). Evolving microbial cells would possess a large surface area to unit volume ratio needed by microorganisms that rely on nutrients of molecular dimensions (27).

The best survival mechanism in bacteria is spore formation followed by vegetative growth when the environmental conditions become favourable. If one had to suggest an early life form capable of surviving in a harsh environment, it would be bacterial spores. Indeed, the arrival of life on Earth of spores travelling

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through space has been suggested (7). Spores would be protected from ultraviolet light and could possibly survive for 4.5 to 45 million years in outer space. This would provide ample time to travel to Earth (26). The origin of the spores and their entry into outer space could have been due to a collision between a planet on which life existed and a meteorite (7). Another possibility is the arrival of complex organic molecules/structures on Earth from outer space. For example, complex buckyballs impacting on the Earth before being destroyed (4). This raises the possibility that other diverse, complex organic molecules also made the journey to the Earth's surface. However, this does not elucidate how the situation on the Earth changed from a prebiotic condition to some type of genetic material such as RNA or PNA (peptide nucleic acid) and if there were numerous or only a few intermediate steps between these transitions.

There is also no absolute consensus on the types of energy sources available during early molecular evolution. Certainly in present day bacteria, the ability to obtain and store energy is central to metabolism, growth (ultimate expression of the physiology of an organism and defined as any increase in the amount of actively metabolic cytoplasm accompanied by an increase in cell numbers, cell size or both) and cell division.

Another view on the emergence of life is that it occurred about 4.2 billion years ago from iron monosulphide bubbles at a submarine hydrothermal vent (19). This type of system acted as a type of hatchery for the first organic cells. This view suggests how geochemical disequilibrium led to the first metabolizing system. It is suggested that a FeS membrane also containing nickel acted as a semipermeable catalytic boundary between the hydrothermal vent liquid and the Hadean ocean. Eventually this membrane was taken over by an organic membrane with also had a transmembrane potential. The origin of enzymes and genetic material is still unknown in this system.

Construction Kit (Elements of the Periodic Table)

During the geochemical evolution of the Earth's chemical construction kit (e.g. periodic table of elements) the Earth would be a reducing environment. Primitive bacteria may have been autotrophs, not heterotrophs, and therefore an energy source such as pyrite derived from hydrogen sulfide and ferrous ions could be a possibility (11, 22).

Huber and Wächtershäuser (11) recently described reactions that could have been the primordial initiation reaction for the chemoautotrophic origin of life. An aqueous slurry of NiS and FeS converted CO and CH_3SH (methane-thiol) into thioacetic acid, which then hydrolyzed to acetic acid. Also, when NiS- FeS was modified with catalytic amounts of selenium; acetic acid and CH_3SH were formed from CO and H_2S. Once you have a supply of acetic acid, you may be on the path to the acetyl-CoA pathway, which is considered an ancient pathway of carbon fixation.

The results of this exciting research, support the view of a hyperthermophilic, chemoautotrophic origin of life in an iron-sulfur environment (11). The earliest organisms may have feed on carbon monoxide and/or carbon dioxide at some type of hydrothermal site.

Mineral Surfaces and Assembly of Genetic Material

Mineral surfaces would be abundant and it is not unreasonable to consider that minerals (2) and microscale molecular environments were central in early molecular evolution. A pre-RNA world may have been an unknown genetic polymer or sheet (9). It is not unreasonable to consider this genetic polymer was assembled on surfaces such as those of minerals, which would be abundant (2). Lahav and Nir (14) also recently suggested a bio-geochemical model for the emergence of template and sequence directed (TSD) synthesis. Since surfaces are virtually ubiquitous on the Earth, they may have been of central importance in early molecular evolution. However, a paucity of information and hypotheses exists on the exact role(s), if any, of surfaces on molecular evolution in the pre-DNA world.

A stable template such as a mineral surface and a means to copy the genetic material would be required. In the absence of enzyme catalysts, physical-chemical catalysts like temperature cycling, metal ions and cathode-anode gradients on surfaces (24) are interesting to speculate about. The appropriate genetic monomer units would still be required as well as the capability to make an exact or near exact copies. It is a possibility that temperature cycling may have been the physical catalyst that drove the reaction(s) in a mechanism that involved denaturation and reannealling of the growing genetic material (23). This process would act as the mechanism to glue complementary oligonucleotides or genetic material together into a stable genetic material with a high degree of constancy yet the ability to mutate or change.

The adsorption of nucleic acids to montmorillonite

clay is known to be pH dependent (10) and also dependent on the length of the DNA (17). Below pH 5.0 (approximate isoelectric point for nucleic acids) adsorption of calf thymus DNA and yeast ribonucleic acid to montmorillonite is greater on internal clay surfaces. Above pH 5.0, DNA adsorption is less and restricted to external clay surfaces (10). Calcium and magnesium can cause a 2-fold increase in adsorption of nucleic acids to clays (10). The presence of KCl can cause an increase in nucleic acid adsorption and expansion of clay lattices (10). Magnesium and calcium would likely be present during early molecular evolution, and may have assisted in the binding of genetic material to mineral surfaces.

In the absence of enzyme catalysis, elongation of genetic material such as RNA may have been catalyzed by bringing monomers close to each other on a mineral surface. Temperature fluctuations may have been common. For example, geothermal hot springs, volcanic activity, deep marine thermal vents, and mineral surfaces exposed to intense sunlight during the day with cooling at night. Lower temperatures would promote adsorption and elongation/assembly of monomers while elevated temperatures would release polymers from the mineral surfaces into a water film, followed by readsorption of the growing polymeric material to clay or mineral surfaces as the temperature decreased. Repeated cycles of heating and cooling may have assisted in elongation and pairing of complementary polymers. Lahav and Nir (14) have also discussed the role of environmental cooling and heating cycles in the early template directed synthesis of emerging life. Eventually the fluctuating environment would not be the driving force and template directed synthesis involving enzymes, genetic material and the chemical construction kit contained in a semi-permeable membrane would emerge.

Joyce and Orgel (12) have summarized possible monomers for assembly of genetic material such as; hydroxy acids, amino acids, phosphomonoesters of polyhydric alcohols, aminoaldehydes and molecules containing two sulfhydryl groups. If ions such as Mg^{2+} and Ca^{2+} were involved, it is probable that side groups necessary to bind these elements were likely phosphate or carboxylate groups (12). Suitable compounds include aspartic acid, glutamic acid, serine phosphate, alpha-hydroxysuccinic acid and citric acid (12).

As the primitive genetic material became encapsulated (cells need an inside and outside) within a membrane, the mineral scaffold or surface could be eliminated (2). If RNA evolution preceded the formation and evolution of enzymes, genetic material may have been maintained by replication of RNA or possibly PNA (peptide nucleic acid) which was a potential prebiotic DNA precursor described by Nielson *et al.* (16). The PNA has a more stable backbone than DNA and can bind to itself more strongly than complementary strands of DNA (4).

Whatever the early genetic material was assembled from, it would eventually require certain characteristics. Evolving genetic material would need to be able to divide between 2 evolving cells. It would need to self-replicate, remain conserved, yet allow for some sequence diversity and have the ability to change.

Transposons and Molecular Evolution

Mobile genetic elements such as transposons in bacteria means that genome organization and evolution is a fluid dynamic process as opposed to a constant genome. Bacterial genomes are capable of relative constancy and change. Genome reorganization as a result of transposition must have been important in molecular evolution. The mobile genetic elements in addition to gene transfer mechanisms (transduction, transformation and conjugation) provides a diverse biochemical tool kit for restructuring bacterial genomes. It is however, not known if these activities were present in early molecular evolution or arrived on the scene much latter. A small mobile length of DNA such as a transposon may have been a suitable structure to assist in the assembly of evolving DNA. Transposition involves the breakage and strand transfer (mediated by the same transposase enzyme) and subsequent formation of phosphodiester bonds. As the evolving bacterial genomes became more complex and approached their optimal or present day sizes, transposition would not be as necessary. Many transposons could have been lost while some bacterial retained selected transposons which we know of today.

From an evolutionary perspective, both mobile DNA and self-catalytic RNA have unique feature that would be valuable in the self-assembly, evolution and diversification of early bacteria.

Replication and Synthesis and Early Genetic Material

It is noteworthy that replication of DNA refers to the extension of the strands of DNA. DNA synthesis refers to the actual increase in the amount of DNA

regardless, of its arrangement in the chromosome. Both replication and synthesis of genetic material would also be central to the molecular evolution of early genetic material. However, both processes are dependent on the presence of enzymes and regulated biochemical pathways. Synthesis and replication of genetic material in the absence of enzymes would still require the necessary building blocks for assembly of the genetic material. A template to provide order would also be necessary as well as a means to enclose the genetic material and provide an inside and an outside to the evolving cell structure. If enzymes were present and catalytic, self-assembly of a cell would certainly be easier. The origin of a pool or library of peptides on mineral surfaces capable of catalyzing peptide bond formation is feasible (14). The selfassembly of a catalytic enzyme is likely no more difficult than self-replicating genetic material. The problem is that the self-assembly of a catalytic enzyme such as a genetic material-polymerase on a mineral surface may assist with elongation of the genetic material but it does not explain how the enzyme self-assembled multiple copies of itself. Alternatively, a primitive enzyme (or possibly one or more multifunctional enzymes with broad substrate specificity and slow catalysis capabilities) assisted with assembly of genetic material and the cell structure. A mineral surface may have acted as a support on which layers of diverse elements and molecules were brought together at the same physical micro-environment and time to allow life to commence. This type of surface assembly would increase in chemical complexity over time until some degree of order emerged due to catalytic and replicative capabilities. Increasing molecular complexity may have been very much at work in early molecular evolution. However, out of complexity, integrated metabolic pathways must emerge while obeying the laws of thermodynamics. In this case, macromolecules taking on conformations that require the least amount of free energy.

There is no explanation as to how the integration of DNA, RNA and proteins inside a cellular structure came into existence on the Earth. The construction kit (selected elements of periodic table) was likely available at specific locations or was transported by wind and water to locations. However, from the construction kit, the compounds necessary for assembly of amino acids, nucleotide bases, ribose and deoxyribose, phosphate and phospholipids for membranes had to self-assemble in a stable manner that permitted cell growth and division. Assembly is one mystery of evolution and bacterial cell division is another.

Energy and Early Evolution

If you remove organisms and oxygen from the Earth's early atmosphere by travelling back in time about 4.9 billion years (7) you are left with mineral surfaces, water vapour and later liquid water, carbon dioxide, most likely nitrogen in the atmosphere, methane, hydrogen sulfide, sulfur dioxide and insoluble iron sulfides. Under these conditions today, bacteria such as *Clostridium aceticum*, capable of producing acetate from H_2 and CO_2 would likely be present. Also, the sulfur metabolizing archaebacter, *Pyrodictium* and *Thermoproteus* are known to acquire energy by the formation of hydrogen sulfide from hydrogen and sulfur, which has been suggested to be a primeval energy source reaction (25).

The origin and subsequent evolution of the first sustainable energy-producing metabolic cycle(s) is central to an understanding of early molecular evolution. It is not unreasonable to suggest that energy producing and storing metabolic reactions may have preceded the assembly of the first genetic material. Integrated metabolic reactions are much easier to understand when enzyme catalysts are present. Enzymes regulate the pace of reactions and also regulate the sequence or order in which the reactions proceed. Otherwise the metabolic reactions necessary for life would proceed to equilibrium (none of the cells properties would change over time) and organisms would not be able to self-assemble. If the organisms were already alive they would spontaneously degrade and die. Equilibrium in living organisms can only be achieved by death. Hence, enzymes and energy would be a central requirement for early bacterial cells to grow and divide thus completing a bacterial cell cycle (period from one cell division to next cell division) (5). The first metabolic reactions would not occur with the assistance of enzymes. However, once enzymes are present, integrated biochemical pathways, replication of the genome (complete set of genetic information in a cell) and cell division all become much easier to explain. Also, some metabolic intermediates are chemically unstable and therefore it is difficult to explain their accumulation in early evolution.

Cell Division

A single bacterial cell is a complex living entity.

It is difficult to hypothesize how primitive bacterial cells underwent cell division and divided cell components into two identical or virtually identical offspring cells, in most likely a chaotic environment. A functional genetic system requires protocells or cells capable of multiplying by division (6). The containment and subsequent cellularization of life allowed the cell to emerge as the basic unit of life. Moreover, the first cell had to self-assemble in an environment that supplied it needs and had a means to divide and partition genes into the offspring cells (13).

Cells need and inside and outside to divide. The fusion of peptide microspheres and simple lipids on a surface may have produced a simple spherical structure surrounding primitive genetic material. The transition from microspheres (8) (protein spheres formed by polymerization of amino acids in hot water) to a cell capable of regulated cell division requires a number of integrated molecular steps.

It is interesting to consider if a primitive microscale cathode-anode gradient separated by a primitive lipid, phospholipid or microsphere structure (Fox (8), protein spheres formed by polymerization of amino acids in hot water) provided a barrier with an electrical gradient that was a precursor of more complex membranes and eventually cell walls.

It is noteworthy that the formation of protein microspheres requires elevated temperatures (as low as 65°C). Elevated temperatures or temperature cycling between light/dark periods would easily fulfil this temperature requirement. An anode reaction under anaerobic conditions may have been created by adsorption of hydrogen sulfide to the mineral surface while hydrogen evolution from iron sulfide surfaces could have created an anode. In addition, the cathode-anode system could have been separated by a lipid, primitive membrane barrier or microsphere on a mineral surface, that was a precursor of a more advanced membrane with a charge differential on either side of the membrane formed by the cathode-anode system (24).

Phospholipid bilayers membranes found in present day bacteria are fluid and flexible unless they undergo a transition to a gel phase (21). Encapsulation of genetic material and biochemical pathways would have had significant advantages in prokaryotic evolution. The cell as the basic unit of life could now survive by exchanges of nutrients and gases with its external environment. Encapsulation also provided the means for containing evolving protoplasm and genetic material. A self-assembling system contained by a fluid membrane would eventually be capable of division once a trigger for cell division evolved.

Phospholipid bilayers can also take on different configurations. This is a necessary feature for cells of different sizes and shapes. Moreover, phospholipid bilayers are continuous in their structure yet have the capability to self seal. They can therefore undergo fission (division) and fusion (joining together) (6) without losing their internal contents to the external environment. Phospholipid bilayers are also easy to form. Mechanical agitation can turn phospholipids into a suspension of vesicular bilayers (6). This type of self-assembling structure could then enclose the evolving biochemical pathways and genetic material. Perhaps, collisions between vesicles or microspheres brought different components into a fused structure. Or perhaps, multitudes of structures gently agitated over surfaces participated in exchanges of molecules and elements at the same physical location. However, this does not explain the origin of the phospholipids and how the necessary elements and molecules would pass into the structure to permit continued evolution. Alternatively, the first membranes could have been more rigid of a different composition, with phospholipids adding to this unknown structure to provide a more fluid, versatile membrane capable of multiple divisions. It is also important to note that membranes grow by accretion- the addition of molecules to an already existing membrane. This is a significant feature in evolution as it means that an ancestral membrane had to only self-assemble once (6). The first membrane could then enlarge and divide by fission. Division of an evolving structure may have been initially uncontrollable. For example, the structure exceeded a threshold volume and the force that holds the vesicle together was exceeded and the vesicle explodes. However, if the structure can divide before it explodes, the internal contents are preserved. The evolving cell would only need to be large enough to maintain a stable structure capable of division.

Eventually, the membrane would produce a biological potential based on a property of the membrane known as capacitance (18). Membrane capacitance is the ability to keep charges separated when there is a certain voltage (mV) transversing it. For example, a transmembrane potential can be approximately 60 mV (20). It would be difficult to explain that electrical potentials appeared in evolving cells before concentration gradients. Differences in concentrations also separate the internal cellular environment from the external surrounding

environment (18). The cell can then maintain a suitable internal environment by integrated metabolism. The cell is an open system, allowing both matter and energy to pass through its boundary, the cell membrane (18). To be more accurate, the cell membrane is differentially permeable. Some substances can not leave or enter the cell.

Since membranes grow by accretion, another possibility can be considered. Suppose that self-assembling primitive membranes, vesicles, or microspheres (primitive encapsulation structure) were present before genetic material such as PNA, catalytic RNA, enzymes and any primitive evolving biochemical cycles. The membranes, vesicles or microspheres acted as the support or surface on which genetic material and the remainder of the cell self-assembled. It is not unreasonable to speculate that the primitive encapsulation structure was one of the first components of the evolving cell(s) to self-assemble as no catalysis is required for formation of the structure. Once this structure was present, genetic material and integrated biochemical cycles may have self-assembled on the inner surface and in the water-filled matrix of the structure in a somewhat protected environment. The evolving, self-assembling structure may have been more protected by the presence of clays attached to the structure or put another way, the evolving structure was attached to clay or mineral surfaces. The early presence of a contained structure could have accelerated the remaining evolution of self-assembly. The evolving pre-cell could then assemble a more complex cytoplasmic membrane capable of being energized and at the same time assembling cytoplasmic components. Genetic material could have assembled on the inner surface of the membrane, vesicle or microsphere structure. Transport of nutrients into the evolving cell(s) could have occurred via simple diffusion and gradually by the evolution and addition of facilitated diffusion and active transport. Eventually, peripheral, integral and transmembrane proteins would be added to the evolving structure. An osmotically protected evolving structure (primitive forerunner of protoplast) may have been possible. It is recognized that diffusion may not have provided all the compounds necessary for the internal assembly of the first pathways and the genetic monomers required for the assembly of genetic polymers. However, the first membranes may have had larger pores and did not exhibit a high degree of selective permeability.

DNA replication in bacterial begins at the origin of replication (*ori* C) which is attached to the inner surface of the cytoplasmic membrane. This relationship between DNA and the cytoplasmic membrane may have had a central role in early molecular evolution, if genetic material was ever assembled on an internal surface of a membrane. A protected internal environment of a fluid, continuous membrane is a possible surface for assembly of genetic material. The fluid nature of the structure would also permit elongating genetic material to coil. Coiling on a rigid structure would be more difficult.

Even today, some nitrifying bacteria contain internal membranes. Eukaryotic cells also require a membrane factory, the Golgi apparatus. Membranes are a central structure for cellular life forms. The earlier primitive membranes, vesicles or microspheres appear in early molecular evolution, the easier it would be for a cell to self-assemble. It is now the task to determine if some type of membrane structure with an inside and outside preceded the self-assembly of genetic material and the first biochemical pathways.

The actual mechanism of ancestral encapsulation may never be elucidated. Whatever the mechanism, the ability to proceed from an encapsulated structure to growth and division occurred. It is also possible the first cell divisions were more akin to budding (offspring cell is formed by pinching off a portion of the parent cell; a cross wall separates the bud from parent cell) and equal offspring cells from cell division evolved from a simpler budding mechanism. Actual cell division and a suitable partioning of cell contents came along latter, as a more complex regulated, process. Budding may have produced aggregates of primitive cells capable of numerous interactions at the same environmental location.

Consider the following question; what were the minimal factors/conditions involved in the self-assembly of the first primitive cell(s) that allowed it to divide by binary fission? This is not a trivial question, considering the origin of life may have occurred numerous times (15) and failed to evolve until both the molecular and environmental conditions were suitable. Bacterial cell division is central to our present understanding of microbiology as well as the origin of life forms capable of cell division. Why a bacterial cell divides is still somewhat of a mystery as complete answers are lacking (3). It has been suggested bacterial cells divide because beyond a threshold cell size, cellular life is not possible, as the rate of metabolism is insufficient for sustained life (3). The cell avoids this condition by dividing which

restores a suitable cell size and surface:volume ratio necessary for normal cell processes (3). Also, when the cell size increases the surface:volume ratio decreases (3).

Another suggestion as to why cells divide is the need for genetic control. This idea states that cells require a specified amount of genome to control a specific amount of protoplasm (3). When a cell reaches a threshold size with increased amounts of protoplasm, the genome is replicated and the cell divides to restore the genome to protoplasm ratio and control is restored over the cells.

A third suggestion is that cell division occurs when sufficient biosynthetic activities have occurred to permit a new cell to exist as an individual entity (3). It is not known if the first primitive cell(s) capable of division functioned in the same manner as present day bacterial cells. One task is therefore to determine the minimal requirements for cell growth and division by the first microorganisms.

The dividing bacterial cells must also obey the rules of cell division (5). 1. Cells shall not divide unless two genomes are present. The genome has to replicate at least once. 2. Cells shall not commence DNA synthesis unless the cell has sufficient cytoplasm. The function of cell cytoplasm with respect to cell growth is to make more cytoplasm (5). This means the evolving bacterial cells had to extract energy from their environment and divide. Table 1 contains a list of suggested factors/conditions required for minimal self-assembly of a primitive cell and sufficient regulated metabolism for division.

Consider the following hypothetical situation (see Fig 1).

1. An elevated temperature environment with 25-30 elements of the periodic table present, that are required for life as well as some simple compounds.
2. Primitive membrane, vesicle or microsphere surface(s) acting a physical, focal point for self-assembly of gentic material and biochemical pathways.
3. Early biochemical cycles for carbon-fixation and energy production and storage as the beginning of integrated metabolism.

It is not unreasonable to consider that some form of evolving biochemical pathways were needed before DNA, RNA and enzymes could be self-assembled. Perhaps these reactions occurred on the surface of a

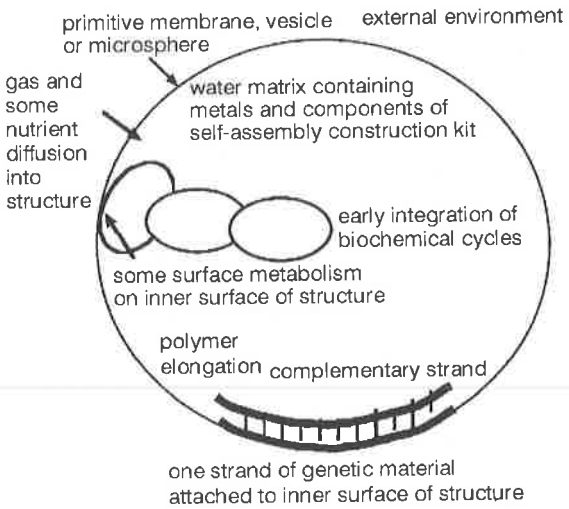

Figure 1. Possible evolution of biochemical pathways and genetic material in a primitive membrane, vesicle or microsphere.

Table 1. Possible requirements of evolving primitive cell(s) necessary for cell division.

1. Replicated genetic material or primitive genome (2 genomes)
2. Compartmentation- an inside and an outside. Microsphere, vesicle or membrane, capable of budding or division without losing the internal cell components. A protected environment such as on or between clay plates is a possibility for such an environment.
3. Sufficient cytoplasm containing evolving, integrated metabolic pathways, necessary chemical construction kit for offspring cells.
4. Increase in cytoplasm, synthesis of genetic material, replication and possible septum formation. Cell septum formation is complex and a simpler mechanism would have been used initially used by evolving cells.
5. Energy source(s) and energy storage capabilities. It is not known if energy was required for the initial cell divisions.

Note: Capsule, flagella, pili, outer membrane such as found in Gram-negative bacteria would not be necessary structures.

membrane, vesicle or microsphere gently bathed in a liquid, geothermal medium such as at the interface between aquatic and solid surface environments. The ability of surface metabolism to assist in the evolution of enzymes and DNA may have been the easier mechanism of cellular self-assembly from the chemical construction kit.

4. The appearance of DNA as mobile genetic material such as transposons or covalently, closed circular (CCC) DNA in the form of plasmids, that are relatively stable at an elevated temperature or under alkaline pH conditions compared to chromosomal DNA. If the early environmental conditions for evolution were harsh, plasmid DNA would be a better candidate in terms of stability. Integration of several plasmids could have lead to a small chromosome. Transposition could have brought genes together in the form of an operon or alternatively, dispersed genes.
5. Further cell membrane assembly and the ability of the cell to undergo cell division and produce 2 offspring cells capable of growth and division. Eventually cell division would lead to aggregates of cells, colonies or biofilms of bacterial cells on surfaces. Some would be dislodged and carried to new locations by flowing water or in the atmosphere as bioaerosols. Many present day bacterial species can be found as members of biofilms. In such an environment, protection from toxic compounds is afforded and transfer of genetic material can be frequent.

Archaebacteria

Archaebacteria (evolutionary distinct prokaryotes that include methanogenic, extremely halophilic and sulfur dependent bacteria, (1)) have adapted to high salt, elevated temperatures and anaerobic conditions. These are the chemical-physical conditions postulated to have been present on the early Earth. If Archaebacteria were some of the first primitive bacteria, it is important to note they have membranes that lack fatty acids, and instead contain hydrocarbon compounds bonded to glycerol by ether linkages (1). These membranes contain polar inner and outer surfaces (glycerol) and a nonpolar interior (1) that tend to spontaneously form lipid bilayers. This feature may have been necessary for primitive evolving microspheres or prokaryotic cells assembling on a mineral surface. The energized membrane is used by the cell to transport ions and uncharged molecules from the external environment into the cell (1).

Summary

It may be that future evolution in bacteria is somewhat limited. Bacteria participate in gene transfer events such as transformation, transduction and conjugation and are also subjected to mutation events. However, it is possible that the single chromosome in different species of bacteria has reached a near maximum size and future bacterial evolution is restricted to fewer changes. For example, metabolic pathways for chemical pollutants in the environment and increasing numbers and species of multiple antibiotic resistant bacteria or emerging plant and animal pathogen microorganisms. Of course, some of these ideas are only speculation.

If any part of the bacterial cell such as DNA, RNA or proteins is removed, the ability of bacterial cells to grow, divide and remain viable in the environment is impossible. Therefore, it is reasonable to suggest that the first cell(s) capable of a division and subsequent growth and additional divisions, must have been relatively advanced in their structure and function. This first cell capable of binary division was likely the universal ancestor from which life evolved and diversified.

As more research is forthcoming on self-assembly and molecular evolution in bacteria, the scientific community may gain new insights into the origin of prokaryotic cell division. It is also possible that the theoretical ideas discussed in this manuscript may be partially or completely incorrect. An integration of scientific information from the physical, chemical and biological sciences and additional research on the early self-assembly of life and how cells divide is needed to stimulate new research and debates on molecular evolution.

ACKNOWLEDGMENTS

Appreciation is expressed to the Natural Sciences and Engineering Research Council of Canada (NSERC) operating grants program for financial support of research in my laboratory.

RESUMO

Evolução molecular em bactérias: divisão celular

A evolução molecular em bactérias é examinada com ênfase na auto-organização de uma célula capaz de divisão primitiva e multiplicação durante o

princípio da evolução molecular. Também se discute a possibilidade de que algum tipo de estrutura de encapsulação tenha antecedido as vias bioquímicas e o agrupamento de material genético. Esses aspectos são considerados sob uma perspectiva evolutiva.

Palavras-chave: divisão celular, evolução

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Revista de Microbiologia (1998) 29:246-250
ISSN 0001-3714

SUGAR-CANE JUICE INDUCES PECTIN LYASE AND POLYGALACTURONASE IN *PENICILLIUM GRISEOROSEUM*

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Submitted: February 07, 1997; Returned to authors for corrections: March 24, 1998; Approved: August 20, 1998.

ABSTRACT

The use of other inducers as substitutes for pectin was studied aiming to reduce the production costs of pectic enzymes. The effects of sugar-cane juice on the production of pectin lyase (PL) and polygalacturonase (PG) by *Penicillium griseoroseum* were investigated. The fungus was cultured in a mineral medium (pH 6.3) in a rotary shaker (150 rpm) for 48 h at 25°C. Culture media were supplemented with yeast extract and sucrose or sugar-cane juice. Sugar-cane juice added singly to the medium promoted higher PL activity and mycelial dry weight when compared to pectin and the use of sugar-cane juice and yeast extract yielded levels of PG activity that were similar to those obtained with sucrose-yeast extract or pectin. The results indicated that, even at low concentrations, sugar-cane juice was capable of inducing pectin lyase and polygalacturonase with no cellulase activity in *P. griseoroseum*.

Key words: pectin lyase, polygalacturonase, pectinases, *Penicillium griseoroseum*, sugar-cane juice.

INTRODUCTION

Pectic enzymes have been tested for retting processes based on the ability of certain pectolytic microorganisms to liberate cellulose fibers from the stems of fibrous plants (15).

Among pectic enzymes, pectin lyase (EC 4.2.2.3) is the most interesting, as it is the only one capable of depolimerize pectins without altering their esterification level (16) and to cleave α-1,4 bonds of highly esterified pectin without the previous action of other enzymes (1). Most commercial enzymatic complexes used today contain cellulases that attack cellulose fibers making them less resistant to industrial processing.

Other inducers than pectin have been studied aiming to reduce the production costs of pectinases. *Penicillium griseoroseum* synthesizes low amounts of cellulases (6) and produces pectin lyase in media containing sucrose and yeast extract (YE) (3), methylxanthines (13), or tea extract (4,12). Sugar-cane has a low pectin concentration (around 0.2%) but is rich in sucrose (approximately 16%). The aim of this work was to investigate the effects of sugar-cane juice on the production of pectin lyase (PL) and polygalacturonase (PG) by *P. griseoroseum*, since it is inexpensive and readily available in Brazil.

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MATERIALS AND METHODS

Microorganism and cultivation conditions

Penicillium griseoroseum was originally obtained from seeds of forest trees at the Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. Inoculum was produced by culturing *Penicillium griseoroseum* on oatmeal-agar for 9 days at 25°C. Mineral medium (50 ml, pH 6.3, consisted of (g/L): KH_2PO_4, 8.0; K_2HPO_4, 2.48; $MgSO_4.7H_2O$, 1.1 and $(NH_4)_2SO_4$, 1.0) was supplemented with 0.03% yeast extract (YE) and sucrose (0.1 to 2.0%) or sugar-cane juice to a final concentration of sucrose ranging from 0.1 to 2.0% (the sucrose content in sugar-cane juice being assumed to be around of 16%). Sugar-cane juice, 0.4% tea extract (TE) and 0.4% citric pectin were also tested in the absence of YE. The cultures were inoculated with $5x10^4$ spores per ml and incubated on a rotary shaker (150 rpm) for 48 h at 25°C (5). All experiments were carried out in triplicate.

Enzymatic analysis and mycelial growth

Cultures were harvested by filtering through a 400 mesh sieve (37 μm pore size) and growth was determined as mycelial dry weight (MDW) according to Calam (7). PL and PG activities were determined spectrophotometrically (A_{235} and A_{540}), in the culture filtrate, according to Albersheim (2) and by the 3,5-dinitrosalicylic acid method (11) using galacturonic acid as standard, respectively. PL activity unit (U/ml) was defined as nmoles of unsaturated uronides produced per min per ml of culture filtrate (13). PG activity unit (U/ml) was defined as nmoles of galacturonic acid produced per min per ml of culture filtrate (14). Cellulase activity was estimated as described and modified by Halliwell (9), filter paper (1x7 cm) was incubated with 2.0 ml of culture filtrate at 40°C for 1 hour and the glucose formed was measured by 3,5-dinitrosalicylic acid method (11) using glucose as a standard.

RESULTS AND DISCUSSION

The effect of different substrates on the production of PL and PG by *Penicillium griseoroseum* can be seen in Fig. 1. The fungus did not produce a significant amount of PL in the presence of sucrose as the sole carbon source. However, when the medium was supplemented with sucrose and YE or other inducers like pectin, sugar-cane juice (with or without YE) , or tea extract, a significant PL activity was detected (Fig. 1A). Maximum PL activity was achieved when the culture was grown in the mineral medium containing sugar-cane juice and YE (72.16 U/ml). In this case, PL activity corresponded to 2.64 and 1.96 times that obtained with pectin and sucrose-YE, respectively. Sugar-cane juice added singly to the medium promoted higher PL activity and MDW when compared to pectin (Figs. 1A and 1C). However, this did not occur for PG (Fig. 1B). Maximum PG activity was obtained with pectin and YE. The use of sugar-cane juice and YE yielded levels of PG activity that were similar to those obtained with sucrose-YE or pectin, while TE could induce only low levels of enzyme activity (Fig. 1B).

Sucrose at concentrations ranging from 0.1 to 2.0% with 0.03% YE promoted an increase in MDW, though PL and PG activity remained approximately constant (the statistical analysis demonstrated that there was no significant difference ($P>0.05$)) (Fig. 2). The same was not true for sugar-cane juice (Fig. 3). Increasing concentrations of sugar-cane juice (0.1-2.0%), in the presence of 0.03% YE, promoted higher

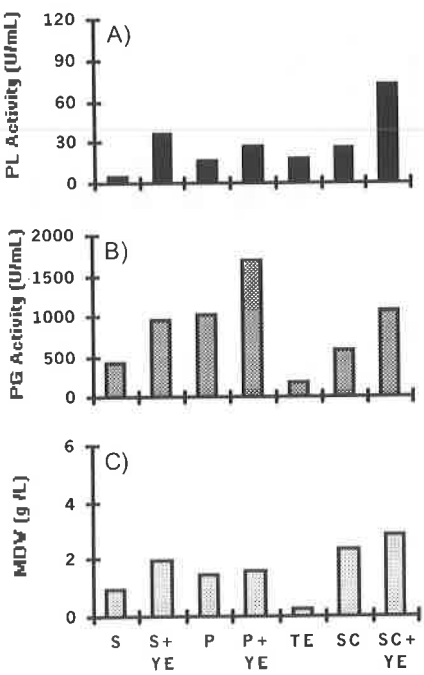

Figure 1. Pectin lyase (PL) activity (A), polygalacturonase (PG) activity (B), and mycelial dry weight (MDW) (C) of *P. griseoroseum* cultured on 0.4% sucrose (S), 0.4% sucrose and 0.03% YE (S+YE), 0.4% pectin (P), 0.4% pectin and 0.03% YE (P+YE), 0.4% tea extract (TE), 0.4% sugar-cane juice (SC), 0.4% sugar-cane juice and 0.03% YE (SC+YE).

PL activity and MDW. The maximum value for PL activity corresponded to 2.9 times that obtained with sucrose and YE added to the culture medium at equivalent sucrose concentrations (Figs. 2A and 3A). PG activity increased as the concentrations of sugar-cane juice were in the range of 0.1 to 0.4% (Fig. 3B).

Sugar-cane juice induced high PL production even in the medium without YE, though with lower activity (Fig. 4A). The higher the concentrations of sugar-cane juice in the medium (up to 0.4%), the higher the amounts of PG synthesized by the fungus (Fig. 4B). MDW increases were directly proportional to the concentration of sugar-cane juice (Fig. 4C).

The results obtained in this study indicate that sugar-cane juice induced PL and PG synthesis in *P. griseoroseum* either in the presence or absence of YE. Differently from sucrose, increasing concentrations of sugar-cane juice promoted increases in PL and PG activities with or without the addition of YE to the medium. Sugar-cane juice and YE seem to have different effects on the induction of PL and PG. A synergistic effect of these compounds on PL and PG activities could be observed when they were added simultaneously to the culture medium. The

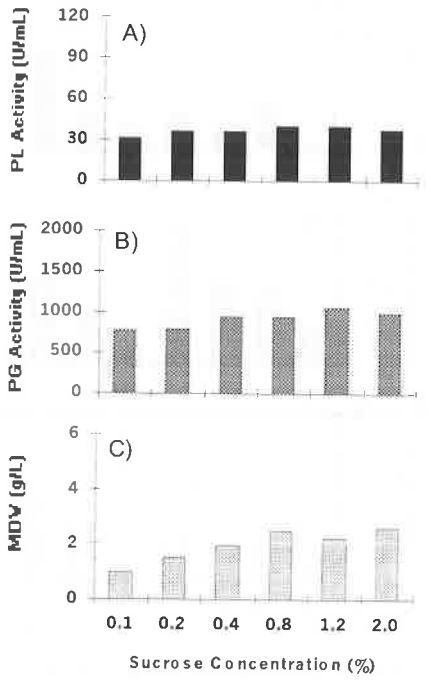

Figure 2. Pectin lyase (PL) activity (A), polygalacturonase (PG) activity (B), and mycelial dry weight (MDW) (C) of *P. griseoroseum* cultured on 0.03% YE and increasing concentrations of sucrose (0.1-2.0%).

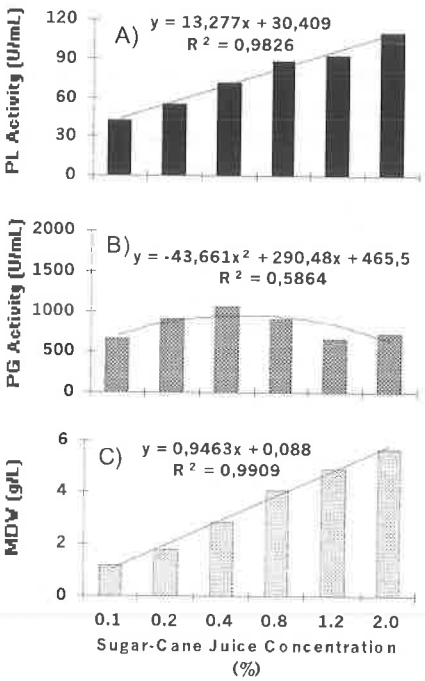

Figure 3. Pectin lyase (PL) activity (A), polygalacturonase (PG) activity (B), and mycelial dry weight (MDW) (C) of *P. griseoroseum* cultured on 0.03% YE and increasing concentrations of sugar-cane juice (0.1-2.0% as sucrose concentrations).

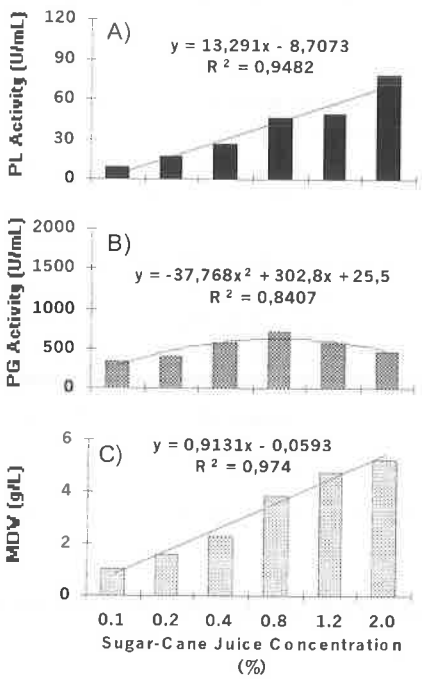

Figure 4. Pectin lyase (PL) activity (A), polygalacturonase (PG) activity (B), and mycelial dry weight (MDW) (C) of *P. griseoroseum* cultured on sugar-cane juice (0.1-2.0% as sucrose concentrations).

results indicate the existence of different induction mechanisms for PL and PG in *P. griseoroseum* cultured on sugar-cane juice. Increasing concentrations of sugar-cane juice promoted linear increases in PL activity (linear function), contrarily to that observed for PG activity (polynomial function) (Figs. 3 and 4). The PG synthesis could be partially repressed when sugar-cane juice at sucrose concentrations above 0.4% was added to the culture medium (catabolite repression) (14).

According to Leme and Borges (10), sugar-cane contains 11-18% sucrose, 0.15-0.25% pectic substances, gums and mucilages, 0.3-0.6% nitrogenous compounds (aminoacids, xanthinic compounds), 5.5% cellulose, among other constituents. *P. griseoroseum* is capable of producing PL when grown in media containing sucrose and YE (3), methylxanthines (13), or tea extract (4,12). When 0.1-2.0% sugar-cane juice (as sucrose concentrations) were added to 50 ml of mineral medium, the amount of pectic substances present were very low (0.00125-0.025%, considering 0.2% pectic substances in sugar-cane juice). Baracat *et al.* (4) showed that the addition of 0.0001-0.005% of citric pectin to the culture medium promoted no significant induction of PL by *P. griseoroseum*. Minussi *et al.* (12) reported that best PL activity was obtained when *P. griseoroseum* was cultured in 0.35% pectin and 0.05% sucrose in comparison to the treatment with 0.4% pectin added singly to the medium. Media containing sugar-cane juice are much richer and therefore are more suitable for higher mycelial mass production. Thus, the authors suggest a synergistic effect brought about by the simultaneous addition of pectin substances, sucrose, and xanthinic compounds present in sugar-cane juice, allied with the high mycelial mass production, for the high PL production by *P. griseoroseum* cultured in sugar-cane juice.

To verify the existence of cellulases, *P. griseoroseum* was grown at 25°C for 144 h on 0.4% sugar-cane juice and 0.03% YE. MDW and the activities of PL, PG, and cellulase were determined. No cellulase activity was detected in the culture filtrate during 144 h, which are in according with Brumano *et al.* (6). PL activity had a slight increase after 120 h of cultivation. MDW were approximately constant after 48h of growth, while PG activity had a decrease (Figure 5). Geöcze *et al.* (8) also observed that there was a fall in PG production when *Penicillium expansum* was cultured in citrus pectin supplemented with YE, reaching maximal enzyme activity after 36 to 48 h of culture. These authors suggered that the amino acids present in the yeast extract may be repressing PG synthesis.

Our results indicated that, even at low concentrations, sugar-cane juice added singly to the medium was capable of inducing PL and PG in *P. griseoroseum*. Sugar-cane juice, when compared to other pectinase inducers, seems to be an economical alternative for the industrial production of PL and PG.

ACKNOWLEDGEMENTS

The authors wish to thank the Brazilian Agencies, FINEP and CNPq, for the financial support.

RESUMO

Caldo de cana induz pectina liase e poligalacturonase em *Penicillium griseoroseum*

Na produção de enzimas pécticas estudou-se a utilização de outros indutores além de pectina visando a redução de custos. Os efeitos de caldo de cana na produção de pectina liase (PL) e poligalacturonase (PG) por *Penicillium griseoroseum* foram investigados. O fungo foi cultivado em meio mineral (pH 6,3) em um agitador rotatório (150 rpm) por 48 h a 25°C. O meio de cultura foi suplementado com extrato de levedura e sacarose ou caldo de cana. A adição de caldo de cana ao meio de cultivo promoveu maior atividade de PL e peso do micélio seco quando comparado com a adição de pectina. Na presença de caldo de cana e extrato de levedura observou-se atividade de PG semelhante ao obtido na presença de sacarose e extrato de levedura ou pectina. Os resultados indicaram que, mesmo em baixas concentrações, caldo de cana foi capaz de induzir pectina liase e poligalacturonase, sem atividade de celulase, em *Penicillium griseoroseum*.

Palavras-chave: pectina liase, poligalacturonase, pectinases, *Penicillium griseoroseum*, caldo de cana

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Revista de Microbiologia (1998) 29:251-253
ISSN 0001-3714

CHROMIUM UPTAKE FROM AQUEOUS EFFLUENTS BY IMMOBILIZED BAKER'S YEAST

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Submitted: April 21, 1997; Returned to authors for corrections: May 05, 1997; Approved: September 19, 1998

SHORT COMMUNICATION

ABSTRACT

Baker's yeast immobilized in alginate was used to take up chromium from effluents. Chromium in aqueous solutions were used in different concentrations. To evaluate the viability and efficiency the baker's yeast for chromium uptake from effluents three experiments done in two differents reactor systems: first in system 1 at 17.5 ml/s and with 10, 20, 25 and 30 mg/l Cr; second in system 2 at 38.7 ml/s with 20 mg/l Cr; third in system 2.1 at 6.65 ml/s and with 20, 30 and 40 mg/l Cr. The efficiency of chromium uptake varied between 86 and 100 %.

Key words: Baker's yeast, chromium, uptake

Heavy metal pollution is an important problem in environmental degradation, stimulating interest in new methods using microorganism to remove heavy metals from effluents. Various microbes have shown potential to sequester and concentrate heavy metals from aqueous environments (13). Biosorption includes passive adsorption of heavy metals at binding sites on the envelopes of cells and metabolically mediated uptake (8). The term biosorption is contested since it encompasses metal adsorption, chemical deposit and intracellular uptake by living cells. These reactions are metal and microorganism type dependent. The composition of the medium may also have a direct effect on both passive adsorption and metabolic uptake (4). The mechanism of metal ions removal by microorganisms shows that the cell wall is the primary site of metal ion accumulation and also that the uptake of heavy metal cations is not mediated by metabolic processes, taking place in dead and living cells (10). These processes may be subject to early saturation and are also sensitive to flow composition and pH.

Commercial pressed baker's yeast (Itaiquara) was immobilized in spherical globules of sodium alginate (15 mg/l) containing glucose, according to the conventional methodology (2). Chromium solutions were prepared by using chromium chlorine in different concentrations from 10 to 40 mg/l of water. These different reactor systems were used: in system 1 a glass tubular reactor (h = 100 cm ; d = 4 cm) and layer size of 35 cm were used, with a flow rate of 17.55 ml/s; in system 2, the tubular reactor was modified (h = 102 cm ; d = 11 cm), with a layer size of 25 cm and flow rate of 38.7 ml/s. In system 2.1, a flow rate of 6.65 ml/s was used. The process consisted of repassing

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5 liters of chromium chlorine solution in different concentrations through the reactor system, collecting samples at 30 min intervals for posterior analysis. Experiments 1 to 4 were done in system 1 (10, 20, 25 and 30 mg/l Cr), experiment 5 in system 2 (20 mg/l Cr) and experiment 6, 7 and 8 in system 2.1 (20, 30 anf 40 mg/l). The collected samples were preserved adjusting pH to < 2 with nitric acid and were analyzed by atomic absorption spectroscopy (1).

In most of the experiments, modifications in color and odor of the recycled solutions and of the immobilized cells were noted. There were also viscosity and pH increases in the recycled solutions and these changes may have occurred when the immobilized yeasts began to decompose.

In all experiments, the reduction of the metal concentration in solution occurred with larger intensity in the initial stages. In experiment 4 (system 1, 30 mg/l Cr), an increase occurred in the metal concentration at the end of the process, probably because microbial adsorption capacity had been exhausted and the metal was released back into the medium. Results in system 2 were similar to system 1. In experiments 6, 7 and 8 (system 2.1), lower flow rates (6.65 ml/s) were used and resulted in better efficiency.

In experiment 1 (system 1), most of the reduction occurred in the first hour, from 10 mg/l to 1 mg/l, going to zero in 2 hours. In experiment 2, during 30 min, reduction occurred from 20 mg/l to 7.38 mg/l, attaining 2.76 mg/l in 2.5 hours. The efficiencies were 100% and 86.73% respectively. In experiment 3, after 5.5 hours, a reduction from 25 mg/l to 2.0 mg/l, occurred with efficiency of 92%. In experiment 4, a reduction from 30 mg/l to 2.51 mg/l in 7.5 hours, with an efficiency of 91.6% was observed (Fig. 1).

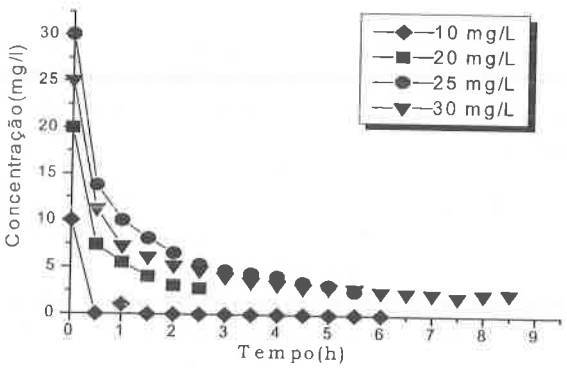

Figure 1 - Uptake of chromium from effluents by baker's yeast immobilized in alginate in a 4 cm diameter by 100 cm tubular glass reactor, layer size of 35 cm and flow rate of 17.55 ml/s.

Fig. 2 shows that in experiment 5 (system 2), reduction of chromium content from 20 mg/l to 2.1 mg/l in 5 hours, with efficiency of 88.65% was observed. The experiments conducted with 6.65 ml/s flow rate (system 2.1) had 100% in chromium removal efficiency and the experiment 7 (initial concentration = 30 mg/l), showed no reduction of chromium content in some cases, as showed in Fig. 2.

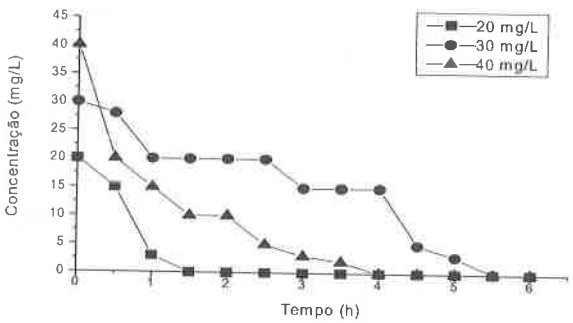

Figure 2 - Uptake of chromium from effluents by baker's yeast immobilized in alginate in a 11 cm diameter by 102 cm tubular glass reactor, layer size of 25 cm and flow rate of 6.65 ml/s. Experiments 6 to 8 in system 2.1.

In this kind of process, there are many interfering variables and on these we have studied changes metal solution circulation flow, reactor size, immobilized cells layer size and metal solution concentration.

The removal efficiency obtained was from 86.73 to 100%. System 2.1 presented the most efficiency and the shortest process time, 100% in 1.5 hour, but system 1, starting with 10 mg/l chromium concentration, also showed 100% of retention efficiency within 2 hours. It is possible to remove heavy metals from aqueous solutions using immobilized cells of baker's yeast in a fixed layer reactor.

RESUMO

Utilização de leveduras de panificação na remoção de cromo em meio aquoso

Leveduras de panificação imobilizadas em alginato, foram utilizadas com o objetivo de promover a remoção de cromo presente em efluentes. Trabalhou-se com soluções de cromo de diferentes concentrações. A fim de avaliar a viabilidade e eficiência do uso de leveduras de panificação na remoção de cromo, três experimentos foram realizados em dois diferentes sistemas de reatores: o

primeiro no sistema 1 com 17,5 ml/s e 10, 20, 25 e 30 mg/l Cr; o segundo no sistema 2 com 38,7 ml/s e 20 mg/l Cr; o terceiro no sistema 2.1 com 6,65 ml/s e 20, 30 e 40 mg/l Cr. A média das eficiências de retenção do cromo variaram entre 86 e 100%.

Palavras-chave: leveduras de panificação, cromo, remoção

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Revista de Microbiologia (1998) 29:254-258
ISSN 0001-3714

EFFECT OF AERATION ON BIODEGRADATION OF PETROLEUM WASTE

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Submitted: October 15, 1997; Returned to authors for corrections: April 16, 1998;
Approved: January 20, 1999

ABSTRACT

Large amounts of oily sludge are generated as residues by the oil industry, representing a real problem for refineries. This work studied the technical viability of treating oily sludge biologically, through stimulation of native microorganisms, at bench scale. Such microorganisms were able to grow in a medium containing oily sludge as the only carbon and energy sources. Two oily sludge concentrations were studied, 5% (v/v) and 10% (v/v), with a C:N ratio of 100:1. Higher microbial populations were observed in the first case. Substrate inhibition and/or toxic effect took place in the second case. The importance of aeration on the microbial activity and on the biodegradation of the residue was ascertained. In terms of n-paraffins, pristane and phytane consumption, maximum global efficiency of 76.9% (w/w) was achieved, in a medium containing 5% (v/v) of oily sludge. Bacteria of the genus *Pseudomonas* predominated. Two yeast species were also identified and two filamentous fungi were isolated.

Key words: petroleum waste, biodegradation, aeration

INTRODUCTION

The oil industry is responsible for the generation of high amounts of oily and viscous residues, which are formed during production, transportation and refining. Such residues, called oily sludges, are basically composed of oil, water, solids, and their characteristics, such as varied composition, make their reutilization very difficult, and confer on them high recalcitrance.

The marked stability of the multiphase system is due to the adsortion of oil on the solid particles, producing a highly protective layer, since they tend to deposit in the bottom of tanks. Additionally, the polar fractions promote charge repulsion, imparing the formation of a homogeneous phase (15). From a chemical point of view, this recalcitrance can be ascribed to the presence of aromatic, polycyclic aromatic hydrocarbons (PAHs) and complex compounds with a very high molecular weight, such as asphaltenes. In addition to that, some of these compounds act as solvent of microbial membranes.

It is estimated that, approximately, 1% of the total oil processed in a refinery in Rio de Janeiro (Brazil) is discarded as oily sludge. This has been accumulated in storage tanks for several years.

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Due to the high energy costs, the potential risk of air pollution and the persistence of PAHs, incineration is not recommended. Similarly, inadequate disposal of a such very toxic residue in landfills, encourage the search of other alternatives. Biotreatment can be applied, using the following methods: Composting, Landfarming and Biopile (9). All of them exploit soil biodiversity; however they have the disadvantage of needing long process times and there is the risk of contaminating air and aquifers by leaching. They also demand large areas and are affected by climate. An interesting alternative to circumvent these problems is the use of a bioreactor, since optimum process conditions can be easily controlled, allowing higher quality final effluent in shorter times. However they might have high costs. Taking advantage of such potential, the Valero Refining Company developed, for the first time on an industrial scale, a process using a bioreactor for treating oily sludge. One of the refineries, Corpus Christi, published an economic study, comparing biotreatment with incineration for a capacity of 2000 t/year residue. In a non-optimized system, in terms of oxygen utilization, chemical cost and waste quality, a lower cost for the biotreatment was achieved, making it economically competitive and environmental friendly (16).

Bacteria, yeasts and filamentous fungi have been reported as transforming agents because of their ability to degrade a wide variety of xenobiotic substances, commonly found in wastes from the oil industry. Being able to use these substances as the only carbon and energy source, microorganisms are powerful alternatives to conventional methods in resolving environmental problems. Prince and Sambasivam (17) point out several advantages of biological treatment: mineralization promotes permanent destruction of these residues, eliminating the hazard of further contamination and leading to high acceptance by public opinion. It can also be coupled with other processes, increasing global treatment efficiency. Optimization can be achieved by exploiting several biological phenomena, such as: microbial organization into consortia (12,14), cometabolic actions (7,13), bioaugmentation (18, 21), capacity of adaptation (20), and the possibility of genetic manipulation (8). Moreover, adhesion mechanisms and water/oil interfacial area are of great importance, affecting directly the uptake of hydrocarbon by cells and the extent of biodegradation (5,19).

This work encompasses the study of the effect of aeration on oily sludge biodegradation by native microorganisms, previously adaptated in a medium containing oily sludge as the only carbon and energy source.

MATERIALS AND METHODS

Oily sludge characterization: The characterization was performed in raw oily sludge, aqueous and organic phases. Total nitrogen and ion concentration were determined in the aqueous phase by Chemiluminescence (4) and by HPIC (Dionex, HPIC-AS4A column), respectively. Total nitrogen concentration was also evaluated in the organic phase by a modified Kjeldahl Method (1). Paraffins, aromatic hydrocarbons, resins and asphaltenes analyses were performed by TLC-FID (Iatroscan MK-5), and total polycyclic aromatic hydrocarbons by UV absortion in the wave length range of 220 - 450 nm (10). Distillation range (3) and dynamic viscosity (2) were also determined.

Biomass: The inoculum size and microbial growth were evaluated by total enumeration of colony forming units (CFU), in TSA medium, whose composition is as follows (in g/l): glucose, 10; yeast extract, 2; meat peptone, 5; agar, 15; NaCl, 5 (pH=7.2).

Isolation: The Streak plate method was used with different media (TSA and MacConkey for bacteria, and Sabouraud for yeasts and filamentous fungi).

Identification: Bacteria were the only group which, so far, have been identified, using the Identification Kit API 20 NE, manufactured by BioMérieux.

Inoculum preparation: A medium containing 5%(v/v) of oily sludge, 30 ml of mineral medium (Bushnell-Haas - g/l: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $FeCl_3$, 0.05; pH=7.0) and 17.5 ml of water, in a 500 ml conical flask, was placed in a rotary shaker (170 rpm, 30°C) for two days. 10 ml of this medium were transfered to a flask, containing the medium, and again incubated. This procedure was repeated, every two days for six days. The final culture was used to start the biodegradation process.

Experimental procedure: The biodegradation essays were carried out with two different oily sludge concentrations (v/v): 5% (MA) and 10% (MB). Both media were prepared to give a C:N ratio of 100:1. Water and mineral medium were added in the same proportion as in the medium for inoculum preparation. Evaporation was evaluated through a control,

containing 3g/L of sodium azide (MA* and MB*). The experiments were performed in flasks, closed with caps, through which they could be saturated with oxygen every 24 hrs for 5 days. The system was shaken for two days at 150 rpm. The microbial activity was estimated by CO_2 emission, over 42 days, employing gas chromatography (HP 5890II, Porapack - 2.5 m x 1/8" column). n-paraffins (nC-10 to nC-34), pristane and phytane concentrations (in µg/ml) were determined by GC, employing DB5 - 30m x 0.32mm column (J&W). The biodegradation efficiency based on respiration rate was calculated as accumulated CO_2 during the whole time period, using by the following expression:

$$EB(\%) = CO_{2\ bio} \times 100/C_i$$

$$CO_{2\ bio} = 2 \times CO_{2\ total} - CO_{2\ control}$$

where:

EB = Efficiency of biodegradation (% µmols/µmols)

$CO_{2\ bio}$ = µmols of CO_2 produced by microbial activity

$CO_{2\ total}$ = µmols of total CO_2 accumulated

C_i = µmols of CO_2 equivalent to initial carbon (theoretical value)

$CO_{2\ control}$ = µmols of CO_2 accumulated in the control (abiotic system)

The biodegradation efficiency was also evaluated by the decrease in the concentration of n-paraffins, pristane and phytane.

RESULTS AND DISCUSSION

Before the adoption of any technological strategy for treating a residue, its characterization is required. Table 1 displays the composition and main physical properties of the oily sludge from Duque de Caxias Refinery (REDUC).

It can be seen that the oily sludge possesses limited amounts of nitrogen and phosphate, indicating the necessity for fortifying the media with nutrients (11). Most of the nitrogen is not available, since it is part of complex structures, relatively inaccessible to microorganisms.

Although the concentration of asphaltenes is not very high, the predominance of aromatics and the high concentration of polycyclic aromatic hydrocarbons, usually found in this kind of residue (6), confer on it high toxicity.

The range of distillation (289 to 550°C) shows the preponderance of heavy compounds of considerable stability, low solubility in water and consequently marked recalcitrance. This high viscosity, which does not hamper microbial attack on the oil, points to the importance of mechanical agitation.

Table 1: Oil sludge characterization

Analyses		Solids	Aqueous phase	Organic phase	Raw oily sludge
Conc.	(%w/w)	7	24	69	100
Ions	(mg/L)				
NO_3^-		—	45.0	nd	10.8
PO_4^{3-}		—	< 0.03	nd	—
NH_4^+		—	25.6	nd	6.2
Total N	(%w/w)	nd	0.4	0.2	0.1
S.A.R.A.	(%w/w)				
Saturates	—	nd	28.0	19.3	
Aromatics	—	nd	44.0	30.4	
Resins		—	nd	21.0	14.5
Asphatenes		nd	7.0	4.8	
Total PAHs	(%w/w)	—	nd	13.51	9.32
Viscosity	(cP)				
30°C, 3 rpm		—	nd	nd	885.5
50°C, 12 rpm		—	nd	nd	207.7
70°C, 30 rpm		—	nd	nd	70.8

The microbial capacity to grow, using the oily sludge as sole source of carbon and energy can be observed in Fig. 1, which shows the daily CO_2 production versus time. It is clear that the interruption of oxygen feed to the media (points indicated by arrows), led to a dramatic decrease in the rate of CO_2 evolution, denoting a significant decrease in microbial activity. Between the 23rd and 25th days, for instance, the rate of gas evolution decreased from 2.5 µmol/day to 0.8 µmol/day.

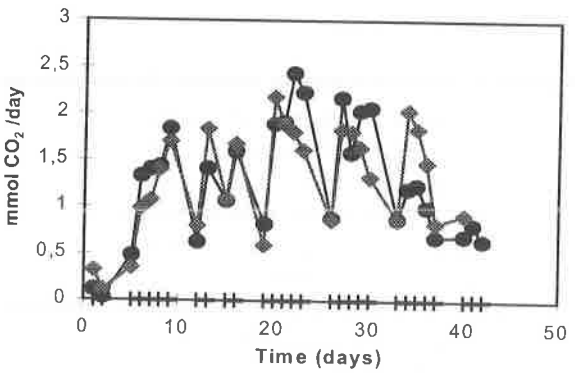

Figure 1 - Evolution rates of CO_2 in the media **MA** - 5% (v/v) of oily sludge (•), **MB** - 10% (v/v) of oily sludge (◆) and controls - **MA*** (-) and **MB*** (+).
Arrows = air feed interruption

Low biodegradation efficiencies were achieved for 10% sludge (v/v) (EB_{21days}=8.9% and EB_{42days}=20.7%). This may be due to substrate inhibition and/or toxic effects. The biodegradation efficiencies for 5% (v/v) were higher (EB_{21days}=17.4% and EB_{42days}=39.0%). These results were corroborated by higher microbial population in the last case (Fig. 2).

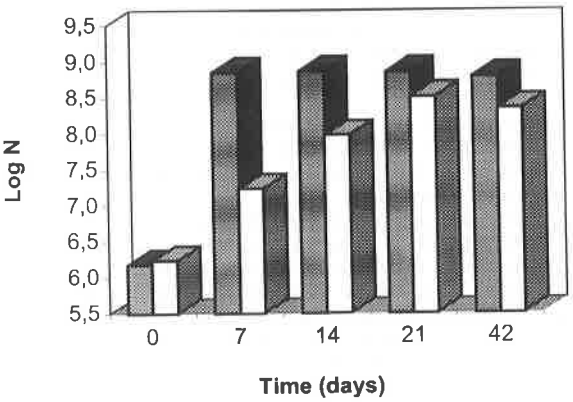

Figure 2 - Microbial growth in the media **MA** - 5% (v/v) of oily sludge (■) and **MB** - 10% (v/v) of oily sludge(□). Microbial population (N) in CFU/ml.

The uptake of paraffins, particularly pristane and phytane, resulted in higher biodegradation of both compounds in medium MA (33.7% w/w and 10.2% w/w, respectively) and low utilization in medium MB (0.0% w/w and 0.2% w/w). According to Watkinson and Morgan (22), pristane is widely employed as an internal standard for analyses of hydrocarbon samples, as it has a considerable degree of persistence. Thus, the significant consumption of this substance shows the potential of the biotreatment system. The consumption of paraffins as a whole, for medium MA, can be seen in the chromatograms in Figs 3 (initial), 4 (intermediate) and 5 (final). There is a decrease in the peaks of a wide range of compounds, from nC-10 to nC-34. The global efficiences of biodegradation were 76.9% (w/w) and 50.6% (w/w) in medium MA and MB, respectively.

Because of analytical limitations, PAHs were not determined, but attempts will be made, in future experiments, to overcome this problem.

The identification of native bacteria showed a predominance of the genus *Pseudomonas*, which was expected since this genus has been commonly found in affected areas (9). So far, the folowing bacteria species have been identified: *Pseudomonas cepacia, Pseudomonas aureofaciens, Pseudomonas picketti,*

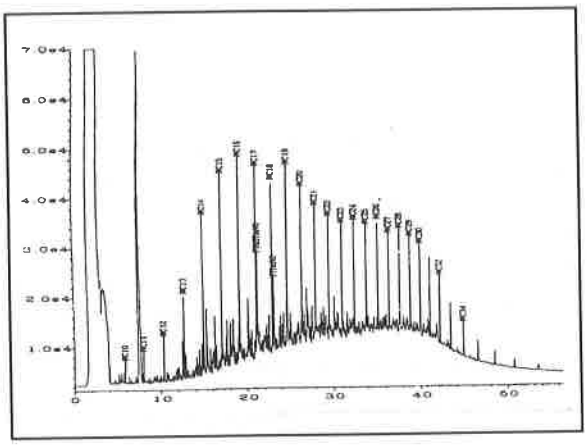

Figure 3 - Medium MA: initial analysis of *n*-paraffins, pristane and phytane (t=0)

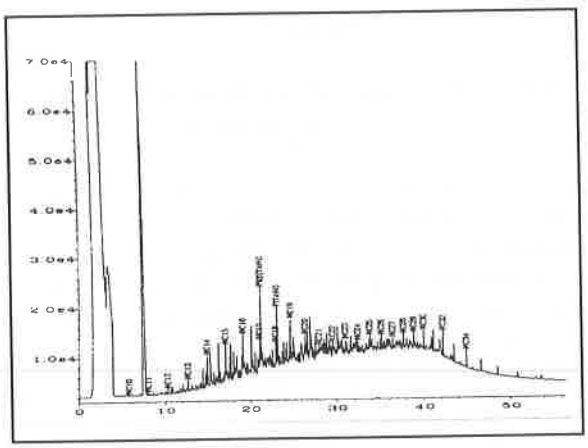

Figure 4 - Medium MA: intermediate analysis of *n*-paraffins, pristane and phytane (t=21 days)

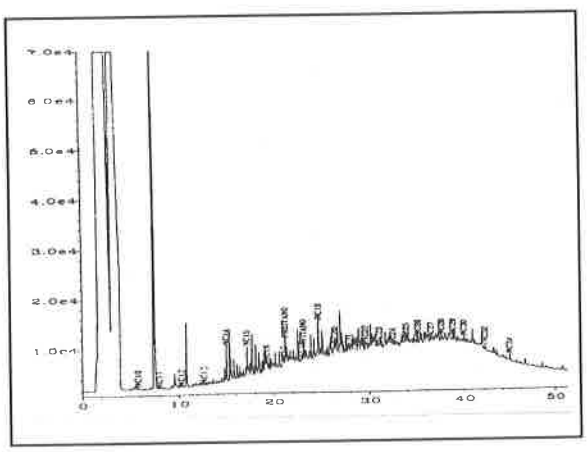

Figure 5 - Medium MA: final analysis of *n*-paraffins, pristane and phytane (t=42 days)

Flavobacterium indologenes, Xanthomonas maltophilia and Ochrobactrum anthropi. Two yeast species, *Candida tropicalis* and *Rhodotorula mucilaginosa*, were also identified and two filamentous fungi were isolated, although they have not been identified yet. Once more, the importance of aeration became evident, since all bacterial species are strictly aerobic microorganisms. Such identification will allow the adoption of strategies in the optimization of oily sludge biotreatment.

ACKNOWLEDGMENTS

This work was supported by PETROBRAS, through grants to the first author and for laboratory infrastructure facilities.

RESUMO

Efeito da aeração sobre a biodegradação de resíduo de petróleo

Grandes quantidades de borra oleosa são geradas como resíduos pela indústria do petróleo, representando um problema real para as refinarias. Neste trabalho foi estudada a viabilidade técnica do tratamento biológico de borra oleosa, conduzido a partir do estímulo de microrganismos nativos, em escala de bancada. Tais microrganismos foram capazes de crescer em meio contendo borra oleosa como única fonte de carbono e de energia. Duas concentrações deste resíduo foram estudadas, 5% (v/v) e 10% (v/v), para uma relação C:N de 100:1. Maiores densidades microbianas foram observadas na primeira condição. Por outro lado, inibição pelo substrato e/ou efeito tóxico ocorreram na segunda condição. Foi comprovada a importância da aeração sobre a atividade microbiana, assim como sobre a biodegradação do resíduo. Em termos de consumo de n-parafinas, pristano e fitano, a eficiência global máxima atingida foi de 76,9% (p/p), em meio contendo 5% (v/v) de borra oleosa. O procedimento de identificação mostrou a predominância de bactérias do gênero *Pseudomonas* e de leveduras dos gêneros *Candida* e *Rhodotorula*. Dois fungos filamentosos também foram isolados, estando, no momento, sujeitos a procedimentos de identificação.

Palavras-chave: borra oleosa, biodegradação, aeração

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Revista de Microbiologia (1998) 29:259-264
ISSN 0001-3714

PRODUCTION OF ALPHA-AMYLASE IN ACID CHEESE WHEY CULTURE MEDIA WITH AUTOMATIC PH CONTROL

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Submitted: November 27, 1997; Returned to authors for corrections: April 24, 1998;
Approved: September 17, 1998

ABSTRACT

The influence of aeration and automatic pH control on the production of α-amylase by a strain of *Bacillus subtilis* NRRL 3411 from acid cheese whey was studied. Tests were carried out in a rotary shaker and in mechanically stirred fermenters. α-amylase was analysed according to DUN's method. Oxygen absorption rate was determined by Cooper's method. Cell oxygen demand was determined as oxygen consumption in a Warburg respirometer. The level of dissolved oxygen was measured by means of a galvanic silver-lead electrode. Results suggest the possibility of industrial use of acid cheese whey as a carbon source for α-amylase production, since the yield was similar to that produced with lactose. The highest α-amylase levels 10,000 DUN/ml units were not attained at higher aeration rates -431 $mLO_2/L.h$-. The indicated value correspond to a 96 h process with automatic pH control at 7.5. These conditions resulted in double concentration of α-amylase. The enzyme production was directly related to growth in the form of cell aggregates.

Key words: *Bacillus subtilis,* α-amylase, acid cheese whey, automatic pH control.

INTRODUCTION

The microbial amylases have found wide scale industrial application. These in enzymes are used in starch-processing, baking and in the textile industry.

The production of α-amylase, using strains of *Bacillus*, is influenced by both nature and concentration of carbon and nitrogen sources. It has been shown that *Bacillus subtilis* NRRL 3411 produces higher enzyme concentration in a lactose medium than in starch, saccharose or glucose. (6)

Since lactose is the most expensive of these carbon sources, we considered the possibility of using acid cheese whey, a by-product of the dairy industry.

Acid cheese acid whey is a readily available substrate and a powerful poluent. Its use in the production of liquid stabilized enzyme preparations could represent a way of reducing pollution whilst providing a low cost carbon source medium at industrial level.

The aim of this work is to improve the production of α-amylase. Using a previously selected culture medium, a comparative study was carried out in Erlenmeyer flasks and in a fermenter at different aeration rates. Enzyme production at different pH values was studied and an automatic pH control process developed.

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MATERIALS AND METHODS

Microorganism. A strain of *Bacillus subtilis* NRRL 3411, maintained as spores in peat (see Table 1), was used. Stock cultures were prepared as follows: one part of river sand previously washed, pH adjusted to 6.8-7, was thoroughly mixed with high water holding capacity peat (100%) 100 g of this peat can absorb 100 g of water, and yet remain as a fine dust with all its characteristics. The mixture was adjusted to 12% humidity content, and sterilized in five-gram portions, for 3 hours at 121°C. The mixture was then impregnated with a 3 ml spore suspension in medium 2 (see Table 1). Tubes were hermetically sealed with either a threaded cap or a plastic film cover over cotton caps, and stored at 5°C.

Media. Culture media are shown in Table 1. Experiments were carried out using lactose or acid cheese whey as a carbon source (6).

Table 1. Composition of culture medium.

Composition (g/l)	Sporulation (1)	Inocula (2)	Process (3)
KH_2PO_4	0.5	1	1
K_2HPO_4	0.5	1	1
$CaCl_2$	0.06	-	0.1
$CaCO_3$	-	4	-
$MgSO_4.7H_20$	0.2	0.2	0.3
$FeCl_3.6H_20$	-	-	0.006
$(NH_4)_2SO_4$	-	8	-
Peptone from meat (Britania N° 1)	4	10	-
Casein	-	-	32
Yeast extract	0.5	5	6
Lactose	-	-	30
Soluble starch	10	20	-
Agar	15	-	-
pH	7	7	6.8

Inocula. Each flask was seeded with a peat-kept spore suspension in 5 ml sterilized distilled water, previously exposed to a 10 min 100°C thermal shock. (3,6,12)

Cell growth. Microbial growth was quantified by optical density measurements (650 nm) and by dry weight. A 10 ml sample was centrifuged at 2,200 g for 20 min. Precipitate was washed with distilled water, resuspended in water and dried at 100°C until constant weight was reached.

Determination of α-amylase. DUN's method was used to determine the α-amylase activity of cultures: 10 ml of soluble starch solution at 1% were added to 1 ml of the cell-free solution (7.2 pH), and incubated at 40°C for 10 min. The enzyme reaction was stopped by adding 10 ml HCL 0.1N solution. 1 ml of this solution was then added to 10 ml of iodized iodine solution. The optical density was measured at 660 nm. A blank, substituting the enzyme solution for distilled water, was carried out. One DUN enzyme unit is defined as the quantity of enzyme that causes 1% reduction in the intensity of the blue colour obtained by mixing the iodine solution and the starch solution at 40°C for 1 min. (1).

Consumption of carbon source. To determine lactose concentration in the culture medium, Miller's spectrophotometric method (8), which measures reducing sugars, was used. A standard curve was obtained with lactose concentration between 50 and 330 ppm, using a 550 nm wavelength.

Cell oxygen demand. Cell oxygen demand was determined by means of a Warburg respirometer at 28°C. The variation in the gas quantity is measured at constant volume. Any change in the gas quantity is measured by a pressure change in a manometer (11).

Oxygen absorption rate. The oxygen absorption rate (OAR) was measured using the sulphite method (2). The oxygen absorption by solution of sodium sulphite with cupric ion as catalyst may be used as measures of oxygen solution rate. The principle of such method is that the rate of oxidation of the sulphite is limited only by the rate of the oxygen transfer from gas to liquid.

Dissolved oxygen. Dissolved oxygen was measured with a sterilizable silver-lead galvanic electrode.

Operating conditions. Inocula were produced in 250 ml Erlenmeyer flasks containing 50 ml of medium N° 2. Production was carried out in 500 ml Erlenmeyer flasks containing 100 ml of medium N° 3 in a rotary shaker at 250 rpm and 2.5 cm eccentricity. For the preparation of the culture media, the medium components peptone, yeast extract, ammonium sulphate, phosphates and sulphates were sterilized separately of the carbon source and carbonates, at 121°C for 20 min. After sterilization all the components were mixed. It was necessary to sterilize the medium components separately to avoid any alteration and/or interaction which might modify the pH in a significant way.

Experiments were carried out in media containing either lactose or whey, with concentration expressed as lactose at 30 g/l. The whey used was a by-product from mozzarella cheese production. This raw material

was provided by Cooperativa Láctea (a dairy products manufacturing plant) Santa Rosa, Province of La Pampa, Argentina. To avoid alteration during transport, whey was stabilized with 0.1% (100 vol) hydrogen peroxide. In the laboratory, whey was heated to boiling point for 50 min to precipitate most of the protein, and then cooled and vacuum filtered. It was stored frozen to allow the availability of a raw material with constant composition. As whey provided by the manufacturing plant contained in the order of 40 g/l lactose, it was diluted with distilled water to give 30 g/l final concentration. Whey provided the sole carbon source.

Tests carried out in fermenters were performed at different agitation rates: 100, 200, 300, 400 and 500 rpm using a 1 L/L. min aeration rate. Equipment used was similar to a 5-liter New Brunswick unit, with two turbines, one as a foam break. Monitors were used to measure and control pH, temperature and agitation rates, measure dissolved oxygen partial pressure, and control foam by automatic addition of an antifoam silicone agent through a peristaltic pump. For tests with automatic pH control, a system operating with an Ingold sterilizable electrode and automatic addition of 5N sulphuric acid solution through peristaltic pumps was used. Controls were performed at different pH levels: 6.5, 7 and 7.5. The processes began at a pH value of 6.8.

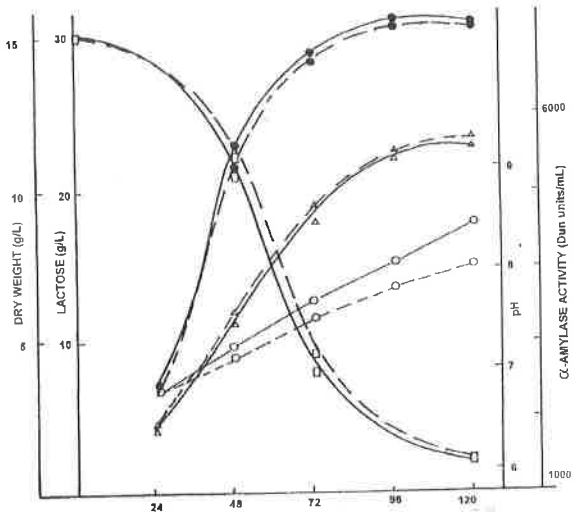
RESULTS

Fig. 1 shows the results obtained in previous experiments carried out in rotary shaker (6). The medium contains lactose as carbon source and a similar medium where lactose is replaced by acid cheese whey (Medium 3, Table 1).

Production of α-amylase from this microorganism is associated with growth and reaches its maximun level during the stationary phase (5). Changes in the measured parameters followed a similar pattern reaching enzyme values of approximately 5500 DUN/ml units. In all tests in which acid cheese whey was used, pH change was moderate with a tendency towards less alkaline values.

In both media, microorganisms grew as aggregates. Cell oxygen demand (maximum values) were in the order of 230 ml O_2/L.h. On the basis of these results, all further tests were performed using cheese whey as carbon source.

Table 2 shows the results obtained at 100, 200, 300, 400 and 500 rpm in a mechanically stirred



——— Lactose Medium
--------- Whey Medium

● Dry weight, □ Lactose, O pH, Δ α-amylase activity.

Figure 1. Production of α-amylase from *Bacillus Subtilis* NRRL 3411 growing on lactose and whey medium in a rotary shaker. (250 rpm and 2.5 cm stroke).

fermenter. The highest enzyme levels are obtained at 300 rpm. Here again, the microorganism develops as cell aggregates as in the stirred flasks. In general, the process was completed in less time using fermenters, with rapid change of pH, which becomes more noticeable as the agitation rate is increased.

At 100 and 200 rpm growth is lower, and in these conditions, for higher agitation rate, the maximum dry weight values are similar to those at 300, 400 and 500 rpm.

Table 3 shows oxygen dissolution values. It is evident that the value at 300 rpm is similar to that determined in the rotary shaker. Even though the values for dissolved oxygen increase from 400 rpm, enzyme concentrations do not behave likewise.

On the basis of previous experiments, where it was shown that a similar concentration is reached in fermenters as in rotary shakers, and accounting for a rapid change of pH towards very alkaline values, new experiments were performed with a pH control system. pH control processes were carried out at 6.5, 7 and 7.5, reaching the highest enzyme concentrations at pH 7.5, (Fig 2), doubling the enzyme concentration and reaching a value of 10,000 DUN/ml. units. These tests were carried out at 300 rpm. Biomass concentration as well as lactose consumption throughout the process were similar to those for tests without pH control.

Table 2. Oxygen absorption rate (OAR) in a mechanically stirred fermenter at different agitation rates, using 1 L/L.min.

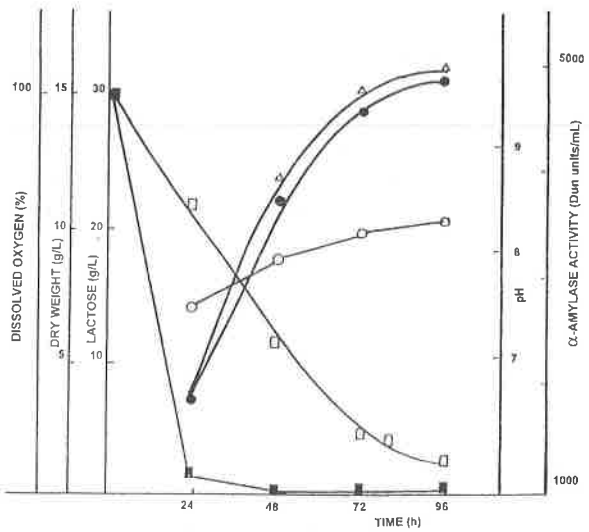
RPM	HOURS																					
	24						48						72						96			
	Lactose g/l	Dry Weight g/l	pH	Amylase U.DUN/ml	Oxygen dissolved %	Lactose g/l	Dry Weight g/l	pH	Amylase U.DUN/ml	Oxygen dissolved %	Lactose g/l	Dry Weight g/l	pH	Amylase U.DUN/ml	Oxygen dissolved %	Lactose g/l	Dry Weight g/l	pH	Amylase U.DUN/ml	Oxygen dissolved %
100	26	2	6.9	200	0	16	6	8.3	500	0	13	8	8.3	700	0	12	9	8.5	1,000	0
200	25	3.5	7	500	0	15	7	7.8	1,500	0	10	10	8.2	1,900	0	8	11	8.4	2,800	0
300	22	4	7.5	1,800	5	11	10	7.9	4,100	0	3	15	8.2	4,600	0	2	16.5	8.3	5,000	0
400	20	4.1	7.7	1,600	20	10	11	8	3,800	10	3	16	8.5	4,300	10	2.5	16.2	8.6	4,700	20
500	17	5	7.8	1,800	30	9	11.5	8.2	3,800	20	2	16	8.7	4,200	20	2	16.5	8.9	4,200	40

Also, here the cell oxygen demand (maximum value) was in the order of 230 ml O_2/L.h, (Fig. 3). The same cell aggregate type of growth is observed.

The described results express the averages of triplicate experiments.

Table 3. Production of α-amylase from *Bacillus subtilis* growing on whey medium in a mechanically stirred fermenter at different agitation rates; using 1 L/L.min.

RPM	O.A.R. (ml O_2 /L.h)
100	67.02
200	184.32
250	270.72
300	431.06
400	630.46
500	1,224

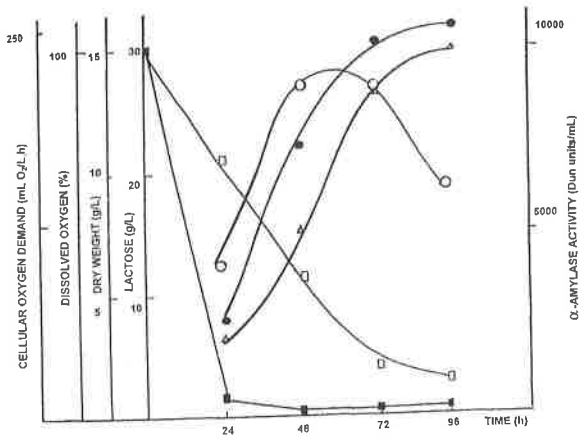


● Dry weight, □ Lactose, O pH, Δ α-amylase activity, ■ Dissolved oxygen.

Figure 2. Production of α-Amylase from *Bacillus Subtilis* NRRL 3411 growing on whey medium in a mechanically stirred fermenter (300 rpm and 1 L/L.min).

DISCUSSION

If we consider the results obtained in stirred Erlenmeyers, it becomes evident that whey can be used as source of lactose to obtain α-amylase. Media containing whey show lower changes in pH than those containing lactose. This behaviour can be attributed to the proteins contained in this by-product (10),



● Dry weight, □ Lactose, O pH, Δ α-amylase activity, ■ Dissolved oxygen, O Cell oxygen demand.

Figure 3. Production of α-amylase from *Bacillus Subtilis* NRRL 3411 growing on whey medium in a mechanically stirred fermenter. (300 rpm and 1 L/L.min), with automatic pH (7.5) control.

namely lactoalbumin and lactoglobulin, which do not precipitate in the de-proteinization treatment which removes whey casein.

From fermentors results, it can be inferred that growth limitation at 100 and 200 rpm could be due to lack of oxygen, as shown by cell demand and oxygen dissolution values in Table 3. It can be observed that higher agitation rates produce higher biomass.

Even though it is necessary to supply oxygen for growth, an increase in stirring conditions allows for an improved enzyme production level up to 300 rpm. This behaviour could be related to different variables which can be considered separately. In general, it is observed that an increase in the agitation rate produces a shorter fermentation with a marked pH change towards alkaline values limiting enzyme production (4). An increase in agitation rate over 300 rpm changes the growth pattern of the cell aggregates. The decrease of enzyme levels at high stirring values could be related to enzyme denaturization (7) and to a change in the relationship O_2- CO_2 (9).

Tests carried out with automatic pH control indicate that a pH of 7.5 has a considerable influence on enzyme production, since double the α-amylase values were obtained for aeration conditions at low oxygen concentrations.

It can be concluded that, under conditions in which the highest enzyme concentration is obtained, pH plays a fundamental role in the accumulation of α-amylase. It is also worth mentioning that retaining the aggregated form of growth, possible up to 300 rpm, ensures a high production of α-amylase.

ACKNOWLEDGEMENTS

We thank the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Facultad de Ciencias Exactas y Naturales - UNLPam for financial support.

RESUMO

Produção de α-amilase em soro ácido de queijo como meio de cultura, com controle automático do pH.

O presente artigo teve o objetivo de relatar a produção de α-amilase usando uma estirpe de *Bacillus subtilis* NRRL 3411, usando soro de queijo como fonte de carbono. Foi determinada a influência da aeração do meio de cultura bem como o controle automático do pH. As determinações de α-amilase foram realizadas pelo método de DUN e a taxa de absorção de oxigênio em diferentes condições de aeração pelo método de Cooper. A demanda celular de oxigênio foi estabelecida em um respirômetro de Warburg e o nível de oxigênio dissolvido por meio de eletrodos galvânicos prata-chumbo. Os resultados indicam a possibilidade de uso do soro de queijo como fonte de carbono pois foram similares aos obtidos com lactose. Melhores rendimentos, 10.000 unidade DUN/ml, foram obtidos em 96 h de processo com aeração média -431 mLO_2/l.h- e com controle operativo do pH a 7.5, condições estas que permitiram dobrar a atividade de amilase no caldo. Finalmente, foi observado que a obtenção de α-amilase está relacionada com a maneira de crescimento dos microrganismos nos agregados celulares.

Palavras-chave: *Bacillus subtilis,* soro ácido de queijo, controle automático do pH.

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Revista de Microbiologia (1998) 29:265-271
ISSN 0001-3714

HIGH BIODEGRADATION LEVELS OF 4,5,6-TRICHLOROGUAIACOL BY *BACILLUS* SP. ISOLATED FROM CELLULOSE PULP MILL EFFLUENT

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Submitted: February 02, 1998; Returned to authors for corrections: April 24, 1998; Approved: July 23, 1998

ABSTRACT

An aerobic Gram positive spore-forming bacterium was isolated from cellulose pulp mill effluent. This microorganism, identified as *Bacillus* sp. and named IS13, was able to rapidly degrade the organic chlorinated compound 4,5,6-trichloroguaiacol (4,5,6-TCG) from a culture containing 50 mg/l, which corresponds to about $3x10^4$ times the concentration found in the original effluent. The biodegradation of this compound, usually found in cellulose pulp mill effluents, was evaluated by spectrophotometry and gas chromatography analysis. During 4,5,6-TCG decreasing, the lack of by-products had shown by such analysis lead to verify the possibility of either adsorption or absorption of 4,5,6-TCG by the cells, instead of real biodegradation. There were no traces of 4,5,6-TCG after lysozyme and SDS cell disruption. Vigorous extraction was applied before spectrophotometry analysis and there was no release of residual 4,5,6-TCG. Plasmid isolation was attempted by using different protocols. The best results were reached by CTAB method, but no plasmid DNA was found in *Bacillus* sp. IS13. The results suggest that genes located at the bacterial chromosome might mediate the high decrease of 4,5,6-TCG. The importance of this work is that, in being a natural ocurring microorganism, *Bacillus* sp. IS13, can be used as inoculum in plant effluents to best organochlorinated compounds biodegradation.

Key Words: biodegradation, 4,5,6-trichloroguaiacol, aerobic bacteria and DNA.

INTRODUCTION

In the last few decades, the production and use of organochlorinated compounds has increased substantially. Although these compounds are responsible for many environmental problems, they are still heavily utilised due to their industrial importance (21).

Some microorganisms have adapted to living on areas highly contaminated with natural or xenobiotic organochlorinated compounds (14). Frequently, these organisms possess the capability to metabolize such substances to utilising them as a source of carbon and nitrogen (3,19).

The bacterial cells are characterized by large metabolic and physiological machinery which allow microorganisms to inhabit hostile ecological niches (20). The employment of such properties to solve

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environmental problems can be a possible alternative in effluent treatment.

The cellulose bleaching process is responsible for the production of many chlorinated compounds that are unavoidably spilled in the environment. Many of such substances are toxic and very difficult to microbial metabolization. 4,5,6- trichloroguaiacol is one of these compounds, which are frequently present in cellulose pulp mill effluent, and is correlated with several flora and fauna problems. Therefore, 4,5,6-trichloroguaiacol was chosen as a model compound to study the biodegradation process of chloro aromatic substances.

Biodegrading bacterial metabolism is directly involved with enzyme production either coded by genes located in plasmids or chromosomes (5). Many biodegradation-related plasmids are able to codify complete xenobiotic biodegrading pathways or at least part of them (8,17). Several bacterial isolates from different locations have shown to harbor similar catabolic plasmids, suggesting an intense genetic transfer among bacteria in nature (7). On the other hand, chromosome-encoded genes may also be involved with biodegradation. The presence of such genes in the bacterial chromosome can be justified by the natural occurrence of organochlorinated compounds (4). Throughout the evolution, microorganisms had to survive with this kind of environmental pressure, thus having to develop biological self-defense strategies. The utilization of recombinant DNA technics may be applied in the search for genes involved with biotransformation process, and the understanding of the functional mechanisms of these DNA elements is very important if industry is willing to apply such capabilities to solve pollution problems.

The aims of this research is to identify and study microorganisms capable of biodegrade organochlorinated compounds present in a local industry and verify the existence or not of plasmids possibly involved with this function.

MATERIALS AND METHODS

Bacterial strains

Bacillus sp. IS13 was isolated from a local cellulose pulp mill effluent. The identification was performed based in the following characteristics: spore-forming capability, Gram staining, and aerobic growth. The control bacterium, *Bacillus subtilis*, MC1 was obtained from the Microbiology Department of Federal University of Rio Grande do Sul, Brazil.

Chemicals

4,5,6-trichloroguaiacol (4,5,6-TCG) was obtained from Hélix Biotech Co., Canada. 3,4,5,6-tetrachloroguaiacol (3,4,5,6-TeCG) was gently provided by Dra. Maria do Carmo Ruaro Peralba of The Chemistry Institute of The Federal University of Rio Grande do Sul. All other chemicals used were of analytical grade.

Culture conditions

The cultures were grown in 250 ml Erlenmeyers flasks containing 150 ml of LB medium, pH 7.0 supplemented with 50 mg/l of 4,5,6-TCG (maximum water dissolved concentration of 4,5,6-TCG reached). They were incubated in rotatory shaker at 150 rpm and 37°C. The organochlorinated compound was dissolved previously in distillated water, pH 5.5, by shaking at 150 rpm and 37°C for 48 hours.

CFU determination

Bacillus subtilis MC1 (control bacterium) and *Bacillus* IS13 were cultured overnight at 37°C in LB broth. The cultures were dilluted with destillated water and plated on pure LB and LB supplemented with 50 mg/l of 4,5,6-TCG. The CFU/ml was determinated by colony formation.

Biodegradation analysis

Decrease of levels of 4,5,6-TCG was analysed by spectrophotometry (spectrophotometer Shimadzu UV-160A) and gas chromatography (HP 5890 chromatograph equipped with a PONA column 50 m x 0.2 mm x 0.5 µm, and FID detector). The gas chromatography analysis were performed utilising N_2 as a carrier gas with a flow of 1.5 cm^3/min., and the temperature program was 185°C isothermally for 30 minutes. The quantitative analysis was made by using tetrachloroguaiacol as internal standard. The results of both methodologies were compared.

Spectrophotometry analysis. Samples of 1 ml were collected from a culture of *Bacillus* sp. strain IS13 at 0, 3, 6, 12, 20 and 24 hours of incubation. To the sample, 2 ml of hexane were added and shaked in vortex for 60 cycles of 1 second each. 1 ml of the hexane layer was dispensed into a plastic spectrophotometer cuvette and analysed at 296 nm. Samples were also extracted with acetone, dichloromethane and petrolleum ether, but the best results were obtained with hexane.

Gas chromatography. Samples of 20 ml from the same culture described as above were extracted during 5 min. with 20 ml of hexane. The hexane phase was collected and completely dried by N_2 stream. The 4,5,6-TCG extracted was re-dissolved in 0.1 ml of hexane. The samples were derivatized with diazomethane. A sample of 50 µl from a solution of 10 mg/ml of 3,4,5,6-tetrachloroguaiacol was used as standard control. A 1 µl was injected in the CG. For these experiments samples were collected after biomass evaluation by optical density (600 nm).

Cellular lysis. Cellular lysis and vigorous shaking were carried out to verify absorption and adsorption of 4,5,6-TCG respectively, as follows.

Adsorption test. 3 samples of 1 ml each of a 24 hours culture were collected and added with 2 ml of hexane. Extraction was performed applying 2 minutes of intermittent shaking on vortex with maximum agitation power. Then, 1 ml of hexane layer was collected and analysed in a spectrophotometer at 296 nm wave length. The results were compared with standard extractions (shaking 60 times /1 second pulse).

Absorption test. 3 samples of 1 ml of a 24 hours culture were dispensed into Eppendorf tubes and centrifuged at 8000 g by 2 minutes. The supernatant was extracted with 2 ml of hexane and analysed in a spectrophotometer at 296 nm wave length. The cellular phase was ressuspended with 1 ml of distilled water and centrifuged at 8000 g. To the pellet were added 0.2 ml of STET buffer (8% w/v sucrose, 0.1% v/v Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0). Cellular lysis was carried out by 5 minutes incubation at room temperature after addition of 20 µl of lysozyme solution (10 mg/ml). Cell suspensions were boiled for 45 seconds and extracted with 2 ml of hexane by shaking in vortex 60 times of 1 second pulse. Cellular lysis was also performed by alkaline lysis with SDS (12) following extraction and analytical procedures as described above.

DNA plasmid extractions

DNA plasmid extraction was attempted by six different methodologies: Kado and Liu (12); Anderson and Mckay (2); Del Sal (7) with some modifications, alkaline lysis (16) and boiling lysis (16); and pulsed field eletroforesis (6, 11) with some modifications. The best results were obtained by CTAB method which was performed as follows. *Bacillus* sp. IS13 was inoculated in LB medium containing 50 mg/l of 4,5,6-TCG and grown overnight. Bacterial cells were centrifuged at 8000 g by 2 minutes. The supernatant was discharged and the pellet ressuspended in 0.2 ml of STET buffer. The suspension was incubated at room temperature for 5 minutes after addition of 4 µl of lysozyme (50 mg/ml). Tubes were boiled for 45 seconds and centrifuged for 10 minutes. A toothpick was used to remove lysed cellular debris and 8 µl of CTAB (cetyl trimethyl ammonium bromine 5%w/v) (Sigma Co.) was added before centrifuging at room temperature for 5 minutes. The pellet was ressuspended in 0.3 ml of NaCl 1.2 M and ressuspended with a micro pipette. 2 µl of RNAase (20 mg/ml) and 2 µl of Proteinase K (20 mg/ml) were added and incubated at room temperature during 20 minutes. Next, samples were incubated at 65°C for 90 minutes and cooled again to room temperature. One or two extractions with 1:1 phenol-chloroform (phenol pH 8.0 / chloroform v/v) were carried out. The solution was re-precipitated by centrifuging by 10 minutes after addition of 0.75 ml of ethanol. The pellet was rinsed with 70 % ethanol, dried under vacuum and ressuspended in 20 µl of distilled deionized water.

RESULTS AND DISCUSSION

4,5,6-TCG Consumption

The *Bacillus* sp. IS13 was isolated from cellulose pulp mill effluent while *Bacillus subtilis* MC1 (control bacterium) came from nosocomial samples. These bacteria share very similar morphology, cell wall and growth characteristics. Both *Bacillus* sp., strain IS13 and *B. subtilis* MC1, were cultured in LB medium supplemented with 50 mg/l of 4,5,6-TCG, but only *Bacillus* IS13 was able to degrade this chemical. The biomass concentration reached in such medium was 1.7×10^8 (*Bacillus* sp. IS13) and 7.9×10^7 CFU/ml (*B. subtilis* MC1) after 24 hours of culture. The concentration of 4,5,6-TCG in LB medium remained unaltered in the control without bacterial cells after 7 days of incubation at 37°C (Fig. 2). The bacterium control (*B. subtilis* MC1) did not show the same behavior of the isolated (*Bacillus* sp. IS13) when cultured with 4,5,6-TCG. Since 4,5,6-TCG concentration was not modified by cellular absorption (no 4,5,6-TCG was found in lysed cells), either cellular adsorption (no 4,5,6-TCG was found after vigorous cellular agitation) or evaporation, the high decrease of the 4,5,6-TCG concentration was interpreted as being a consequence of *Bacillus* sp. IS13 metabolism. The experimental results

demonstrated that 50 mg/ml of 4,5,6-TCG do not sustain perceptive biomass increament (results not shown) as a unique carbon source. Based on culture analysis, the microorganism *Bacillus sp.* IS13 showed the capacity to degrade near all 4,5,6-TCG by co-metabolism with LB medium components from a culture with 50 mg/l in less than 12 hours as demonstrated by spectrophotometry at 296 nm (Fig. 1) and by gas chromatography (Fig. 2). Alkaline and acid extractions (14) were performed to identify biodegradation by-products as anysoles, veratroles, catechols and seringols. After gas chromatography analysis no by-products were detected, suggesting chemical instability or rapid biodegradation of such compounds. Similar results were obtained by Häggblom *et al.* (10) in the biodegradation of 3,4,6/3,5,6-TCG and 4,5,6-TCG by *Rhodococcus* sp. and

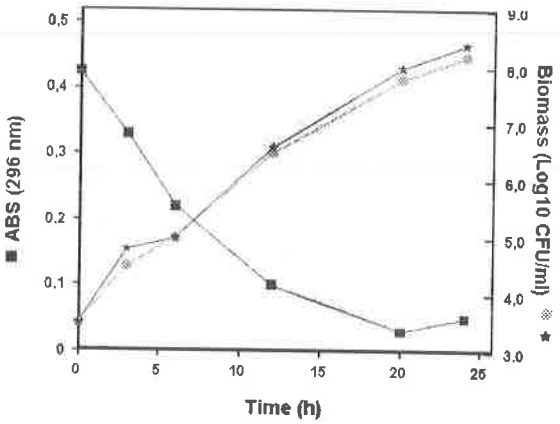

Figure 1. Spectrophotometry analysis of the decrease 4,5,6-TCG (■) and biomass increase (●) by *Bacillus sp.* IS 13 in pure LB (★) and LB +50 mg/l of 4,5,6-TCG. ABS (296 nm) of 0.4=50 mg/l of 4,5,6-TCG.

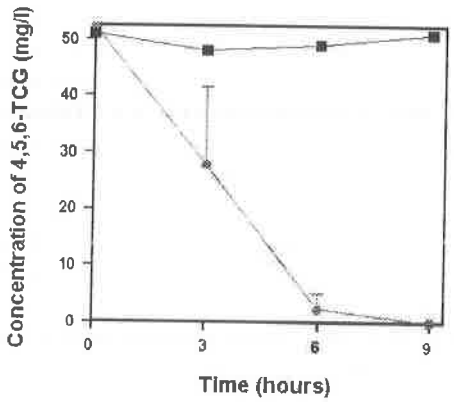

Figure 2. (●) Gas chromatography analysis of the decrease of 4,5,6-TCG by culture of *Bacillus sp.* strain IS 13 in LB +50 mg/l of 4,5,6-TCG. After 9 hours, 4,5,6-TCG were not detected. (■) Control without bacteria.

Mycobacterium sp. In their work, they were also unable to detect metabolites from TCG biodegradation assays. Dichloroanysoles and dichlroveratroles have been reported as by-products of the biodegradation of chlorinated phenols and guaiacols, respectively (15). The difficulty to recovery such metabolites could be explained by volatility (9) or transformation in less toxic and rapid biodegradable compounds. The 3,4,6/3,5,6-TCG and 4,5,6-TCG biodegradation rates in such work were approximately 2.27 mg/48h. In other experiments of the same authors (10), 3,4,6/3,5,6-TCG was biodegradated at levels approximately of 0.0006 mg/h. Neilson *et al.* (13) described an anaerobic consortium that could reduce 100 µg/l of 4,5,6-TCG in 24 hours of culture. Allard *et al.* (1), working with the biotransformation of di- and trichlorocatechols by an anaerobic bacteria consortium, demonstrated reductions ranging from 60 to 100 µg/l of these compounds in culture periods varying from 8 to 50 days. Somewhat higher degrees of biotransformation were observed for *Rhodococcus* sp. and *Acinetobacter* sp. which were able to biotransform about 100 µg/l in 10 hours of 2,6-dibromophenol by O-methylation. Results in the present work contrast with several previous researches by the fact that *Bacillus* sp. IS13 was able to decreases much higher concentrations (50 mg/l) of 4,5,6-TCG, in a short period of time (less than 12 hours). Such span of time corresponds to the exponential growth phase of the tested bacterium (Fig. 1) and suggests a correlation between biomass increament and the decrease of 4,5,6-TCG. Bacterial control (*Bacillus subtilis*) was able to survive but not to reduce the 4,5,6-TCG concentration under same culture conditions. Some other researchers have been identifying very efficient microorganisms, which were able to biodegrade chloro aromatic compounds, others than 4,5,6-TCG. Zaitsev *et al.* (23) isolated a strain of *Rhodococcus opacus* GM-14 that utilizes 4-chlorophenol and 3-chlorophenol at concentrations of 250 mg/l and 100 mg/l respectively. Wu *et al.* (22) studied an anaerobic consortium isolated from sludge granules able to degrade 40 to 60 mg/l of pentachlorophenol per day. Although different bacteria have been identified as related to biodegradation, the genus *Bacillus* is one of the most frequently. Such genus is known to possess a very efficient capacity to adapt itself to new environments due to its broad range of enzymatic metabolism (18). Such metabolic versatility can be utilized to break down the structure of toxic molecules allowing

microbial survival even in the presence of uncommon compounds. Moreover, it is known that the higher capability of degradation showed by *Bacillus* sp. IS13 could be induced by the long exposure of these microorganisms to effluents containing many different kinds of organochlorinated compounds. Such selective pressure could have activated genetic routes responsible for transformation of 4,5,6-TCG.

Adsorption and absorption tests

The experiments demonstrated that transformation of 4,5,6-TCG was related with biomass increment supported by growth on LB medium, being the decrease of the organochlorinated compound due to co-metabolism. During biomass increment, detection of 4,5,6-TCG biodegradation by-products were not observed, neither by scanning spectrophotometry nor by gas chromatography. This fact lead to the verification of the possibility of 4,5,6-TCG adsorption or absorption by cells, instead of being metabolized Fig. 3, shows the amount of 4,5,6-TCG after several cycles of incubations and different extractions methods. Very low concentration of 4,5,6-TCG was found at intact and lysated cells after 24 hours of incubation, demonstrating no absorption of the 4,5,6-TCG. To verify adsorption possibility, vigorous agitation was applied during extraction, showing only traces of 4,5,6-TCG after the same time of incubation. Spectrophotometer analysis of pure hexane (without 4,5,6-TCG) demonstrated same values for recovered cultures after 24 hours of incubation. Such results suggest that there were no significative of 4,5,6-TCG absorbed or adsorbed on the cells, which could be interpreted as a false positive biodegradation result. Gas chromatography confirmed spectrophotometry results showing the same behavior for *Bacillus* sp. IS13 cultures growing in LB medium supplemented with 4,5,6-TCG.

DNA plasmid extraction

Once *Bacillus* sp. IS13 was characterized as a potencial bacterium for bioremediation processes, it was decided to search for DNA plasmids that could contain genes responsible for biodegradative activity. DNA extractions were attempt more than 30 times by six different methodologies. Performing the CTAB method (7), it was possible to identify two plasmids from *Pseudomonas* sp. (results not shown) and also to extract DNA from *Bacillus* sp. strain IS13. The results showed that this strain seems to lack any plasmidial DNA structures. The absence of plasmids

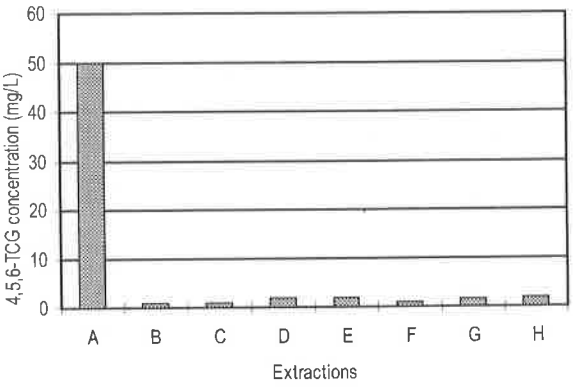

Figure 3. Absorbance at 296 nm relative to 4,5,6- TCG concentration in LB medium. A) Standard extraction of culture of *Bacillus* IS13 at 0h. B) Standard extraction of culture of *Bacillus* IS 13 after 24h of incubation. C) Vigorous extraction of culture of *Bacillus* IS13 after 24h of incubation. D) Cellular phase of culture of *Bacillus* IS13 after 24h of incubation. E) Liquid phase of culture of *Bacillus* IS13 after 24h of incubation. F) SDS lysis after 24h of incubation. G) Lysozyme lysis after 24h of incubation. H) Hexane X hexane (without 4,5,6-TCG).

suggests that the biodegradation capacity of *Bacillus* sp. strain IS13 is chromosome-encoded. The chromosomic presence of genes able to transform organochlorinated compounds can be explained by the necessity of the bacterial cells to detoxify its surrounding environment in order to survive. Toxic xenobiotic and, especially, toxic natural compounds were frequently present in nature with microorganisms during evolution. This fact has always imposed a selective pressure, justifying the presence of stable biodegrading routes in the bacterial metabolism. The biodegradation potential of *Bacillus* sp. IS13 must be further verified to allow its possible utilization in bioremediation of polluted effluents and locations.

CONCLUSIONS

Bacillus sp. strain IS13 is a highly adapted bacterium with great potential to biodegrade 4,5,6-TCG and possibly other related chlorinated compounds. Such microorganism can be further studied to be utilized in the industrial effluent treatment and decontamination of natural areas. Results in this work also suggest that the genes responsible for 4,5,6-TCG biodegradation may be located in the bacterial chromosome. Such characteristics could be interesting to industrial effluent treatment due to biodegradation active stability.

RESUMO

Altos níveis de biodegradação do 4,5,6-tricloroguaiacol por *Bacillus* sp. isolado de efluente de indústria de polpa de celulose

Isolou-se uma bactéria gram positiva, esporulada a partir de efluente de fábrica de polpa de celulose. Esse microrganismo, identificado como *Bacillus* sp. e nomeado IS13, foi capaz de degradar rapidamente o composto orgânico clorado 4,5,6-tricloroguaiacol (4,5,6-TCG) presente em meio de cultura a uma concentração de 50mg/L. Essa concentração equivale a $3x10^4$ vezes mais 4,5,6-TCG que a concentração encontrada no efluente original. A biodegradação desse composto foi analisada por espectrofotometria de varredura e cromatografia gasosa. A falta de sub-produtos de degradação sugeriu a verificação da possibilidade de adsorção e absorção celular do 4,5,6-TCG ao invés de biodegradação propriamente dita. Não foram encontrados traços de 4,5,6-TCG após lise celular com lisozima e SDS e não houve desprendimento desse composto após agitação vigorosa. Logo, o desaparecimento do 4,5,6-TCG do meio de cultura analisado foi interpretado como biodegradação devido ao metabolismo do *Bacillus* sp. IS13. A partir desse microrganismo, buscou-se isolar plasmídeos utilizando diferentes protocolos. Os melhores resultados foram obtidos através do método do CTAB, porém não encontraram-se plasmídeos no isolado IS13. Os resultados sugerem que a alta taxa de degradação do 4,5,6-TCG é mediada por genes presentes no cromossomo bacteriano. A importância desse trabalho encontra-se na possibilidade de utilização do *Bacillus* sp. IS13 como inóculo em plantas de efluentes industriais, a fim de biodegradar compostos orgânicos clorados presentes nesses locais.

Palavras-chave: biodegradação, 4,5,6-tricloroguaiacol, bactéria aeróbia, DNA.

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Revista de Microbiologia (1998) 29:272-275
ISSN 0001-3714

DRUG RESISTANCE OF YEASTS ISOLATED FROM OROPHARYNGEAL CANDIDIASIS IN AIDS PATIENTS

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Submitted: September 02, 1997; Returned to authors for corrections: April 17, 1998; Approved: September 09, 1998

ABSTRACT

Candida spp was isolated from 59 (68.60%) out of eighty six samples of oral mucosa of AIDS patients. The identification, based or the production of a germ tube and chlamydospores, and on the assimilation and fermentation of carbohydrates, revealed 52 strains (88.13%) of *C. albicans*, 4 (6.77%) of *C. tropicalis* and 3 (5.08%) of *C. krusei*. The susceptibility of these strains to amphotericin B, flucytosine, itraconazole, fluconazole and ketoconazole was determined using the agar dilution method. Comparing the minimum inhibitory concentration values found in the susceptibility test with the serum levels achieved by these drugs, only 8.47% and 5.08% of the yeasts strains proved to be resistant to amphotericin B and flucitosyne, respectively. A high frequency of strains resistant to azole derivatives (25.42%, to itraconazole, 45.76%, to ketoconazole and 66.10% to fluconazole) was observed.

Key words: *Oropharyngeal candidiasis*, yeasts, *in vitro* resistance, antifungal drugs.

INTRODUCTION

Oral candidiasis is the most ordinary fungal infection in human immunodeficiency virus (HIV) positive patients. It is considered of high prognosis value in the development of AIDS (6,7). *Candida albicans* is the most frequent species. However other species, such as *C. tropicalis*, *C.glabrata*, *C.krusei* and *C. parapsilosis,* have been also identified in the oropharyngeal cavity (20).

The development of resistance to antifungal agents, observed in previously susceptible yeast isolates, as well as recurrence of oropharyngeal candidiasis following treatment with different antifungal drugs, are highly relevant factors which should be taken into account to determine the use of *in vitro* susceptibility tests in the microbiology laboratory routine (9,17).

Dilution tests in solid and liquid media, for determination of the minimum inhibitory concentration (MIC) of antifungal drugs against yeasts, have been proposed by Alves and Cury (1).

The aim of this work was to determine the resistance of 59 strains of *Candida spp.*, isolated from the injured oral mucosa of AIDS patients to amphothericin B, flucytosine, fluconazole, ketoconazole and itraconazole.

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MATERIALS AND METHODS

Samples

Samples were collected from the oral mucosa of 86 AIDS patients treated at the Tropical Diseases Hospital, Goiânia, Goiás State, Brazil. Samples were maintained up to one year on Sabouraud dextrose agar kept at 25°C.

Microorganisms

The identification of yeast isolates was carried out through the production of germ tubes and chlamydospores, as well as through carbohydrates assimilation and fermentation tests, according to Kreger van Rij (12). Fifty nine isolates were included in this study.

In vitro *susceptibility tests*

Each isolate was tested by the dilution method in buffered Yeast Nitrogen Base (YNB) agar, using five antifungal drugs: amphotericin B (Squibb), fnucytosine (Roche), fluconazole (Pfizer), ketoconazole (Janssen) and itraconazole (Janssen).

Dilution method in agar. Amphotericin B (AmB) and flucytosine were dissolved in 1.0 ml distilled sterilized water whereas the azoles were dissolved in 1.0 ml dimethylsulfoxide (DMSO), to a final concentration of a 25600 µg/ml. Solutions were diluted 1:20 in broth (YNB-6.7%, L-asparagine - 1.5% and phosphate buffer-0.01M-pH-7.0) and from this, two-fold dilutions were carried out in the same broth. Each dilution was mixed with YNB agar in order to get an antifungal range from 128µg/ml to 0.25 µg/ml (1,13).

Inoculum and MIC. Prior to testing, each isolate was grown at least twice on Sabouraud dextrose agar to ensure optimal growth. Colonies were suspended in 5 ml physiological solution, vortexed for 15 s, and the cell density was adjusted to a 0.5 McFarland standard, using a spectrophotometer at 530nm (16). Three µl of this suspension were transfered to plates containing the medium and the drugs in different concentrations. A control plate, free of drugs, was included. The MIC was determined as the lowest concentration in which there was no cell growth.

Determination of resistance

Candida isolates were classfied as resistant or susceptible, according to the MIC values when compared to serum levels achieved by amphotericin B, flucytosine, fluconazole, ketoconazole e itraconazole (2,19). Strains with following MIC results were considered resistant: >8.0µg/ml for ketoconazole, itraconazole and fluconazole; >64µg/ml for flucytosine and >µ2g/ml for amphotericin B.

RESULTS

From the 86 samples collected from the oropharyngeal mucosa of AIDS patients bearing whitened lesions, 59 *Candida* spp strains were isolated, including, revealing 52 strains (88.13%) of *C. albicans*, 4 (6.77%) of *C. tropicalis* and 3 (5.08%) of *C. krusei*.

The MIC_{50} (inhibition of 50% of grown) and MIC_{90} (inhibition of 90% of grown) values for each drug are displayed in Table 1.

Table 1. Minimum inhibitory concentration of five antifungal agents, against 59 clinical isolates of *Candida* species[a], determined in YNB agar medium.

Antifungal Agent	*Candida* species	MIC (µg/ml) Range	50[b]	90[b]
Itraconazole	*C. albicans* (n=52)	0.25-64	8.0	64
	C.tropicalis (n=04)	0.25-8.0	8.0	8.0
	C.krusei (n=03)	0.5-8.0	8.0	8.0
Fluconazole	*C.albicans* (n=52)	0.25->128	>128	>128
	C.tropicalis (n=04)	1.0->128	>128	>128
	C.krusei (n=03)	>128	>128	>128
Ketoconazole	*C.albicans* (n=52)	0.25-128	16	128
	C.tropicalis (n=04)	0.25-128	8.0	128
	C.krusei (n=03)	0.25-128	16	128
Flucytosine	*C.albicans* (n=52)	0.25->128	0.50	4.0
	C.tropicalis (n=04)	0.25->128	128	>128
	C.Krusei (n=03)	0.25-16	16	16
Amphotericin B	*C.albicans* (n=52)	0.50-4.0	1.0	2.0
	C.tropicalis (n=04)	0.50-2.0	2.0	2.0
	C.krusei (n=03)	1.0-2.0	2.0	2.0

[a] All isolates were tested in triplicate
[b] MICs encompassing 50 and 90% of all isolates tested

Comparing the MIC values of each drug, it was observed that 25.42% and 45.76% of the isolates were resistant to itraconazole and ketoconazole, respectively. The highest resistance was observed for fluconazole, i.e., 66.10%. All the isolates of *C. krusei* were resistant to fluconazole, with MIC>128µg/ml, while 75% of *C. tropicalis* isolates were resistant to this drug. A high susceptibility of *Candida albicans* and *C. krusei* isolates to AmB and flucytosine was recorded, which presented MIC < 2 and MIC <64, respectively. Among the isolates of *C. tropicalis*, 75%

were resistant to flucytosine. The frequency of yeast isolates that were resistant to the antifungal drugs tested is shown in Fig.1.

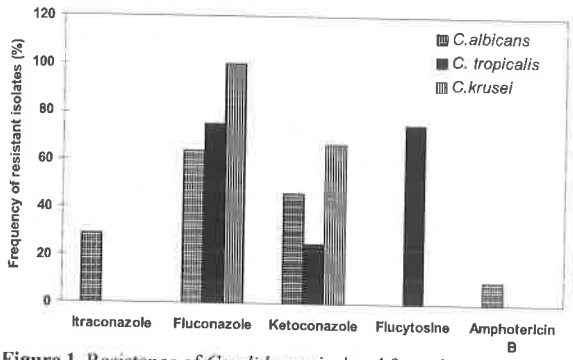

Figure 1. Resistance of *Candida spp* isolated from the oral mucosa of AIDS patients to antifungal agents.

DISCUSSION

Identification of 52 *Candida albicans* isolates (88.13%), out of the 59 yeasts isolated from the oral mucosa in AIDS patients, showed the prevalence of this species among *Candida sp. C. albicans* can be often found either in healthy individuals or in HIV positive asymptomatic patients (4).

Oropharyngeal candidiasis, diagnosed in more than 75% of AIDS patients, leads to some discomfort in food chewing, causing a stress in the immunological state of the host, due to bad nutrition (7). Diagnosis, followed by efficient treatment is necessary and the *in vitro* susceptibility tests are important for a correct therapy. Oropharyngeal candidiasis in AIDS patients has proved to be difficult to treat (9).

In this study, most of the *C. albicans* and *C. krusei* isolates presented low MIC values for AmB and flucytosine however, 75% *C. tropicalis* isolates were found to be resistant to flucytosine. Rodero *et al* (18) noted that 82% yeasts, isolated from the oropharyngeal mucosa in HIV positive patients, were susceptible to flucytosine, when a resistance level higher than 12.5µg/ml was considered. Bonifácio e Souza *et al* (5) observed inhibition of growth of *C. albicans* isolated from clinical materials by 2 µg/ml AmB. The situation seems to be different in AIDS patients. An increased frequency of *C. albiccns* serotype B in this type of patients was observed and this may correlate with a higher incidence of resistant strains (8). The increased resistance of *C. tropicalis* isolates to flucytosine observed in this study can be related to strains less sensitive to the drug during AIDS.

Azole derivatives, however, presented different results. High resistance was observed to fluconazole and ketoconazole, while only 25.42 % of the isolates were resistant to itraconazole. All *C. krusei* isolates were resistant to fluconazole, and 75% of *C. tropicalis* were resistant to this drug and to ketoconazole. Gallagher *et al.* (9)observed that 54% of the *C. albicans* isolates from HIV$^+$ patients exhibited lower susceptibility to ketoconazole. Resistance to fluconazole in *C. albicans* appear to be a less common phenomenon than in other *Candida* species. Comparative *in vitro* studies have consistently shown that *C. krusei* strains show notably higher fluconazole MICs than *C. albicans* (15). *C. krusei* exhibits innate resistance to fluconazole, and fluconazole prophylaxis has been associated with an increased incidence of *C. krusei* infection in some centers (20).

Studies on the genotypic characterization of *C. albicans* have demonstrated that the recurrent oral candidiasis, mainly in AIDS patients, is caused by the emergence of fluconazole resistant strains (3). Recurrences after treatment with azole derivatives, mainly fluconazole, in HIV patients have been explained by the persistence of *Candida* colonies in the lesion area after the clinical cure, or by a fungistatic but not fungicidal action of the drug (10,14).

The mechanism of resistance to azoles is related to their mode of action (11,14). Azoles are inhibitors of P450 cytochrome dependent on C14 demethylase, an important enzyme which synthesizes the ergosterol of the fungal cell. Resistance of yeasts to these drugs may be due to alterations in the C14 demethylase or to a lower capacity of the azoles to bind to P450 cytochrome oxidase.

Considering the emergence of isolates resistant to antifungal drugs, particularly to fluconazole, *in vitro* susceptibility tests, based on the MIC values, are necessary for the choice of the appropriate therapy.

RESUMO

Resistência à drogas de leveduras isoladas de candidíase orofaríngea em pacientes com AIDS

Entre oitenta e seis amostras da mucosa oral de pacientes com AIDS, 59 (68,60%) foram positivas para leveduras do gênero *Candida*. A identificação, feita pela produção de tubo germinativo e clamidósporos e através de assimilação e fermentação de hidratos de carbono, revelou 52 cepas (88,13%) de *C.albicans*, 4 (6,77%) de *C. tropicalis* e 3 (5,08%) de *C.krusei*. Avaliação destas leveduras para

susceptibilidade *in vitro* frente a anfotericina B, flucitosina, itraconazol, fluconazol e cetoconazol, foi realizada pelo método de diluição em ágar. Comparando-se os valores de concentração inibitória mínima encontrados com os níveis séricos alcançados por estes antifúngicos verificou-se que apenas 8,47% e 5,08% das 59 leveduras foram resistentes a anfotericina B e flucitosina, respectivamente. Foi registrada uma percentagem de cepas resistentes aos derivados azólicos, sendo 25,42% ao itraconazol, 45,76% ao cetoconazol e 66,10% ao fluconazol.

Palavras-chave: candidíase orofaríngea, leveduras, resistência *in vitro*, antifúngicos.

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Revista de Microbiologia (1998) 29:276-281
ISSN 0001-3714

CHARACTERIZATION OF SELECTED STRAINS OF MUCORALES USING FATTY ACID PROFILES

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Submitted: September 16, 1997; Returned to authors for corrections: April 09, 1998;
Approved: September 19, 1998

ABSTRACT

The fatty acid profiles of several fungi of the order Mucorales (Zygomycetes), including *Backusella lamprospora* (Lendner) Benny and R.K. Benj., *Benjaminiella youngii* P.M. Kirk, *Circinella simplex* van Tieghem, *Cunninghamella blakesleeana* Lendner, *Mortierella ramanniana* (Möller) Linnem., *Mucor circinelloides* f. *janssenii* (Lendner) Schipper, *Mycotypha microspora* Fenner, *Rhizomucor miehei* (Cooney and R. Emerson) Schipper and *Rhizomucor pusillus* (Lindt) Schipper, and of *Volutella* sp. Fr., from the class Ascomycetes, were qualitatively analysed by gas-liquid chromatography in order to determine the taxonomic value of these chemotaxonomic markers. The fatty acids present in all strains were palmitic (16:0), oleic (18:1), linoleic (18:2) and γ-linolenic (18:3) acid, with the exception that the latter was not found in *Volutella* sp. Chemotaxonomic markers for some species and genera were obtained, including a non-identified fatty acid, FAME8 (minimum and maximum retention times of 27.92 and 28.28 minutes) for *Rhizomucor miehei* CCT 2236 and *Rhizomucor pusillus* CCT 4133, and FAME3 (minimum and maximum of 16.53 and 16.61 minutes) for *Benjaminiella youngii* CCT 4121. The chemotaxonomic marker of the order Mucorales was the fatty acid 18:3ω6, confirming previous data from literature. The results of the present study suggest that qualitative fatty acid analysis can be an important chemotaxonomic tool for the classification of fungi assigned to the order Mucorales (Zygomycetes).

Key words: taxonomy, chemotaxonomic marker, fatty acids, Mucorales

INTRODUCTION

The order Mucorales, class Zygomycetes, is comprised mainly by saprobic fungi with a cosmopolitan distribution, including some opportunistic parasites of plants or animals (8), and spoilage molds, such as *Rhizopus* spp. The Mucorales have important economical applications, including the production of microbial rennet by *Rhizomucor miehei*, and lipases by *Rhizomucor miehei* and *R. pusillus*, both for dairy industry; fumaric acid for paper industry by *Rhizopus* spp. (7), and fatty acids for pharmaceutical (precursors of prostaglandins) and food industries by *Mortierella* spp. and *Mucor* spp. (18).

The classification and identification of fungi are traditionally based on morphological criteria.

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However, the study of closely related, non-sporulating fungi and of those with unknown life cycle is limited by scarce differential morphological features (2). Biochemical and molecular methods, such as fatty acid profiles, protein and nucleic acid sequencing have been of great value to overcome these limitations (3,23).

Fatty acids are useful taxonomical markers which have been widely used in the classification of several groups of microorganisms (27). However, fatty acid composition has been applied to the characterization of relatively few fungal taxa, including *Ganoderma australe* (15), *Mortierella* spp. (1), phytopathogenic (10,11,12) and endomycorrhizal fungi (6,19).

Reproducible fatty acids profiles can be obtained under rigorous control of growth temperature, incubation time and culture media formulation (4, 14, 18). The early studies applying fatty acids analysis as a taxonomic tool for filamentous fungi were accomplished in the 60's by Shaw (21, 22, 23, 24). This author investigated the presence of γ-linolenic fatty acid in fungi from different classes, delimiting two taxonomic groups on that basis (21); one group included representatives from the class Oomycetes, Zygomycetes and Chytridiomycetes, and the other representatives from the class Ascomycetes and Basidiomycetes. Similar results were observed in later studies (30), where Zygomycetes were characterized by the presence of ω6 series of C18 polyunsaturated fatty acids, in particular γ-linolenic acid.

The classical characterization of Mucorales includes morphological characteristics and cultural features (1,2,6). The objective of this study was to assess the potential of fatty acid profiles in the differentiation of selected species belonging to the order Mucorales (Zygomycetes), focusing mainly on economically important taxa (*Mortierella* spp., *Mucor* spp. and *Rhizomucor* spp.).

MATERIALS AND METHODS

Fungi. *Backusella lamprospora* (CCT 3480; collected in Belém PA, Brazil), *Benjaminiella youngii* (CCT 4121, originally IMI 336111), *Circinella simplex* (CCT 4260; collected from roots of *Cariniana* sp. in the Atlantic forest in Estação Ecológica Juréia-Itatins, Peruíbe SP, Brazil), *Cunninghamella blakesleeana* (CCT 4123, originally IMI 200337), *Mortierella ramanniana* (CCT 4428; collected from *Araucaria angustifolia* root in São Francisco de Paula RS, Brazil), *Mucor circinelloides* f. *janssenii* (CCT 4396), *Mycotypha microspora* (CCT 4126, originally IMI 282443; CCT 4127, originally IMI 108621), *Rhizomucor miehei* (CCT 2236, originally NRRL 3420), *Rhizomucor pusillus* (CCT 4133; isolated from shoyu (soybean sauce) in Campinas SP, Brazil), *Volutella* sp. (CCT 2995; collected from soil under the Atlantic forest in Estação Ecológica Juréia-Itatins, Peruíbe SP, Brazil) were preserved by freeze drying at the Culture Collection Tropical (CCT), Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas SP, Brazil.

Culture methods. Fungal strains were cultivated on Potato Carrot Agar medium (25) for 7 to 10 days at 28°C. Biomass for fatty acid analysis was grown in 250 ml Erlenmeyer flasks containing 100 ml of Saboraud Dextrose Broth (20), seeded with inocula from solid cultures, and incubated at 28°C on a rotatory shaker at 150 rpm for 3 to 4 days. Biomass was harvested by filtration using 8 µm cellulose acetate membranes (Millipore) and lyophilized.

Extraction and analysis of fungal fatty acids. Fatty acids were extracted and derivitised as recommended in the protocols of the MIDI chromatographic system (20), with minor modifications. The extraction procedure included a saponification step using 1.0 ml of NaOH 3,75 M - methanol 50% (1:1, v/v) and 100 mg of powdered dry biomass. The tubes were mixed by vortexing for 5 to 10 s, and heated in a boiling water bath for exactly 5 min. Tubes were further vortexed for 5 to 10 s, returned to the boiling water bath for an additional 25 min, then cooled to room temperature. The saponificate was acidified and methylated by addition of 2.0 ml of HCl 6.0 N (titrated) - 100% methanol (13:11, v/v), followed by vortexing for 5 to 10 s, heating for 10 ±1 min at 80°C, and rapid cooling. The fatty acid methyl esters (FAMEs) were extracted by adding 1.25 ml of hexane - methyl *tert*-butyl ether (1:1, v/v) and gentle mixing by continuous inversion for 10 min. The top phase containing the organic solvent with the extracted FAMEs was removed with a Pasteur pipette and transferred to a new set of tubes. A final washing step was performed by addition of 3.0 ml of NaOH 0.3 M and gentle mixing by continuous inversion for 5 min. Approximately 2/3 of the organic phase were transferred to chromatographic vials, sealed and stored at -20°C until analysis.

Prior to FAMEs analyses, the samples were evaporated under nitrogen and resuspended in 50 µl of hexane. The analyses were carried out in triplicate

in a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and a split/splitless injector using a 30 m x 0.32 mm, 0.25 μm internal diameter, polar Omegawax ™ 320 capillary column (Supelco). The carrier gas was helium at a flow rate of 20 $cm^3 s^{-1}$ and a split-ratio of 40/1. The temperature of the injector was 250°C and that of the flame ionization detector was 250°C. The oven temperature after sample injection (2μl) was 20 min at 160°C, increasing to 200°C at 5°C/min and held at this temperature for 15 min.

The chromatograms were recorded in a Shimadzu Model C-R4A Chromatopac integrator. The identification of individual FAMEs was based on the retention times relative to a mixture of FAMEs standards (Matreya, Inc. n° 4232), including lauric acid (12:0), myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), palmitoleic acid (16:1), margaric acid (17:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), γ-linolenic acid (18:3 ω6), nonadecanoic acid (19:0), arachidic acid (20:0), gadoleic acid (20:1), eicosadienoic acid (20:2), dihomo-γ-linolenic acid (20:3), arachidonic acid (20:4), behenic acid (22:0). The identified and non-identified fatty acids which reproductively amounted up to at least 1% of the total peak area in the samples were used for taxonomic characterization of the strains in a tabular format.

RESULTS AND DISCUSSION

The fatty acid profiles of ten representative strains belonging to the order Mucorales (Zygomycetes), and one asexual representative of the class Ascomycetes, *Volutella* sp., were analysed. One of the Mucorales strains, *Cunninghamella blakesleeana* CCT 4123, was analysed in duplicate in order to evaluate the fatty acid profile variability present in different independent cultures of the same organism. The analyses were carried out in triplicate and the resulting profiles were qualitatively identical, with some variation in the amounts of individual compounds. These results suggest that the standardization of the growth conditions used are limited to the generation of qualitative fatty acid data. Quantitative analysis of fatty acid data would require the analysis of a larger number of replicates and the use of classical and non-parametrical statistics in the analysis of the data.

Out of twenty fatty acids present in the fungi studied, ten could not be identified by comparison with the fatty acid standard mixture used; these were named FAME1 to FAME10 according to their retention times (Table 1). The qualitative fatty acid profiles, including identified and non-identified fatty acids, are presented in Table 2. Palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2) and γ-linolenic acid (18:3) were present in all strains analysed, with the exception that the latter was not present in *Volutella* sp. Pentadecanoic acid (15:0), margaric acid (17:0), nonadecanoic acid (19:0), arachidic acid (20:0), gadoleic acid (20:1), dihomo-γ-linolenic acid (20:3) and arachidonic acid (20:4) were absent in all strains analysed. The remaining fatty acids were discontinuosly distribuited among the strains analysed (Table 2).

Table 1. Retention times of identified and non-identified fatty acids.

Fatty acids*	Common name	Retention times (t_R, minutes) (min – max)
12:0	Lauric acid	3.16 – 3.18
14:0	Myristic acid	5.48 – 5.51
FAME1	ND	10.26 – 10.36
16:0	Palmitic acid	10.70 – 10.81
16:1(9)	Palmitoleic acid	11.67 – 11.76
FAME2	ND	14.68 – 14.80
FAME3	ND	16.53 – 16.61
FAME4	ND	21.13 – 21.26
18:0	Stearic acid	21.98 – 22.04
18:1(9)	Oleic acid	22.76 – 22.96
FAME5	ND	24.25 – 24.35
18:2(9,12)	Linoleic acid	24.79 – 24.91
FAME6	ND	25.75 – 25.86
18:3(6,9,12)	γ-Linolenic acid	25.91 – 26.02
FAME7	ND	27.13 – 27.24
FAME8	ND	27.92 – 28.28
FAME9	ND	28.89 – 28.98
20:2(11,14)	Icosadienoic acid	32.32 – 32.44
22:0	Behenic acid	32.74 – 33.16
FAME10	ND	36.72 – 36.90

*FAMES 1 to 10: non-identified fatty acids. ND: not determined.

The fatty acid profiles obtained were, in most of the cases, comparable to data derived from the literature. The fatty acid profile of *Mucor circinelloides* f. *janssenii* CCT 4396 was identical to that of *Mucor circinelloides* CBS 478.70, identified by Westhuizen *et al.* (30), except for the presence of 20:2 in strain CCT 4396. Strains of *Rhizomucor miehei* CCT 2236 showed the same fatty

Table 2. Fatty acid profiles of selected fungi from the order Mucorales and the class Ascomycetes.

Strains	Fatty acids (relative proportional average, in %)																		
	12:0	14:0	FAME1	16:0	16:1	FAME2	FAME3	FAME4	18:0	18:1	FAME5	18:2	FAME6	18:3	FAME7	FAME8	FAME9	20:2	22:0	FAME10
B. lam [1]	3.6	4.05	1.7	17.65	1.4	2.5		3.7	2.4	14.65		11.3		18.75	2.6		4.6	3.7	3.85	3.4
B. you [2]	2	4.3		4.7			2.7			28.7	4.2	19.8		30.5			2.9			
C. bl A_1 [3]			3.1	10.3		4.4		5.9		8		9.8	9.2	11.7	2.8		14.8	10.6		9.3
C. bl A_2 [4]			2.8	12.5		4.05		4.9		10.7		15.2	7.6	15.15	4		9.05	7.15		6.85
C. sim [5]	3.6	4		21	1.7	2.4		3.2	8.2	18.2		6.8	4.9	5.7	2.2		6.1	6.7		5.2
M. ram [6]		3.55		24.6	1.15	1.8		2.2	2.15	33.15		6.85	3.25	7.45	2.55		5.25	3.2		2.7
M. cir [7]		4.2		12	11.1			2		18.3	10	6.1		20.8	2		6.2	4.5		2.6
M. mi A [8]	2.05	4.2	1.9	18.25	3.6	2.85		3.55		17.3		17.5	5.05	5.45	2.15		6.05	5.45		4.55
M. mi B [9]	5.7	4.2	3.3	10.1	4.2	4.7		6		8.7		9	8.6	6.6	4.4		9.5	8		6.9
R. mie [10]			3.45	5.7	1.45	4.35		5.65		7.15		7.55	8.1	5.8	4.1	7.3	10	8.1	13.75	7.2
R. pus [11]			1.6	14.75	1.55	2.25		2.9	2.6	14.15		14.3	5.85	3.25	3	7.75	4.75	4.55	12.2	4.3
Volu [12]				28.3					8.3	41.9		18.5			3					

[1] *Backusella lamprospora*; [2] *Benjaminiella youngii*; [3] *Circinella simplex*; [4] *Cunninghamella blakesleeana* A_1; [5] *Cunninghamella blakesleeana* A_2; [6] *Mortierella ramanniana*; [7] *Mucor circinelloides* f. *janssenii*; [8] *Mycotypha microspora* A; [9] *Mycotypha microspora* B; [10] *Rhizomucor miehei*; [11] *Rhizomucor pusillus*; [12] *Volutella* sp.

acid profile as the strains analysed by Sumner and Morgan (26) and Westhuizen *et al.* (30). The 16:0, 16:1, 18:1, 18:2 and 18:3ω6. The 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3ω6 fatty acids present in *Rhizomucor pusillus* CCT 4133 had already been verified in other *Rhizomucor pusillus* strains by Sumner and Morgan (26), Mumma *et al.* (17) and Westhuizen *et al.* (30).

Rhizomucor pusillus CCT 4133 and *Rhizomucor miehei* CCT 2236 could be differentiated on the basis of their fatty acid profiles by the absence of 18:0 in the latter. The non-identified FAME8 was restricted to these two species only, and thus could be considered as a chemotaxonomic marker for the genus *Rhizomucor*.

Backusella lamprospora CCT 3480 was characterized by a fatty acid profile including all the identified fatty acids present in the other fungi. The presence of 22:0 was restricted to this fungus and to the strains of *Rhizomucor* analysed.

Amano *et al.* (1) differentiated representatives of the subgenus Micromucor and Mortierella by the occurrence of polyunsaturated C20 fatty acids in the latter group. The C20 polyunsaturated fatty acids were characteristically absent in the strains of *Mortierella ramanniana* var. *ramanniana*, which belong to the subgenus Micromucor. The fatty acid profile of *Mortierella ramanniana* var. *ramanniana* was composed by 16:0, 18:0, 18:1, 18:2 and 18:3ω6 (1). In a later study, Westhuizen *et al.* (30) identified 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3ω6, 20:0, 20:1, 20:2 and 20:4 in *Mortierella ramanniana* var. *ramanniana* CBS 243.58. Nevertheless, the presence of fatty acids from the C20 series in strain CBS 243.58 may have been influenced by growth temperatures used in the study. CBS 243.58 was grown at 21°C, whereas strains analysed by Amano *et al.* (1) were grown at 28°C. *Mortierella ramanniana* CCT 4428 was also grown at 28°C and presented a similar composition of identified fatty acids to the CBS 243.58 strain, except by the occurrence of small amounts of 20:2 (approx. 3.2% of relative proportional average) in the former strain. The discrepant results from the different studies indicate that synthesis of C20 fatty acids by *Mortierella ramanniana* is temperature dependent.

A smaller number of fatty acids were detected in *Benjaminiella youngii* CCT 4121. FAME3 (Table 2) was found exclusively in this strain, suggesting that this fatty acid may be a potential chemotaxonomic marker for *Benjaminiella youngii*.

The fatty acid profile of *Mycotypha microspora*

CCT 4126 (A) was identical to that of *Mycotypha microspora* CCT 4127 (B), showing that there are no differences between strains of the same species. Similarly, the fatty acid profiles of *Cunninghamella blakesleeana* CCT 4123, derived from duplicate cultures, showed no significant difference, demonstrating the reproducibility of the method. The presence of the fatty acids 16:0, 18:1, 18:2 and 18:3 in the *Cunninghamella blakesleeana* CCT 4123 was previously observed by Shaw (22) in *Cunninghamella blakesleeana* IMI 6387.

One species of the genus *Volutella,* included in this study as a reference organism different from the studied Zygomycetes, showed a profile with the smallest number of fatty acids. This organism was the only one that did not present the fatty acid 18:3(ω6), g-linolenic acid, characteristic of the Zygomycetes fungi, in accordance with earlier reports (14,16,30).

In general, the major Ascomycetes and Basidiomycetes fatty acids are 16:0, 18:1 and 18:2(ω6) (9,10,14,15). Ascomycetes and their asexual representatives can be characterized by the presence of 18:3(ω3), which does not occur in Zygomycetes, or by a profile composed of up to 18:2(ω6) fatty acid (13,23,30). From the identified fatty acids of *Volutella* sp. CCT 2995 it can be observed that, as a member of Ascomycetes, this strain is characterized by the absence of 18:3(ω6) and the presence of 18:2(ω6), corroborating the previous investigations.

According to Amano *et al.* (1), the fatty acids can provide valuable information to establish an accurate taxonomic system for fungi. However, the fatty acid profiles, as any other taxonomic character, should not be considered isolatedly. In combination with taxonomic information from different sources, including morphological, molecular and biochemical data, the analysis of fatty acid profiles can be considered a valuable tool for the polyphasic taxonomy of fungi (5,13,28,29).

The results of the present study suggest that qualitative fatty acid analysis can be an important chemotaxonomic tool for the classification of fungi assigned to the order Mucorales (Zygomycetes). Fatty acid profiles allowed the differentiation of closely related species assigned to the same genus (*Rhizomucor miehei* CCT 2236 and *Rhizomucor pusillus* CCT 4133) whereas strains from the same species (*Mycotypha microspora* CCT 4126 and 4127), as well as duplicate cultures of the same organism (*Cunninghamella blakesleeana* CCT 4123), showed identical profiles.

Chemotaxonomic markers that could be potentially used for the identification at species and genus were also found. A non-identified fatty acid, FAME8, was only detected in the strains of the genus *Rhizomucor,* whereas non-identified fatty acid FAME3 was characteristic for *Benjaminiella youngii*. The fatty acid 18:3ω6 was confirmed as chemotaxonomic marker for strains belonging to the order Mucorales studied in the present investigation.

ACKNOWLEDGMENT

MS was supported by a M.Sc. grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We would like to thank Dr. Tania Akiko Anazawa, from the Food Science Department of FEA/UNICAMP, for valuable assistance with chromatographic analyses. The authors are also indebted to Dr. Ludwig H. Pfenning for suggestions and guidance.

RESUMO

Caracterização de linhagens de Mucorales através do perfil de ácidos graxos

O perfil de ácidos graxos de *Backusella lamprospora* (Lendner) Benny e R.K. Benj., *Benjaminiella youngii* P.M. Kirk, *Circinella simplex* van Tieghem, *Cunninghamella blakesleeana* Lendner, *Mortierella ramanniana* (Möller) Linnem., *Mucor circinelloides* f. *janssenii* (Lendner) Schipper, *Mycotypha microspora* Fenner, *Rhizomucor miehei* (Cooney e R. Emerson) Schipper e *Rhizomucor pusillus* (Lindt) Schipper, da ordem Mucorales (Zygomycetes), e *Volutella* sp. Fr., da classe Ascomycetes, foram analisados qualitativamente por cromatografia gás-líquida, tendo como objetivo determinar o valor taxonômico destes marcadores quimiotaxonômicos. Os ácidos palmítico (16:0), oléico (18:1), linoléico (18:2) e γ-linolênico (18:3) foram encontrados em todas as linhagens, com exceção do último, o qual não foi encontrado na linhagem de *Volutella* analisada. Foram obtidos marcadores quimiotaxonômicos para algumas espécies e gêneros estudados, incluindo um ácido graxo não-identificado, FAME8 (tempos de retenção mínimo e máximo de 27,92 e 28,28 minutos) para *Rhizomucor miehei* CCT 2236 e *Rhizomucor pusillus* CCT 4133 e FAME3 (tempos de retenção mínimo e

máximo de 16,53 e 16,61 minutos) para *Benjaminiella youngii* CCT 4121. Para a ordem Mucorales, o marcador quimiotaxonômico obtido foi o ácido graxo 18:3ω6, confirmando dados da literatura. Os resultados do presente estudo sugerem que a análise qualitativa do perfil de ácidos graxos pode ser uma ferramenta importante na classificação de fungos da ordem Mucorales (Zygomycetes).

Palavras-chave: taxonomia, marcador quimiotaxonômico, ácidos graxos, Mucorales.

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Revista de Microbiologia (1998) 29:282-285
ISSN 0001-3714

INTRA AND EXTRACELLULAR NUCLEASE PRODUCTION BY *ASPERGILLUS NIGER* AND *ASPERGILLUS NIDULANS*[1]

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Submitted: September 30, 1997; Returned to authors for corrections: February 17, 1998; Approved: July 23, 1998

SHORT COMMUNICATION

ABSTRACT

Intra and extracellular nuclease production by strains of *Aspergillus niger* and *Aspergillus nidulans* was estimated using a modified DNAse test agar and cell-free extract assays. Differences in the production of nucleases by *A. niger* and *A. nidulans* were observed. These observations suggest that the DNAse test agar can be helpful for a quick screening for some types of nucleases in filamentous fungi. The assays using cell-free extracts can also be useful for initial characterization of other types of nucleases.

Key words: Nucleases, *Aspergillus niger, Aspergillus nidulans,* filamentous fungi

Nucleolytic enzymes have several important biological roles, such as nutrition (pancreatic enzymes), protection (restriction enzymes), repair and recombination mechanisms or DNA transport. Nucleases can be inconvenient when genetic manipulation techniques are used. The frequency of genetic transformation can decrease dramatically if the vector is degraded by nucleases. Mink *et al.* (7) observed an increase in transformation efficiency of *Saccharomyces cerevisiae* and *Aspergillus nidulans* when protoplasts were obtained with an endonuclease-free enzyme preparation. Transient gene expression in rice tissues was also increased when plantules were exhaustively washed before the vector was added (2). Many nuclease inhibitors such as heparin (11) and spermidine (2) have been used in genetic transformation of fungi and other organisms.

Aspergillus niger and *A. nidulans*, despite being considered taxonomically closely related, show very different transformation frequencies in heterologous and in homologous systems. As an example, transformation of *A. nidulans* with the homologous *oli*C 31 gene resulted in frequencies of 10 to 70 transformants/μg DNA (12). In the transformation of *A. niger* with the correspondent *oli*C 3 gene (13), frequencies were lower than 2 transformants/μg DNA. In order to investigate the basis for this difference the levels of nuclease production was compared in a few strains of both *A. nidulans* and *A. niger*. A modified DNAse test agar originally described by Jeffries *et al.* (5) mainly for assaying bacterial nucleases was used to assay extracellular nucleases. Two other methods were used to assay nucleases in cell-free extracts.

[1] This paper is dedicated to the memory of Dr. Renato Bonatelli Jr. who was the supervisor of the author at the Departamento de Genética e Evolução, Universidade de Campinas, SP, Brasil.

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Strains of *A. niger* used were: lgp 73 (*pabA1, nicA1, olvA3, lgpB73* – Laboratório de Genética de Microrganismos, UNICAMP, Brazil) and wild type 350 (provided by M. Devchand, Allelix Inc., Canada). *A. nidulans* strains were biA1 methG1 (*biaA1, methG1* - provided by I. Baracho, UNICAMP, Brazil) and T580 (*pyrG*) (provided by M. Devchand, Allelix Inc., Canada). *Aspergillus* Minimal Media - MM (9) and Complete Media - CM were used. CM is MM supplemented with 2 g peptone, 1.5 g hydrolysed casein and 0.5 g yeast extract per litre; vitamins are added as required. Incubation temperatures for *A. niger* and *A. nidulans* were 30° and 37°C, respectively.

For the DNAse test agar, strains were inoculated in CM or MM agar plates, supplemented with 2 g/l of herring sperm DNA (HS DNA-Sigma). Strains were also grown in MM without inorganic phosphate. After 3 days of incubation, precipitation of nucleic acids was performed by adding 1N HCl to the plates for 5 minutes. Formation of hyaline halos around the colonies indicated DNAse activity, in contrast to an opaque background for unhydrolysed DNA. Nuclease S1 from *Aspergillus oryzae* and an endonuclease from *Escherichia coli* (Sigma) were spotted on the plates prior to addition of HCl as controls for the DNA degradation and precipitation.

A modification of a microtiter dish assay (1) was performed using a cell-free extract. About 10^6 protoplasts, obtained as described by Hamlym *et al.* (4), were resuspended in 500µl reaction buffer (TM - Tris-Cl 50 mM pH8, $MgCl_2$ 10 mM) and ultrasonicated by inserting a small tip into the suspension (3 cycles of 45 W for 10 sec.). After a 2 min. centrifugation at 12,000 g, the supernatant was collected. The wells of a microtiter dish were filled with 100 µl of TM containing 100 mg HS DNA/ml. Fifty microliters of the cell-free extract was applied to the first well and 1:2 dilutions were done for the next 10 wells. After overnight incubation at 37°C one microgram of ethidium bromide was added to each well. The plate was placed on a UV light box and photographed through a red filter. Degradation of DNA was followed by the reduction in fluorescence intensity. Electrophoretic analysis of DNA exposed to extracts (from *A. niger* lgp73 and *A. nidulans* T580) was performed using three micrograms of a 5.8 kb plasmid or five micrograms of chromosomal DNA (from *A. niger* lgp73 strain). Incubation was done with 50 µl of the cell-free extract in the presence of TM at 37°C. Aliquots (10 µl) were taken from the reaction mixture at different intervals (from 5 to 45 minutes), electrophoresed in a 0.8 % agarose gel which was photographed on a UV light box, through a red filter. Degradation of DNA was detected by the formation of smaller fragments, as compared with high molecular weight chromosomal or plasmid DNA.

The possibility that nucleases could be interfering with transformation efficiency of strain lgp 73 was first suggested because of unsuccessful attempts to isolate intact DNA from mycelia using 25mM EDTA in the extraction buffer. When the concentration of EDTA was increased 4-fold, intact chromosomal DNA was obtained, suggesting an inhibition of enzyme activity on DNA.

The DNAse test agar was used to detect extracellular nucleases. Hydrolysis halos were not detected when the strains were grown on Complete Media or Minimal Media supplemented with HS DNA, possibly due to excess of phosphate sources in those media. Phosphate-repressible nucleases have been isolated from *A. nidulans* (6) and *Neurospora crassa* (3). For this reason the composition of the Minimal Medium was modified so that the main source of phosphate was derived from HS DNA (no inorganic phosphate added).

Table 1 shows the sizes of halos around colonies grown on modified MM. Growth of all colonies was normal in this medium but halos were formed only around *A. niger* colonies. *A.niger* wt350 produced more nucleases than lgp 73 as shown by the ratio halo/colony (Table 1). To evaluate if diffusion was the reason for the difference observed, the plates were kept at 4°C for 4 days after the regular incubation times. The resulting hydrolysis halos were larger around *A. niger* colonies but no halos were observed around *A. nidulans* colonies (not shown). These results indicate that extracellular nucleases were not produced or secreted in the *A. nidulans* strains in the conditions tested.

If during the process of protoplast production some cells are disrupted nucleases may be released that could influence transformation efficiency. The microtiter dish assay was performed to detect differences in the amount of other nucleases in *A. niger* and *A. nidulans*. Protoplasts were washed twice before sonication, so most of the extracellular nucleases of the protoplast forming mixture were eliminated at this point. Both *A. nidulans* and *A.niger* cell-free extracts had high activity on HS DNA in the microtiter dish assay and differences in the amount of enzyme were not detected (not shown). The ability of the enzymes contained in the cell-free extracts to degrade intact double stranded DNA was also evaluated by electrophoresis. Fig. 1 shows the

Table 1 - Size of DNA hydrolysis halos in MM DNAse test agar for two *A. niger* and *A. nidulans* strains.

STRAINS	Halo (cm)	Colony (cm)	ratio Halo/ Colony
A.niger lgp 73	4.1	2.3	1.8
A. niger WT350	3.6	1.5	2.4
A. nidulans T580	0.0	2.8	-
A. nidulans biA_1 $methG_1$	0.0	2.8	-

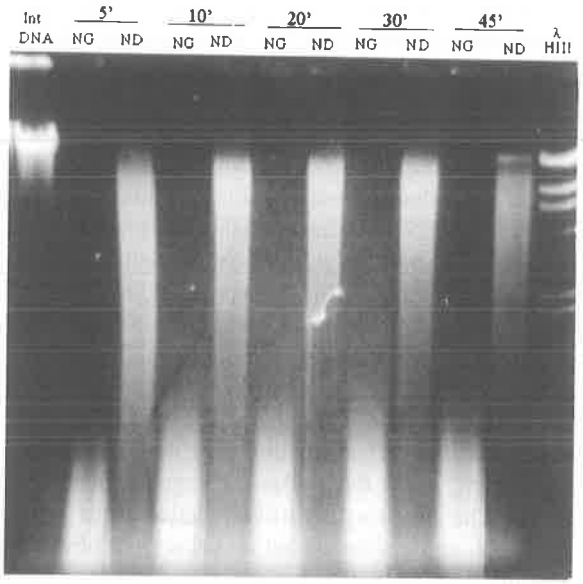

Figure 1 - Electrophoresis of chromosomal DNA after exposure to cell-free extracts of *A. niger* (NG) and *A. nidulans* (ND). lgp 73 DNA was exposed to NG and ND cell-free extracts at diferent time intervals (5 to 45 min. as shown above the lanes); Int DNA: intact lgp 73 DNA; λ HIII: lambda DNA digested with *Hind* III.

digestion of fungal chromosomal DNA by nucleases contained in the extracts of *A. niger* and *A. nidulans* at different time intervals. Degradation of DNA was observed in samples from both species within the first 5 minutes of incubation and was not significantly increased after 45 minutes. The specificity of the nucleolytic enzymes contained in the extracts was different between the two species: the DNA fragments resulting from *A. nidulans* digestion were very large, ranging from about 20 Kb to less than 1 kb and *A. niger* enzymes produced fragments around 1 kb in the first 5 minutes of reaction. When plasmid DNA (5.8kb) was used fragments were very small and were not detected in a 0.8% agarose gel (not shown).

These results show that there is a difference in the production and/or secretion of extracellular nucleases between the *A. niger* and *A. nidulans* strains tested and that the specificity of other nucleases is also different between the two species. As some of *A. nidulans* (6) and *N. crassa* (3) nucleases the extracellular nucleases detected by the modified DNAse test agar in *A. niger* seem to be repressed by high phosphate levels. In addition, the *A. niger* nucleases detected here do not act as a modification/ restriction system since degradation was observed with DNA from the *A. niger* lgp73 strain. Even though *A. niger* is a very important fungus used for production of several enzymes (8), nuclease production has not been analysed intensely in this species. The *Aspergillus oryzae* S1 nuclease is widely used in molecular biology protocols and recently, the gene for the nuclease O of *A. oryzae* was cloned (10). The characterization of the nucleases detected in this work could be of interest for biotechnology.

It can also be suggested from this study that the higher production of nucleases by *A. niger* strains can be one of the causes for the lower transformation efficiencies generally observed for this species. Several parameters have been considered for improving transformation efficiency in filamentous fungi. However, the production of nucleases by the manipulated fungus has not been given much consideration. To confirm the implication of intrinsic nucleases on transformation efficiency in *A. niger* however a more comprehensive analysis including strains of different backgrounds will be necessary. The DNAse test agar can be helpful for a quick screening of strains that produce large amounts of extracellular nucleases. This screening would avoid the use of such strains in transformation or point out to precautions such as handling of protoplasts at lower temperatures, additional washes of protoplasting cells or use of nuclease inhibitors.

RESUMO

Produção de nucleases intra e extracelulares por *Aspergillus niger* e *Aspergillus nidulans*

A produção de nucleases intra e extracelulares foi estimada em algumas linhagens de *Aspergillus niger*

e *Aspergillus nidulans* usando um teste para DNAse em placa e um ensaio com extratos livres de células. Pôde-se observar diferenças na produção de nucleases entre *A. niger* e *A. nidulans*. Estas observações sugerem que o teste de DNAse em ágar pode ajudar na detecção rápida de nucleases extracelulares em fungos filamentosos. Os ensaios usando extratos livres de células podem também servir na caracterização inicial de outros tipos de nucleases.

Palavras-chave: Nucleases, *Aspergillus niger, Aspergillus nidulans,* fungos filamentosos.

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Revista de Microbiologia (1998) 29:286-288
ISSN 0001-3714

USE OF REMAZOL BLUE DYED AVICEL FOR THE DETERMINATION OF CELLULOLYTIC ACTIVITY IN BASIDIOMYCETES

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Submitted: January 26, 1998; Returned to authors for corrections: April 09, 1998; Approved: January 11, 1999.

ABSTRACT

A modified method for direct determination of cellulolytic activity using Avicel colored with Remazol Brilliant Blue R (RBBR) in Agar test tubes is discussed. Refinements were introduced in a simple method for quantitation of cellulase activity, based on the release of dye from Avicel-RBBR medium by the enzymatic hydrolysis. Modifications in Avicel-dye preparation were enhanced and a spectrophotometer for direct OD measurement in agar test tubes used. The use of a spectrophotometer improved the precision of the collected data, since absorbance measurements could be done at the maximum wavelenght for RBBR (595 nm).

Key words: Avicel, cellulase, Remazol Blue, *Lentinus edodes, Pleurotus ostreatus*.

INTRODUCTION

The majority of the tests for detection of cellulolytic activity of microorganisms is based on the formation of a clear halo around a colony growing in opaque agar plate medium. Even when the medium is well dispersed, these halos are difficult to detect (1-4,8,10,11). The visualization of these halos can be improved by the use of Congo Red dye, due its capacity to interact with a wide range of polysaccharides (10). However, the use of this dye may be limited by the interference of hydrolysis products or by the colony diameter, which occasionally may coincide with the halo diameter. These limitations are solved using a colony growth inhibitor and Phosfon to increase contrast between halo and opaque agar (8). The methods for detection of cellulolytic activity in agar plate media are very laborious and need great handling, increasing the risk of contamination by other microorganisms.

Smith, (10) proposed a quantitative method for cellulase in cellulose-azure, using test tubes with 0.75% agar covered by a dye-cellulose agar medium. The dye was released to the basal medium during cellulose degradation by the cellulase produced in the medium. Poincclot and Day, (6) determined cellulase activity by means of solubilization of Remazol Brilliant Blue R (RBBR) from dyed chromatography paper. Schmidt and Kebernik, (7) described a methodology to quantify cellulase and hemicellulase activities in agar tube cultures, using cellulose and hemicellulose colored according procedures of Ng and Zeikus, (5) and detection in a photometer at 578 nm.

The present work reports results of the

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determination of cellulolytic colored by a modified Wood method (11) dispensed in test tubes, and a spectrophotometer at 595 nm for measurements of maximum RBBR.

MATERIALS AND METHODS

Chemicals. Microcrystaline cellulose (Avicel® - Merck), Remazol Brilliant Blue R (RBBR - Sigma), Malt Extract Agar (Difco).

Cellulase assay media. Basal medium was prepared with 0.75% agar in phosphate buffer pH 5.5 (10); upper medium contained 1% Avicel-RBBR (obtained according to procedure described bellow) plus 1.5% agar and nutrients (7).

Microorganisms. Strains from Laboratório de Bioquímica, Universidade Federal Rural de Pernambuco, included *Pleurotus ostreatus* (Jacq. et Fr.) Kummer, strains P015, P017 and P021; and *Lentinus edodes* (Berk.) Sing., strain L022.

Equipment. Spectrophotometer Meternik model SP850.

Procedure. The dyed cellulose was obtained using a modified method of Ng and Zeikus, (5) and Wood, (12). Ten grams of Avicel® were suspended in distilled water at 50°C, and then 100ml of 0.7% RBBR were added. The mixture was maintained under vigorous agitation at the same temperature by approximately 35 min. During this interval, 20g Na_2SO_4 were added in small portions. Afterward, 10 mg Na_3PO_4 were added to increase the pH around 12 and to fix RBBR to cellulose molecules as well. The suspension was maintained under agitation by one aditional hour. The dyed cellulose (Avicel-RBBR) was filtered and washed repeatedly with distilled water 60°C until the filtered material became showed colorless. The washed Avicel-RBBR was rinsed with acetone and ether, and dried in vacuum or in stove at 45°C. Three mililiters of basal medium were added into test tubes, previously standardized with distilled water in a spectrophotometer, and autoclaved at 121°C for 20 min. The upper medium (Avicel-RBBR) was autoclaved separately in a 200 ml aspirator flask. A stirring bar was placed into aspirator flask before autoclaving. The test tubes with basal medium were placed into a ice cold bath, and the upper Avicel-RBBR medium was added until 0.7 mm above the basal medium using a magnetic stirring device.

Enzymatic assay. For enzymatic activity assay, plugs 3mm diameter of the culture on malt extract agar were transferred to tubes containing Avicel-RBBR. Tests, were done using four replicates and three non inoculated tubes as control. Readings were done every two days, up to 50 days.

RESULTS AND DISCUSSION

The measurements of absorbance up to 50 days at 595 nm allowed us to distinguish between low, median and high cellulase active strains. Strains P017, P021 and L022 presented a peak of absorbance followed by decrease, while strain P015 increased continuously through the experiment (Fig. 1). These results are similar to exocellobiohidrolase and endoglucanase activities (13) obtained using the same strains. Readings at 578 nm, done according to Schmidt and Kebernik (7), did not detect the same maximum absorbance, which was 38% lower than that at 595 nm (Fig. 2).

Besides cellulolytic enzymes, *Pleurotus* sp. produces also na extracelullar peroxidase, associated to lignolytic activity, that may cause RBBR discoloration. This oxidase (RBBR-DcO) is H_2O_2-dependent and has an optimal pH activity of 3.5 (9). Enzyme activity decreases rapidly above pH 4.0 (9). The use of buffered basal medium at pH 5.0 and absence of H_2O_2 prevent this type of interference.

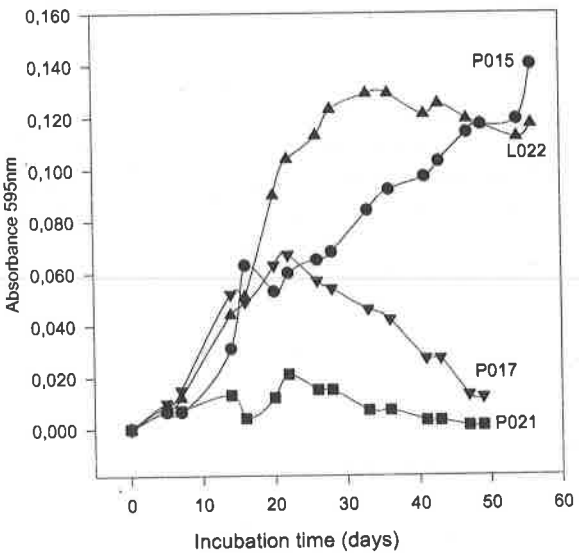

Figure 1. Median values of cellulytic activity of *Pleurotus ostreatus* (Fr.) Kummer, strains P015, P017, P021 and *Lentinula edodes* (Berk.) Pegler, strain L022, on Avicel colored with Remazol Brilliant Blue R in agar in test tubes, against a blank non inoculated medium. Reads were obtained a 595 nm and maximum transmitance was considered with non inoculated blank.

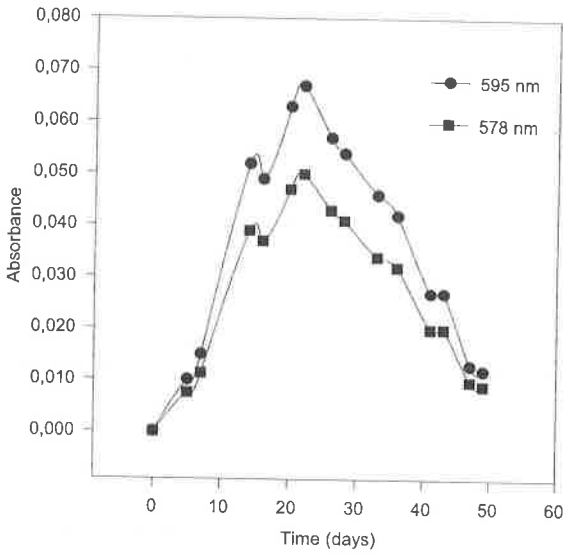

Figure 2. Cellulytic activity of *Pleurotus ostreatus* (Fr.) Kummer, strains P015, on Avicel colored with Remazol Brilliant Blue R, measured at 578 and 595 nm.

The use of a spectrophotometer improved the precision of the method. In addition, the stirring of the upper dyed medium while it is added to the basal medium improves the homogeneity of the misture, avoiding unequal substrate concentration, which may interfere in the cellulase activity.

The use of Wood's modified procedure (12), in stead of the one proposed by Ng and Zeikus, 1980 (5), for cellulase dying, is more rapid and less RBBR consuming and more adequate for determination of cellulolytic activity is fungi.

ACKNOWLEDGMENTS

This research was support by a grant from BNB/ETENE/FUNCI of the Banco do Nordeste do Brasil - BNB.

RESUMO

Uso de Avicel colorida com Remazol Blue para determinação da atividade celulolítica em Basidiomycetos

Neste trabalho, é discutido um método modificado para determinação da atividade celulolítica em fungos utilizando Avicel colorida com Remazol Brilliant Blue R (RBBR), diretamente em tubos com ágar, a partir de refinamentos introduzidos em uma metodologia simples e baseada na liberação deste corante pela ação hidrolítica em Avicel colorida com o RBBR. Foram feitas modificações no preparo da Avicel colorida, bem como um espectrofotômetro para medição direta da absorbância nos tubos com ágar foi usado. O uso de espectrofotômetro aumentou a precisão dos dados coletados, pois permitiu leituras de absorbância no comprimento de onda máximo para RBBR (595 nm).

Palavras-chave: celulase, Avicel, Remazol Blue, *Pleurotus ostreatus*.

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Revista de Microbiologia (1998) 29:289-294
ISSN 0001-3714

THE ROLE OF THE EXTERNAL MYCELIAL NETWORK OF ARBUSCULAR MYCORRHIZAL FUNGI: III. A STUDY OF NITROGEN TRANSFER BETWEEN PLANTS INTERCONNECTED BY A COMMON MYCELIUM

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Submitted: July 07, 1997; Returned to authors for corrections: June 04, 1998; Approved: July 23, 1998

ABSTRACT

An experiment under greenhouse conditions was carried out to evaluate the relative contribuition of arbuscular mycorrhizal fungi (AMF) in the process of nitrogen transfer from cowpea to maize plants, using the isotope ^{15}N. Special pots divided in three sections (A, B and C), were constructed and a nylon mesh screen of two diameters: 40μm (which allowed the AMF hyphae to pass but not the plant roots) or 1μm (which acted as a barrier to AM hyphae and plant roots) was inserted between the sections B and C. Section A had 25.5 mg of N/kg using $(^{15}NH_4)_2SO_4$ as N source. Two cowpea seedlings inoculated with *Rhizobium* sp. were transplanted with their root systems divided between the sections A and B. Ten days later, 2 seeds of maize were sown into the section C which was inoculated with *Glomus etunicatum*. Thirty-five days after transplanting, the maize plants were harvested. AMF inoculation increased dry weight and ^{15}N and P content of maize plant shoots. Direct transfer of ^{15}N via AMF hyphae was 21.2%; indirect transfer of ^{15}N mediated by AMF mycelium network, was 9.6%, and indirect transfer not mediated by AM mycelium network , was 69.2%.

Key words: mycorrhizae, cowpea, maize, nitrogen transfer

INTRODUCTION

Associations between arbuscular mycorrhizal fungi (AMF) and plant roots are usually non-specific in terms of the fungus-plant pairings that are compatible (24). This apparently low host specificity shown by many of the fungi that form AMF, may allow mycelial network of a particular fungus in the soil to be connected directly to the fungal structures within the roots of two or more different plants forming hyphal links between their mycorrhizal roots (25, 26, 28). These links provide a pathway that mediates the inter-plant transfer of nutrients directly through the fungal mycelium. Transfer of nutrients from one plant to another via AM hyphal connections was shown with carbon (8, 20, 21, 22), phosphorus (23, 31) and nitrogen (1, 15, 34).

In intercropping systems with grass and legume plants it is possible that the nitrogen derived from the biological fixation of nitrogen (BFN) by the legume can be transferred to the grass. Brophy *et al.* (3) detected a transfer of 40 kg of N from a legume to a grass, and Rao and Giller (27) observed a high content of N in grass intercropped with a legume. The flow of

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N between plants may happen through several pathways. The legume may release N to the soil through exsudates from the roots, leaves, nodule debris and roots tissues (5, 7, 14). Some works with ^{15}N dilution (4) and subdivided roots (15) have shown increases of N transfer between plants associated with AMF. The AMF may promote a high uptake of N in grass intercropped with a legume, even though there may be no significant differences in the shoot dry weight (10). The N transferred may be enough to improve the growth of the receiver plants (9). The nitrogen transfer between plants mediated by the AMF may occur by the following pathways: 1) direct transfer (DT) of nitrogen through mycelium which interconnects the roots of two plants; 2) indirect transfer (IT_M), involving leakage of nitrogen from one root, its absorption by AMF hyphae scavenging in the rhizosphere, and transfer to neighbouring plants. In addition to these two pathways, there is a third mechanism which is not mediated by AMF, which involves the leakage of nitrogen from roots of one plant and its subsequent absorption by the roots of neighbouring plants. To evaluate the relative contribution of each pathway to the total N transferred from one plant to another is very important because the direct transfer of N by AMF mycelium reduces the loss of N in the soil (leaching and immobilization) and also could improve the N cycling. Our purpose was to examine the contribution of the three possible pathways to the N transfer process between cowpea (donor plant) and maize (receiver plant), using the isotope ^{15}N.

MATERIALS AND METHODS

Rectangular pots were constructed and each pot was divided longitudinally into three identical sections (2 dm^3 of capacity) A, B and C (Fig. 1). Between sections B and C two procedures were adopted: a) a nylon mesh screen of 1µm was inserted to act as a barrier to AMF hyphae and plant roots; b) a nylon mesh screen of 40µm was inserted to allow the AMF to pass but not the plant roots. A solid barrier was also inserted between sections A and B to prevent any physical contact between the two sections.

Each pot section was filled with a soil: sand substrate (1:2, v/v) previously sterilized with CH_3Br. The substrate properties were: pH 5.3; organic C, 0.41%; total N, 0.041%; P and K content 8 and 19 mg/kg, respectively. The substrate in each pot section was amended with: 50 mg P/kg, 50mg K/kg and 0.6 ton/ha of Ca and Mg in the proportions of 4:1. At planting, 25 mg/kg of N was added into the section A, using as source $(^{15}NH_4)_2SO_4$ with 10% of ^{15}N. Fifteen days later, 0.5 mg/kg of N was once again added into the section A, using the same source, but with 99% of ^{15}N.

Two seedlings of cowpea inoculated with *Rhizobium* sp. strain BR-2001, from the National Centre of Agrobiology Research - EMBRAPA - RJ - Brazil, were carefully planted with their roots divided so that half of their root systems were growing in each of sections A and B. The pots were then placed in a green house.

Ten days later, two seeds of maize (receiver plant) were sown into the section C. The inoculation with AMF was made in half of the pots by adding into the substrate of section C, approximately 20 g of soil containing spores and hyphae of *Glomus etunicatum* (Becker and Gerdemann). In the case of non-mycorrhizal plant pots, approximately 25 ml of filtrate solution (filter paper Whatman n° 1) derived from infected soil, was added into section C to ensure that control plants received a bacterial population comparable with that present in the mycorrhizal medium, but lacking AM propagules.

Thirty-five days after maize planting, the plants were harvested. A sub-sample of cowpea and maize roots was taken to determine the root colonization

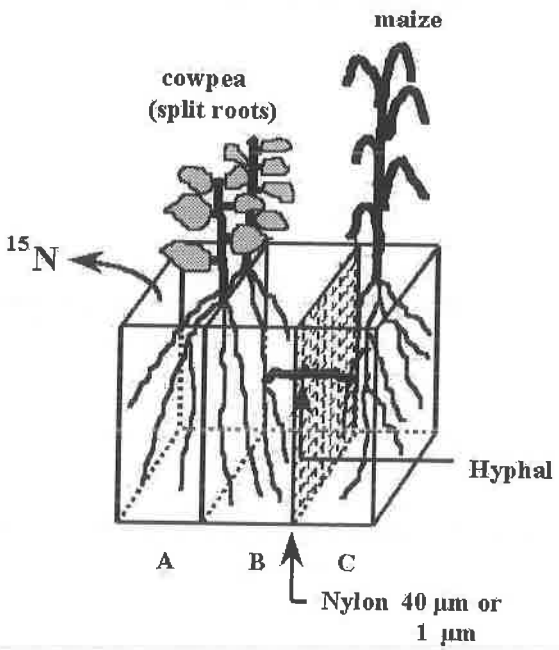

Figure 1. Design of the split pots

by AMF (11). The shoot of maize plants was oven-dried (48h at 80°C), weighed and ground. The ^{15}N content was determined by mass spectrometer and P content colorimetrically by spectrophotometer.

The relative contribution of each of the three processes of N transfer to the total amount ^{15}N transferred to maize was obtained using the procedures of Martins (22) for ^{14}C, as follows:

a) Direct transfer pathway via AMF hyphae (DT):

$$DT\ (\%) = \frac{(N_{M40} - N_{M1})}{N_{M40}} \times 100$$

Where:

N_{M40} = ^{15}N content in maize shoots grown in pots with a nylon mesh screen of 40μm and inoculated with AMF;

N_{M1} = ^{15}N content in maize shoots grown in pots with a nylon mesh screen of 1μm and inoculated with AMF.

b) Indirect transfer pathway mediated by AMF mycelium network (IT_M)

$$IT_M(\%) = \left[\frac{(N_{M1} - N_{NM40})}{N_{M40}} \times 100\right] - DT$$

Where:

N_{M1} = ^{15}N content in maize shoots grown in pots with a nylon mesh screen of 1 μm and inoculated with AMF;

N_{NM40} = ^{15}N content in maize shoots grown in pots with a nylon mesh screen of 40 μm and not inoculated with AMF;

N_{M40} = ^{15}N content in maize shoots grown in pots with a nylon mesh screen of 40 μm and inoculated with AMF.

c) Indirect transfer pathway not mediated by AMF (IT_{NM})

$$IT_{NM}(\%) = \frac{N_{NM40}}{N_{M40}} \times 100$$

Where:

N_{NM40} = ^{15}N content in maize shoots grown in pots with a nylon mesh screen of 40 μm and not inoculated with AMF;

N_{M40} = ^{15}N content in maize shoots grown in pots with a nylon mesh screen of 40 μm and inoculated with AMF.

Data were subjected to an analysis of variance (ANOVA test); least significant differences were calculated by an F test.

RESULTS

The levels of arbuscular mycorrhizal infection in either maize or cowpea plants were satisfactory (Fig. 2). The detection of infection in the roots of cowpea confirmed that transfer of infection had occurred from maize plants. The observation of no colonization in cowpea roots grown in pots with a nylon mesh screen of 1μ, confirmed that this screen was efficient to prevent invasion of external hyphae from section C into B, so that, maize and cowpea plants were not interconnected by a common mycelium. No colonization was observed in non-mycorrhizal treatments.

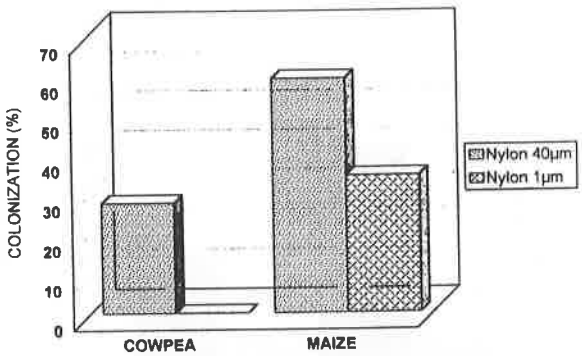

Figure 2. Percentage of cowpea and maize root length infected by *Glomus etunicatum*. Each bar represents the mean of 4 replicates.

The mycorrhizal infection led to a significant increase of both dry weight (Fig. 3) and P content (Fig. 4) of maize shoots. There was no effect due to the barrier between the sections B and C on dry weight of maize shoots. However, P content in the pots inoculated with AMF (+AMF) was significantly lower in the pots with a nylon mesh screen of 1 μm.

The ^{15}N content in maize shoots was not significantly affected by the presence of the imposed barrier (Fig. 5). Mycorrhizal infection led to a significant increase of ^{15}N content in maize shoots.

The relative contribution of each pathway to the total amount of ^{15}N transferred to maize shoots mediated by AMF, including the direct (DT) and indirect process (IT_M), was lower (21.2 + 9.6 = 30.8%) than the contribution not mediated by the fungi (IT_{NM} = 69.2%) (Table 1). Thus the main route by which ^{15}N was transferred from cowpea to maize was via the indirect transfer not involving the AMF, where the ^{15}N may have been exudated from the cowpea root into soil solution being subsequently uptaken by the roots of maize plants.

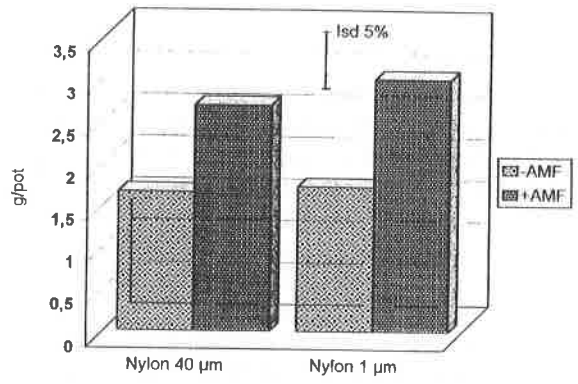

Figure 3. Dry weight of maize shoots. Each bar represent the mean of 4 replicates.

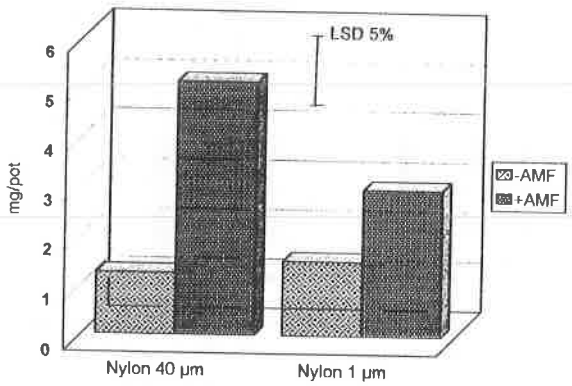

Figure 4. Phosphorus content of maize shoots. Each bar represent the mean of 4 replicates.

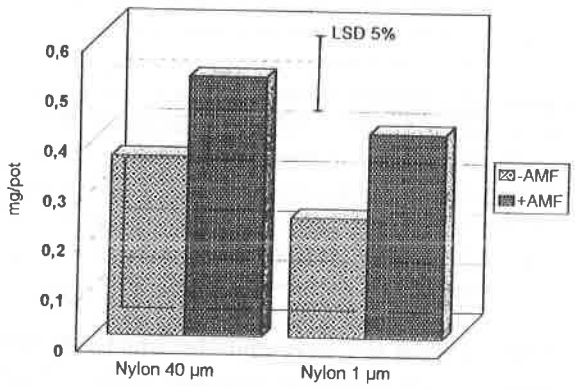

Figure 5. ^{15}N content of maize shoots. Each bar represent the mean of 4 replicates.

Table 1. Contribution of each pathway to the total amount of ^{15}N transferred from cowpea to maize plants.

Mecanisms of Transfer	(%)
DT (Direct Transfer by AMF)	21.2
IT_M (Indirect Transfer by AMF)	9.6
IT_{NM} (Indirect Transfer without AMF)	69.2

$$TD\ (\%) = \frac{(0.52 - 0.41)}{0.52} \times 100 = \mathbf{21.2\%}$$

$$TI_M\ (\%) = \frac{(0.41 - 0.36)}{0.52} \times 100 = \mathbf{9.6\%}$$

$$TI_{NM}\ (\%) = \frac{0.36}{0.52} \times 100 = \mathbf{69.2\%}$$

DISCUSSION

The presence of infection in the roots of cowpea plants in section B of pots confirmed that the AMF had grown from maize roots (section C) to cowpea (section B) (Fig 2). Bethlenfalvay *et al.* (2) and Camel *et al.* (6) have shown that the hyphae can spread from their associated roots into the soil over distances of 6 to 9 cm. Since the onset of sporulation of AMF requires 4-8 weeks and new spores have an endogenous dormancy cycle of 6 weeks to 6 months (33), it seems that the colonization observed in cowpea roots is attributable to growth of mycelium from roots of maize plants.

The external mycelial network of AMF growing from the section C into the section B can promote links between the two plant species. Read *et al.* (30) observed that most plants in seminatural grassland become heavily infected very soon after seed germination. They suggested that infection of the developing root system must arise from contact with mycelium spreading from plants with established infection, and that as a consequence of this pattern of infection, many plants within the community must be inter-linked by mycorrhizal hyphae. This has since been confirmed by studies of the development of infection of seedlings in the field (29).

This work showed that the maize shoot dry weights were higher in mycorrhizal plants than those of their non-mycorrhizal counterparts (Fig. 3). However there is no evidence that this fact is attributable to the N transferred from legume to grass. It is more probable that this plant growth is due to a higher P content

observed in mycorrhizal plants (Fig. 4). Hamel and Smith (12) concluded that the higher growth of maize plants intercropped with soybean under mycorrhizal condition was due mainly to a better P uptake by mycorrhizal plants and the N-transfer to maize plants had low significance to that growth. In practice the N content transferred between plants may not be enough to improve the growth or mineral nutrition of the receiver plant, because perhaps the maize plants need a higher quantity of N than that available from the cowpea plants.

The results revealed that AMF was enable to promote direct transfer of N from donor to receiver plants, because there was an increase of ^{15}N transferred to receiver when they were separated from the donor plants by a nylon mesh screen of 40μm, which allowed only the AMF mycelium to pass. The involvement of AMF in the process of N transfer between plants remains controversial. Some researches have shown a positive effect (17, 19). On the other hand, others have not observed any influence of mycorrhizae in this process (16). Hamel and Smith (13) showed that even though the AMF had established links between plants, they did not increase N transfer. According to Johansen *et al.* (18), the transfer of N from one plant to another mediated by arbuscular mycelium depends on the demand of plants. Tomm *et al.* (32) have also pointed out that this process can be bi-directional depending of the demand of receiver plants.

The transfer of substances between plants mediated by AMF have been also shown for other elements, such as carbon and phosphorus, by using radioisotope techniques. Francis and Read (8) and Martins (22) have demonstrated that the ^{14}C transfer between plants occurred directly via AMF mycelium. Whittingham and Read (35) and Martins and Read (23) showed that direct transfer of ^{32}P between living source and sink plants occurs mainly by hyphae connecting the two plants.

The present study revealed that the AMF can provide channels for transfer of N between plants cultivated under intercropped systems. However, the most significant route by which ^{15}N was transferred from cowpea to maize was by the indirect process not involving the fungus. Further studies are now required to determine if the transferred N is enough to stimulate the growth of the receiver and it is also necessary to evaluate the behaviour of this fungus under other environmental circunstances.

ACKNOWLEDGEMENTS

We are extremely grateful to CNPq (Brazil) for financial support.

RESUMO

Significância do micélio externo dos fungos micorrízicos arbusculares. III. Estudo da transferência de nitrogênio entre plantas interconectadas por um mesmo micélio.

Conduziu-se um experimento em casa de vegetação para avaliar a importância dos fungos micorrízicos arbusculares (FMA) sobre o processo de transferência de nitrogênio de plantas de caupi para o milho, utilizando-se o isótopo ^{15}N. Foram construídos vasos compartimentalizados, compostos de 3 seções (A, B e C) de 2 dm^3 de capacidade. Entre as seções B e C inseriu-se uma tela de nylon com 2 diâmetros de abertura de malha: 1 μm (que atuou como barreira para o micélio fúngico e para as raízes das plantas), ou 40 μm (que atuou como barreira somente para as raízes das plantas). Adicionou-se 25,5 mg/kg de N na forma de $(^{15}NH_4)_2SO_4$ na seção A dos vasos. A seguir, 2 plântulas de caupi previamente inoculadas com *Rhizobium* sp. foram plantadas com seus sistemas radiculares divididos entre as seções A e B. Após 10 dias, duas sementes de milho foram semeadas na seção C, onde se efetuou a inoculação com o fungo *Glomus etunicatum* no orifício de plantio. O experimento foi coletado 35 dias após, e os resultados demonstraram que a presença do FMA aumentou a matéria seca e o conteúdo de ^{15}N e P da parte aérea das plantas de milho. A transferência direta de ^{15}N via hifa fúngica, foi de 21,2%; a transferência indireta mediada pelos FMA, foi de 9,6%; e a indireta não mediada pelos FMA, foi de 69,2%.

Palavras-chave: micorrizas, caupi, milho, transferência de nitrogênio.

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Revista de Microbiologia (1998) 29:295-300
ISSN 0001-3714

EFFECTS OF HIGH TEMPERATURE ON SURVIVAL, SYMBIOTIC PERFORMANCE AND GENOMIC MODIFICATIONS OF BEAN NODULATING *RHIZOBIUM* STRAINS

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Submitted: October 17, 1997; Returned to authors for corrections: February 17, 1998; Approved: September 17, 1998

ABSTRACT

High temperatures can affect the survival, establishment and symbiotic properties of *Rhizobium* strains. Bean nodulating *Rhizobium* strains are considered particularly sensitive because on this strains genetic recombinations and/or deletions occur frequently, thus compromising the use of these bacteria as inoculants. In this study *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains isolated from Cerrado soils were exposed to thermal stress and the strains' growth, survival and symbiotic relationships as well as alterations in their genotypic and phenotypic characteristics were analyzed. After successive thermal shocks at 45°C for four hours, survival capacity appeared to be strain-specific, independent of thermo-tolerance and was more apparent in *R. tropici* strains. Certain *R. leguminosarum* bv. *phaseoli* strains had significant alterations in plant dry weight and DNA patterns obtained by AP-PCR method. *R. tropici* strains (with the exception of FJ2.21) were more stable than *R. leguminosarum* bv. *phaseoli* strains because no significant phenotypic alterations were observed following thermal treatments and they maintained their original genotypic pattern after inoculation in plants.

Key words: bean nodulating *Rhizobium* strains, high temperatures

INTRODUCTION

High soil temperature in tropical regions is one of the major constrains for biological nitrogen fixation in legume crops. Temperatures in these regions average above 40°C (4) may affect symbiotic relationships, nitrogen content and plant production (2,7). These effects are particularly accentuated in *Phaseolus vulgaris* L. (15) which is a very important staple crop in the tropics. Rhizobia strains of *P. vulgaris* L. from different tropical soils vary in heat tolerance (6,11,19) and high temperatures may affect this bacteria's survival, establishment (8) and symbiotic properties (22). Bean nodulating *Rhizobium* strains are considered to be particularly sensitive because of their genetic characteristics. Genes responsible for nodulation and N_2 fixation in these *Rhizobium* strains are located on a single replicon, the symbiotic plasmid (Psym). The genome is complex, containing many reiterated DNA sequences that may provide sites for recombination and genomic rearrangements (5,9). With temperature

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increases, plasmid deletions (25) and genomic rearrangements (22) may occur, resulting in alterations or in loss of symbiotic properties. Consequently, this genetic instability is compromising these *Rhizobium* strains' use in commercial inoculum production. Total or partial plasmid deletions, under high temperature conditions, have been occurring more frequently in sensitive strains (30). However, individual reaction to a given temperature varies within the strains. Using cured plasmid derivatives of *Rhizobium leguminosarum* bv. *trifolii*, Baldani *et al.* (1) showed that some strains were cured at 28°C, others at 39°C and some were not cured even at 44°C. It was also observed that the more heat tolerant *R. leguminosarum* strains retained their Psym even after longer incubation periods at 37°C and others maintained N_2 fixation even at temperatures above 38°C (7).

In order to select stable bean nodulating *Rhizobium* strains for tropical conditions, the objetive of this study was to evaluate the effects of heat on growth, survival, symbiotic performance and genomic modifications in effective *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains isolated from Cerrado soils.

MATERIALS AND METHODS

This study initially analyzed 43 strains of *Rhizobium* isolated from bean plants cultivated in Cerrado soils previously characterized phenotypically as *R. leguminosarum* bv. *phaseoli* (24 strains) and *R. tropici* (16 strains) (16).

Determination of maximum growth temperature in bean nodulating *Rhizobium* strains isolated from bean plants cultivated in Cerrado soils - Isolated colonies of each strain were grown up to a final log phase (10^8 cells/ml) in yeast mannitol medium (YM) at 29°C (26). To determine the maximum growth temperature for each strain, transfers from the initial growth were made. Each inoculum corresponding to 1% of the total volume of the medium was incubated on shaker (Lab-line Orbit Environ - Shaker Model 3527) at a optimum temperature of 29°C, and then at 35, 36, 37, 38, 39, 40, 41 and 42°C in accordance with the method described in Munevar and Wollum (12). Three replicates of each strain were used. The maximum growth temperature was determined as the temperature at which the strain and the control (grown at 29°C) had equivalent growth during the same incubation period (12). Growth was monitored by optical density (OD 600nm).

Survival capacity and *Rhizobium* inoculation tests in bean plants after temperature stress - *R. leguminosarum* bv. *phaseoli* and *R. tropici* strains with different levels of heat tolerance were inoculated in Cerrado soil and were incubated at 45°C in a shaker for 4 hours (0.7 ml of inoculum with 10^8-10^9 cells/g of soil). A second treatment consisted of repeating the above procedure 4 times at 48-hour intervals. To evaluate the survival capacity of the strains following high temperature exposure, viable cells were counted before and after each stress temperature using the pour plate dilution technique in YM agar medium. After each thermal shock, other replicates and their controls, with the same number of cells, were placed in Leonard jars (26) containing asseptically cultivated beans (cv. aporé). The experiments were carried out using four replicates of a completely randomized block design. All plants received N-free solution (7) for 30 days after emergence. The plants' dry weight was measured after drying at 65°C for 48 hours and its nitrogen content determined by microkjeldahl method (23).

Genomic pattern evaluation of *Rhizobium* spp. strains before and after successive exposures to high temperatures and plant inoculation, using Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) - *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains with different levels of heat tolerance isolated from Cerrado soils were grown in YM medium up to the log phase (10^8 cells/ml). The total DNA of *Rhizobium* was isolated using the method described by Sá *et al.* (19) which allowed high quality DNA isolation. Amplification was performed in a thermocycler (ERICOMP) in accordance with the technique reported by Steindel *et al.* (21). After two amplification cycles with denaturation at 95°C for 5 min., annealing at 30°C for 2 min., and extension at 72°C for 30 sec., thirty-three amplification cycles were performed with annealing at 40°C for two min. Final extension was carried out at 72°C for 5 min. Each reaction mixture contained: 7.3 µl of distilled H_2O, 1.0 µl of PCR buffer 10X, 0.5 µl of dNTP (2.5 mM), 0.2 µl of Taq DNA polymerase (Taq Cembiot), 1.0 µl of one decamer primer (Operon Technologies, Inc., Alameda, CA, USA), and 1.0 ng of DNA. The amplification products were eletrophoretically separated on 5% acrylamide gel. The DNA bands were silver stained (20) and photographed.

RESULTS AND DISCUSSION

Bean nodulating *Rhizobium* strains isolated from Cerrado soils varied in their capacity to tolerate heat when incubated at temperatures between 35°C to 39°C (Table 1). This same type of high temperature tolerance have also been reported by several researchers (12,13,14). *R. tropici* strains were more tolerant than *R. leguminosarum* bv. *phaseoli* strains. Among the *R. tropici* strains, 71.4% growth was observed at temperatures ≥37°C compared to only 63% growth in *R. leguminosarum* bv. *phaseoli* strains at the same temperatures. These results are consistent with those obtained by Martinez-Romero *et al.* (10) who reported that besides being more heat tolerant, the analyzed *R. tropici* strains were also more stable because they retained Psym for longer periods of incubation at 37°C.

Table 1. Maximum growth temperature of 24 *R. tropici* strains and 19 *R. leguminosarum* bv. *phaseoli* strains isolated from beans cultivated in Cerrado soils

Species	Maximum temperature (°C)							
	35	36	37	38	39	40	41	42
	% *Rhizobium* strains							
R. tropici	-	28.6	23.8	23.8	23.8	-	-	-
R. leguminosarum bv. *phaseoli*	9.0	28.0	9.0	27.0	27.0	-	-	-

Survival capacity, determined by the number of viable cells after exposure to stress temperatures (45°C for 4 hours), was specific to each strain and to each species independent of their thermo-tolerance (Table 2). These results are more evident when each strains' percent of variation in the number of cells before and after temperature stress is considered (Table 2). For example, BR 322 and SLP 1.3 strains (T. max 39°C) had the same number of cells before and after exposure to one thermal shock therefore the percent of variation was 0, while FJ 2.2 (T. max 36°C) had a drastic reduction in cell numbers, corresponding a 41.9% variation (Table 2). More sensitive strains like SLA 2.2 and FJ 2.21 (T. max 36°C) also had different decreases in the number of viable cells after thermal stress, corresponding to 18.6% and 44.5% average variation, respectively. Mpepereki *et al.* (13) also reported that maximum permissive temperatures and maximum survival temperatures were not significantly correlated in indigenous Rhizobia isolated from tropical soils.

Differences in the effect of high temperatures on strains' symbiotic properties are showed in Table 2. Two strains, SLBR 3.12 and SLP 4.9, lost their ability to nodulate, and two strains, *R. Tropici* - FJ 2.21 and *R. leguminosarum* bv. *phaseoli* - SLA 1.5, had decreased nitrogen fixation levels as measured by plant dry weight and total N after only one thermal shock (Table 2). The latter two strains presented high thermo-tolerance (T. max 39°C) but low survival capacity compared to all other analyzed strains.

Certain strains with contrasting characteristics in relation to temperature were submitted to 4 successive thermal shocks at 48-hour intervals. Their survival capacity was evaluated after each shock and then inoculated in bean plants. Again, the results showed that survival capacity was specific to each strain and species independent of their thermo-tolerance (Table 3). While cell numbers in FJ 2.21 were drastically reduced, corresponding to 77.7% average variation, BR 10,026 and SLP 2.10 were practically not affected (7.0% and 7.5% respectively), and BR 322 had a slight decrease (29.2% variation).

Plant dry weight and number of nodules are shown in Table 3. Among the strains analyzed, the SLP 2.10 and SLA 1.5 strains of *R. leguminosarum* bv. *phaseoli* showed the largest significant differences in dry weight after heat exposure (Duncan 5% of probability). The remaining strains, did not showed statistically significant differences in dry weight and total N accumulation (Table 2). In relation to nodule numbers, significant differences were observed only in *R. leguminosarum* bv. *phaseoli* SLP 2.10 and SLA 1.5 strains. Taken together, these results suggest that the *R. tropici* strains were more stable.

No relationships were evident between thermo-tolerance, survival capacity and N_2 fixation after thermal stress within each species tested under axenic condition. Under such conditions, the number of viable cells apparently did not affect the dry weight and total N accumulation, contrary to what is expected in soils where competition with other strains and microorganisms naturally occurs.

High temperatures also affected the Rhizobia genome (3,27), especially in fast growing rhizobia like bean nodulating strains (22). The effects of high temperatures on genetic modifications were investigated using AP-PCR. According to Welsh and McClelland (28) and Williams *et al.* (29), this method is particularly useful in identifying strains within the same species and in detecting modifications in DNA nucleotides. In addition, AP-PCR provides an efficient assay for genetic variation studies in

Table 2. Survival capacity of *R. tropici* and *R. leguminosarum* bv. *phaseoli* with different thermal tolerance and symbiotic performance in beans after one thermal shock at 45°C for 4 hours.

Rhizobium strain	Max. Growth T °C	Viable cells number/ml (log_{10})			Plant dry weight (g/plant)		Plant total N (mg/plant)	
R. tropici		A	B	C	D	E	D	E
BR 322	39	8.90	8.90	0.0	0.527	0.498	220	198
FJ 2.2	39	8.60	5.00	41.9	0.450	0.446	229	270
SLBR 3.12	39	9.04	5.60	38.0	0.418	+	+	+
SLP 4.9	38	9.18	5.48	40.3	0.520	+	+	+
FJ 2.21	36	8.60	4.78	44.5	0.739*	0.399*	286*	143*
SLA 2.2	36	8.60	7.00	18.6	0.375	0.422	239	270
SLA 3.2	36	9.48	7.00	26.1	0.522	0.511	270	254
R. leguminosarum bv. *phaseoli*								
SLP 2.10	39	9.30	8.30	10.8	0.382	0.387	249	278
SLP 5.8	39	8.90	8.78	1.4	0.394	0.393	273	263
SLA 1.5	39	8.30	5.30	36.1	0.393*	0.283*	314*	201*
SLP 1.3	39	8.30	8.30	0.0	0.485	0.465	218	204
SLP24.1	38	8.95	7.00	21.8	0.449	0.504	253	265
BR 10.026	38	8.60	6.48	24.7	0.330	0.304	265	253
BR 10.028	36	8.85	6.30	28.8	0.375	0.373	292	245
SLP 4.4	36	8.95	6.78	24.3	0.361	0.356	293	288

A: Number of cells before thermal shock
B: Number of cells after thermal shock
C: Average % of variation between A and B
D: Control strain
E: Strain submitted to thermal shock

* Significant differences (Duncan 5% probability) values given represent the mean data of 4 repetitions.
\+ SLBR 3.12 and SLP 4.9 strains lost their ability to nodulate after thermal shock, therefore plant dry weight and total N were not analyzed.

Table 3. Survival capacity of *R. tropici* and *R. leguminosarum* bv. *phaseoli* with different thermal tolerance and symbiotic performance in beans after four thermal shocks at 45°C for 4 hours.

Rhizobium strain	Max. growth T°C	Viable cells number/ml weight (g/plant)						Plant dry (log_{10})		Number of nodule/plant	
R. tropici		A	B	C	D	E	F	G	H	G	H
SLA 2.2	36	8.60	7.00	6.30	7.70	5.95	30.8	0.40	0.47	53	53
SLA3.2	36	9.48	7.00	6.30	5.70	5.60	40.9	0.44	0.43	86	51
FJ 2.21	36	7.60	5.00	3.60	1.70	1.70	77.7	0.60	0.50	77	46
BR 322	39	8.90	8.90	8.30	7.70	6.30	29.2	0.40	0.43	85	50
R. leguminosarum bv. *phaseoli*											
SLP 1.3	39	8.30	8.30	6.90	7.60	6.48	21.9	0.63	0.52	29	63
SLP 2.10	39	9.30	8.30	8.30	8.30	8.60	7.5	0.77*	0.48*	92*	38*
SLA 1.5	39	8.30	5.00	6.48	5.90	5.30	36.1	0.71*	0.44*	100*	60*
BR 10.026	38	8.60	8.48	8.48	8.84	8.00	7.0	0.55	0.49	69	45

A: Number of cells before thermal shock
B: Number of cells after 1st thermal shock
C: Number of cells after 2nd thermal shocks
D: Number of cells after 3rd thermal shocks
E: Number of cells after 4th thermal shocks
F: Average % of variation between A and E
G:Control strain
H:Strain submitted to four thermal shocks

* Significant differences (Duncan, 5% of probability), values given represent the mean data of four repetitions.

microorganisms. In this study, polymorphisms in the amplification of DNA products using the primer S34 (5' GGT TCG ATT GGG GGT TGG TGT AAT ATA 3') (Fig. 1), confirmed at the genetic level, alterations that were observed at the phenotypic level in *R. leguminosarum* bv. *phaseoli* SLP 2.10 (lanes 12 and 13) and SLA 1.5 (lanes 15 and 16). Other strains of this species (SLP 1.3 and BR 10,026) did not have significant differences in dry weight production, (Table 3) but they presented changes in their genomic patterns (Fig. 1). In this case, these alterations probably did not affect genes related to symbiosis or N_2 fixation. The BR 322, SLA 2.2 and SLA 3.2 strains of *R. tropici* did not have phenotypic alterations and they maintained similar PCR banding patterns after high temperature exposure and plant inoculation (Fig. 1). Similar results were obtained with other AP-PCR tests using 6 different primers and the reproducibility of the results was verified in independent experiments.

High genetic variability due to reiterations in the genome of these bacteria caused deletions of certain genome elements at the frequency of 10^2-10^3 (5). Flores *et al.* (5) reported that after cultivating *R. leguminosarum* bv. *phaseoli* CFN 285 strain for one year in a laboratory free of stress factors, nearly 35% of the cells presented differences compared to original cells.

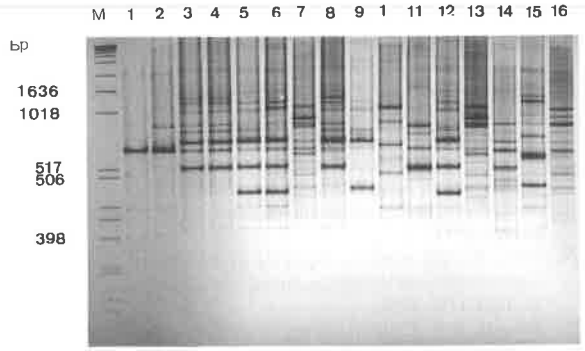

Figure 1. Amplification of genomic DNA from *R. tropici* (1-8) and *R. leguminosarum* bv. *phaseoli* (9-16) strains before (TB) and after thermal shock (TA) with random primer S34. M-DNA marker (1Kb ladder; Bethesda Research Laboratories). 1-BR322 (TB); 2-BR322 (TA); 3-SLA2.2 (TB); 4-SLA2.2 (TA); 5-SLA3.2 (TB); 6-SLA3.2 (TA); 7-FJ2.21 (TB); 8-FJ2.21 (TA); 9-BR10.026 (TB); 10-BR10.026 (TA); 11-SLP2.10 (TB); 12-SLP2.10 (TA); 13-SLP1.3 (TB); 14-SLP1.3 (TA); 15-SLA1.5 (TB); 16-SLA1.5 (TA).

More detailed studies to explore these genetic variations, especially related to high temperature, are currently underway in this laboratory. Results of analysis of some colonies isolated from BR 322 and SLA 2.2 strains of *R. tropici* and from SLA 1.5 and SLP 2.10 strains of *R. leguminosarum* bv. *phaseoli* show variable reactions in nodulation capacity and nitrogen fixation within and between colonies of the same strain following 4 thermal shocks. Some colonies of strains of *R. leguminosarum* bv. *phaseoli* lost their nodulation capacity. Analysis of DNA amplification products from these colonies showed on the genetic level, the variations observed in the phenotypic characteristics and the profiles of the colonies from *R. leguminosarum* bv. *phaseoli* strains were more heterogeneous compared to those of *R. tropici* strains (17).

The results described here indicate significant genetic stability in *R. tropici* strains compared to *R. leguminosarum* bv. *phaseoli* strains. Moreover, the strategies used in this study to evaluate survival capacity, N_2 fixation performance and genetic stability after thermal stress could be useful in selecting efficient, stable *Rhizobium* strains to be used as inoculum for bean plant cultivation in tropical soil conditions.

ACKNOWLEDGEMENTS

The authors thank laboratory technicians Paulo Figueiredo, Renato Simões and Miguel Reis for their collaboration. This work was supported by CNPq and FAPEMIG.

RESUMO

Sobrevivência, fixação de nitrogênio e modificações genéticas em estirpes de *Rhizobium* sp. efetivas na nodulação do feijoeiro, expostas à altas temperaturas.

Altas temperaturas podem afetar a sobrevivência, estabelecimento e as propriedades simbióticas em estirpes de *Rhizobium*. As estirpes capazes de nodular o feijoeiro têm sido consideradas particularmente sensíveis, porque nessas estirpes é comum a ocorrência de recombinações e/ou deleções genômicas comprometendo, muitas vezes, a sua utilização como inoculantes. Neste trabalho, procurou-se avaliar a capacidade de crescimento e sobrevivência em temperaturas elevadas de estirpes de *Rhizobium* efetivas na fixação de nitrogênio no feijoeiro isoladas dos cerrados, bem como avaliar

suas características fenotípicas e genotípicas após choque térmico. A capacidade de sobrevivência à temperaturas elevadas, avaliada após choques térmicos sucessivos (45°C por 4 horas) mostrou ser uma característica própria de cada estirpe, independente de sua termotolerância, que aparentemente foi mais acentuada nas estirpes de *R. tropici*. Algumas estirpes de *R. leguminosarum* bv. *phaseoli* mostraram alterações significativas (Duncan 5% de probabilidade) nas suas características fenotípicas (produção de matéria seca) após choques térmicos e nos seus padrões genômicos evidenciados pela técnica de AP-PCR. As estirpes de *R. tropici* foram aparentemente mais estáveis não sendo detectadas alterações fenotípicas significativas e com exceção da estirpe FJ2.21, após choque térmico e inoculação na planta hospedeira, mantiveram o padrão genômico original.

Palavras-chave: Estirpe de *Rhizobium* associadas ao feijoeiro, temperatura elevada.

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Revista de Microbiologia (1998) 29:301-306
ISSN 0001-3714

PRODUCTION AND ACTION PATTERN OF INULINASE FROM *ASPERGILLUS NIGER*-245: HYDROLYSIS OF INULIN FROM SEVERAL SOURCES

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Submitted: October 17, 1997; Returned to authors for corrections: February 17, 1998; Approved: September 17, 1998

ABSTRACT

A strain of *Aspergillus niger* isolated from soil samples showed great capacity to produce extracellular inulinase. Although the enzyme has been synthesized in presence of monosaccharides, sucrose and sugar cane molasse, the productivity was significantly higher ($p<0.05$) when the microorganism was inoculated in media formulated with dahlia extract and pure inulin, as carbon sources. With regard to the nitrogen source, the best results were obtained with casein and other sources of proteic nitrogen, comparatively to the mineral nitrogen. However, statistic significance ($p<0.01$) only was found between the productivity obtained in the medium prepared with casein and ammonium sulphate. The optimum pH of the purified enzyme for inulin hydrolysis was found between 4.0 and 4.5 and the optimun temperature at 60°C. When treated by 30 minutes in this temperature no loss of activity was observed. The enzyme showed capacity to hydrolyse sucrose, raffinose and inulin from which it liberated only fructose units showing, therefore, an exo-action mechanism. Acting on inulins from several sources, the enzyme showed larger hydrolysis speed on the polissaccharide from chicory (*Cichorium intibus*), comparatively, to the inulins from dahlia (*Dahlia pinnata*) and Jerusalem artichoke (*Helianthus tuberosus*) roots.

Key words: inulinase, *Aspergillus niger*, fructose syrup, inulin

INTRODUCTION

α-D-Fructose is a monosaccharide widely distributed in nature that shows sweetening power 70% higher than sucrose. It consistis in a suitable table sugar, ingredient for some food formulation and in substrate for fermentative processes (13,17,18). It is the principal component of the fructans as the inulin found in the roots of artichoke (*Heliantus tuberosus),* dahlia (*Dahlia pinnata*) and chicory (*Cichorium intibus*) corresponding to 12.5% of its wet weight (1,10,14). According to Barta (1), at this moment, these are the more suitable inulin sources for utilization in industrial scale, whose productivity is estimated, respectively, in 4.5 ton/ha, 2.5 ton/ha and 0.9 ton/ha. Besides, in the last years several authors have showed that other plants of the *Liliaceae* and *Asteraceae* families found in Brazilian savannahs can store amounts up to 85% of fructans in their roots, exhibiting potentiality for economical application as fructose source (7,11).

Enzymatic hydrolysis by inulinases (1-ß-D-

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Fructan-Fructanohydrolase - EC 3.2.1.7) has been proposed as the most promising technique to obtain the fructose syrups from inulin (10,13,18). So, some fungi, yeasts and bacteria have been described as producers of potentially adequated ß-fructosidases, but without a definite valuation for industrial employment. In this work, approximatelly 350 *Aspergillus niger* strains were evaluated by its capacity to synthesize inulinase with potentiality for industrial employment. A strain codified as *Aspergillus niger*-245 showed the best productivity and inulinase thermostability and was selected for the work seqüence. The enzyme was applied on the fructans from several origins and its action, was evaluated.

MATERIALS AND METHODS

Microorganisms and culture media. It was utilized a collection culture of *Aspergillus niger* isolated from soil that has been mantained in slants with Sabouraud-dextrose-agar (Difco) medium in our laboratories. For the screening procedure, 0.1 ml of a spores suspension (4 x 10^7 spores/ml) was inoculated in Erlenmeyers flasks containing 50 ml of dahlia extract (2.0% of total sugars) enriched with 1.5% $(NH_4)_2SO_4$, 0.5% yeasts extrat, 0.5% K_2HPO_4, 0.2% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, and final pH, 5.5. The flasks were agitated in a rotary shaker, for 60 h, at 200 rpm and 28°C. After the mycelia separation by filtration, the filtrate was analysed for enzyme activity. Dahlia extract was replaced by inulin as carbon source, for the studies of the screened strain growth. The fermentation condition as pH, remaining carbohidrates, intra and extracellular enzyme and mycelia dry weight were analysed at each 12 h.

Enzyme assay. Amounts of 0.5 ml of enzyme solution were incubated with 1.0 ml of 2.0% inulin solution in 50 mM acetate buffer, pH 5.0 for 30 min and the reaction stopped by maintenance in boiling water for 5 min.The content of liberated fructose was estimated by the Somogyi and Nelson method (15) and expressed as reducing sugar. The invertase activity was measured by replacing the inulin for a sucrose soluction in the same buffer and concentration and the liberated glucose was estimated by the glucose oxidase method (2). One enzyme unit (EU) was defined as the enzyme amount necessary to liberate 1 µMol of fructose/glucose.min^{-1}, in assay condition .

Inulinase production. For the experiments on the effect of the carbon sources, the media were formulated with 2.0% of glucose, fructose, sucrose, inulin, sugar cane molasse, yam extract and dahlia extract (2.0% of total sugar) and 1.5% of peptones, as nitrogen source. When the effect of the nitrogen source in the enzyme production was investigated, the spores were innoculated in media containing 1.5% of urea, $(NH_4)_2SO_4$, peptones, casein, soy flour and yeast flour, as nitrogen source, and 2.0% inulin (Sigma) was utilized as carbon source. For optimization of the enzyme production, a medium composed with dahlia extract and casein was utilized. From each experimental design, 5 flasks were inoculated and the results, submitted to variance analysis (Tukey test) for verification of statistical significance.

Dahlia and yam extracts. The dahlia and yam extracts were prepared according to the procedure described by Houly *et al.* (10) with some modifications. Small cubes of the peeled tubercules were grounded in a homely miller and the obtained paste was suspended in destiled water in the 1:4 ratio (w:v). This suspension was agitated in magnetic stirring, for 20 min, sieved in a 120 mesh sieve, centrifuged at 3,000 rpm and the total sugar, adjusted to 2.0% in accordance with Dubois *et al.* method (4).

Enzyme characterization. The effect of pH on purified inulinase (not published yet) activity was investigated by measuring the enzyme activity at 50°C, in 50 mM acetate buffer in the pH range from 2.5 to 7.0. For the pH stability determination, aliquots of 0.5 ml of enzyme plus 0.5 ml of the same buffer in mentioned pH range were maintained at 4°C, by 96 h and the residual activity was estimated. The optimum temperature was obtained by measuring the enzymatic activity in 50 mM acetate buffer, pH 5.0 in the temperature range from 35°C to 70°C. For the thermal stability determination of the inulinase, a reaction medium composed with 0.5 ml of enzyme solution and 0.5 ml of 100 mM acetate buffer, pH 5.0, was mantained for 30 min in the same temperature range and the residual activity, measured as described in enzyme assay.

Inulinase action pattern. Aliquots of 0.5 ml of the purified enzyme were incubated, separately in 2.0 ml of raffinose solution (Vetec), sucrose (Reagen), and inulin (Sigma), in a final concentration of 2.0% in 50 mM acetate buffer, pH 5.0, at 50°C. The profile of the enzymatic reaction was followed by thin layer chromatography (TLC) with a solvent system composed by ethyl acetate, acetic acid and distilled water (3:1:1) and the stains revealed as described by

Walkley and Tillman (19). The liberation of glucose from sucrose and fructose from raffinose and inulin was also determined, respectively by the methods of the glucose oxidase (2) and Somogiy and Nelson (15). The values obtained in the raffinose reaction were divided by 2, because to each molecule of liberated fructose, corresponds 1 reducing unit of melibiose.

Hydrolysis of inulin from different sources. For the hydrolysis studies of inulin from different origins, the reaction system was composed by 1.0 ml (14 EU) of purified enzyme, 10 ml of dahlia, chicory or artichoke 5.0% inulin solutions in 50 mM acetate buffer, pH 5.0. The enzymatic action was followed by High Efficiency Liquid Chromatography (HPLC) in a Shimadzu LC-10A chromatography, equipped with a refraction index detector, RID 6-A model, and Supercosyl LC-NH2 column with 250 x 4.6 mm maintained at 20°C. The solvent system was composed by acetonitrile-water (80:20) and the speed of flow of 2.0 ml/min.

RESULTS AND DISCUSSION

Microorganism growth and enzyme production. Fig. 1 shows the growth curve of *Aspergillus niger*-245. It is possible to verify that the biosynthesis of the inulinase happens simultaneously to the log phase of the mycelia growth, starting at the 24 h of incubation and that the largest enzyme liberation happened between 48 and 60 h of fermentation. Such behavior is quite uncommon for this enzyme once, generally, in other microorganisms it reaches the maximum activity in the extra-cellular medium after 72 h of fermentation (3).

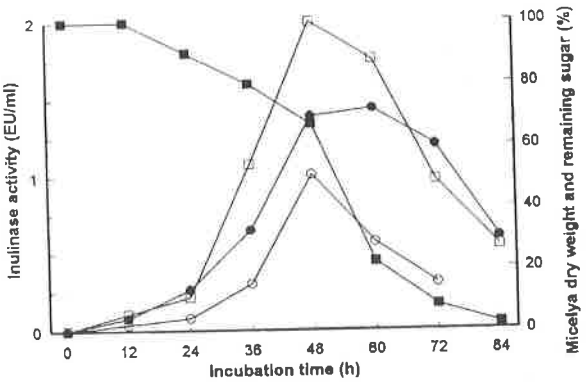


Figure 1. Growth profile and inulinase production by *Aspergillus niger*-245
● Extracel. inulinase ○ Intracel. inulinase
■ Sugar consumption □ Mycelia dry weight

Ohta *et al.* (17) obtained the maximum activity of inulinase of a mutant strain of *Aspergillus niger*, described as high enzyme-producing, after 5 days of inoculation in a medium very similar to the one used in this work, once that medium contained inulin and ammoniun sulfate, respectively, as sources of carbon and nitrogen. Various strains of *Aspergillus* sp, studied by Gupta *et al.* (8), also exhibited maximum inulinase activity in the 9º day of submerged fermentation. The fast enzyme production for the now selected microorganism is a suitable propriety in industrial processes and represents an advantage of this one on the other microorganisms already proposed for such purpose.

Effect of carbon source. As shown in Table 1, the inulinase production in the media formulated with dahlia extract, (that contain inulin) and with the own inulin, its natural inductor, was significantly ($p<0.05$) higher than the one observed in other carbon sources. In spite of the apparent larger productivity in the presence of the extract, it was not registered statistical significance between both. The same table shows, also, that such media favored the biosynthesis of the inulinase activity in detriment of the invertase activity, registering the largest ratios I/S (0.42 to 0.44). That observation was already described by Ferreira *et al.* (6), working with a strain of *Cladosporium cladosporioides*. The smallest I/S ratio obtained in the medium formulated with sucrose and molasse (0.16), can suggest that other invertase molecular forms has been synthesized in those media. Kaur *et al.* (12) isolated four differents invertases from a growth medium of *Fusarium oxysporum* enriched with fructans and sucrose. The production of both activities in the media formulated with monossacarides also suggests that such enzyme activities are synthesized constitutively.

Table 1: Effect of the carbon source on ß-fructosidases production by *Aspergillus niger*-245.

Carbonsource	Inulinase EU + s.d.	Invertase EU + s.d.	I/S ratio
Fructose	1.51 - 0.39	4.16 - 0.69	0.36
Glucose	1.43 - 0,36	3.40 - 0.51	0.42
Sucrose	1.16 - 0.30	7.25 - 1.20	0.16
Inulin	2.92 - 0.50*	6.95 - 0.90	0.42
Yan extract	1.07 - 0.37	2.97 - 0.10	0.36
Dahlia extract	3.68 - 0.50*	8.30 - 0.20	0.44
Molasse	1.41 - 0.38	8.81 - 0.64	0.16

* Statistical significance ($p<0.05$) - described in text
I = Inulinase; S = Invertase.

Effect of nitrogen source. The microorganism was more efficient in the inulinase production when inoculated in media containing proteic nitrogen in relation to those formulated with mineral nitrogen as it can be verified in Table 2. The urea inhibited the synthesis of the enzyme completely, and in relation to the ammonium sulphate, the averages were considerably inferior to all the media formulated with proteic nitrogen. However, statistical significance ($p<0.01$) was only registered between the enzyme production obtained in casein and ammonium sulphate media. In the casein medium was observed the best performance of the microorganism, although, there is no registration of statistical significance in relation to the others formulated with proteic nitrogen. The same table shows that when casein was associated with dahlia extract as carbon source the inulinase production reached 9.9 EU/ml, in a synergistic effect. Synthesis of invertase was not considered in this table because the I/S ratio was not affected by the nitrogen sources. The largest production of inulinase in media formulated with organic nitrogen was, also, described by Ha and Kim (9). Those authors verified that a strain of *Streptomyces* sp presented the largest enzyme productivity in a medium formulated with soy flour compared to others organic and mineral nitrogen sources.

Table 2: Effect of nitrogen source on the inulinase production by *Aspergillus niger*-245

Nitrogen source	EU/ml	S.D.
Ammonium sulphate	1.85*	0.29
Pepton	2.8	0.36
Casein	3.0*	0.83
Soy flour	2.2	0.26
Yeast flour	2.47	0.32
Urea	-	-
Casein**	9.9	0.57

* Statistical significance ($p<0.01$)
** Dahlia extract, as carbon source.

Influence of the pH on the enzymatic activity. In acordance with the Fig. 2, the inulinase from *A. niger* 245 showed whole stability in the pH range from 3.5 to 7.0 and optimum pH between 4.0 and 4.5, coinciding with the results described by Vandamme and Derycke (18) for the enzyme sytnthetized by other strain of *A. niger*. However, Oengen *et al.* (16) obtained the maximum activity for the inulinase from a microorganism of the same group in a range slightly superior, that is 5.0 and 6.0.

For industrial application in obtaining concentrated fructose syrups, enzymes with larger activity in pH range inferior to 5.0, as the one here described, are suitable since they make difficult the bacterial contamination of the process.

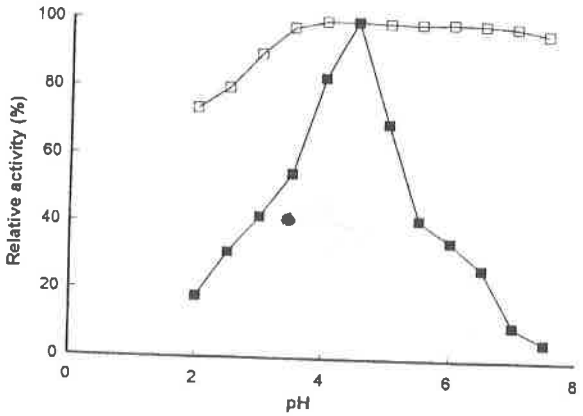


Figure 2: Effect of the pH on the activity and stability of the inulinase from *Aspergillus niger*-245.
■ Activity □ Remaining activity.

Enzyme behaviour in relation to the temperature. As it can be seen in Fig. 3, the enzyme showed the larger activity at 60°C. In this temperature the crude enzyme stayed stable, after 30 min of treatment. It is possible to suppose, however, that the enzyme can support longer that treatment once when submitted for the same time at 65°C, it lost only 8% of its initial activity. This supposition also is supported by the studies of Gupta *et al.* (8). Searching the termostability of the inulinases produced by *Aspergillus sp*, including several *A. niger*, the authors had concluded that the inulinases from this genus, in general, present thermostability higher than several ones from microbial origin.

Action pattern of the enzyme. Fig. 4 shows the reducing sugars (fructose) or glucose liberation from several substrates as function of the reaction time. With regard to the sucrose and its homologous raffinose, practically there was no progression in the reaction after 30 minutes that perhaps can be explained, by the speed with that the enzyme attacks such substrates leading to their prompt exhaustion. From the inulin, the stabilization of the process was only obtained after 180 min and no glucose was found in the reaction medium. Such results are very similar to those produced by the inulinase from *Arthrobacter* sp which showed ability to hydrolyze sucrose, raffinose and inulin, however with activity 5 times

higher on the fructooligosacharides, when compared to the inulin (5).

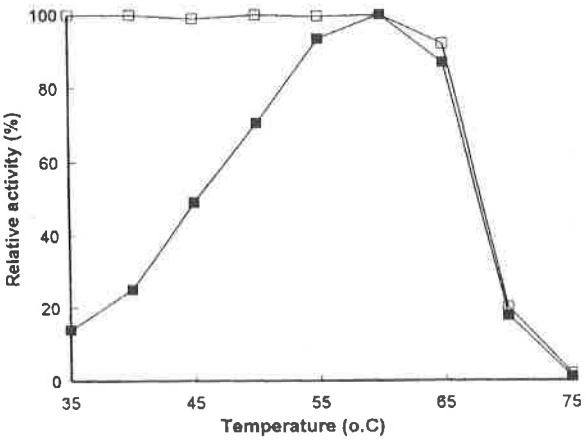


Figure 3: Effect of temperature on the activity and stability of the inulinase from *Aspergillus niger*-245.
■ Activity □ Remaining activity.

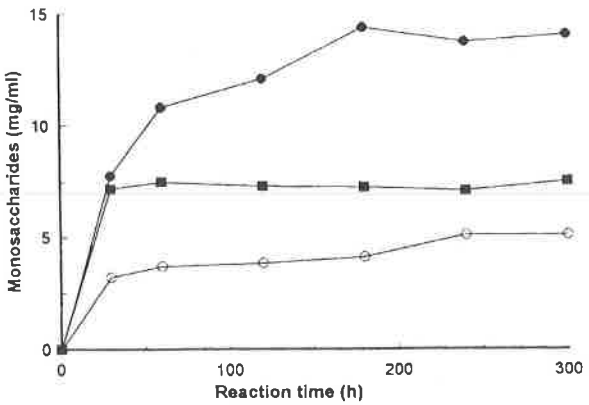
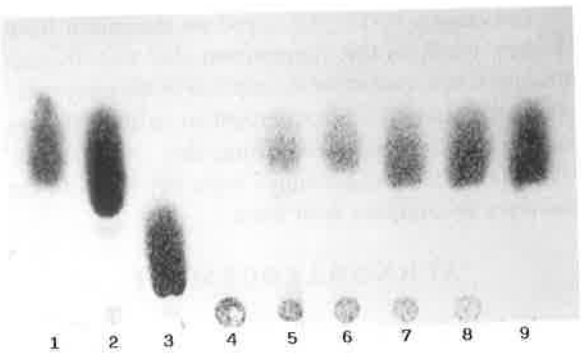


Figure 4: Action of the inulinase from *Aspergillus niger*-245 on several substrates.
● Inulin ○ Raffinose
■ Sucrose

The obtaining of fructose as the only hydrolysis product from inulin and its liberation from the sucrose and raffinose suggest an exo-action mechanism of the enzyme. This action pattern was better evidenced in the chromatogram of the Fig. 5. To the progress of the reaction it corresponds a intensity increasing of the stains produced by fructose, proportionally, to the decrease in the intensity of the stains related to the inulin, confirming, therefore, the expressed results in Fig. 4.



1- Fructose, 2- Glucose, 3- Sucrose, 4- Inulin 0', 5- Inulin 30' 6- Inulin 60', 7- Inulin 120', 8- Inulin 180', 9- Inulin 240'.

Figure 5: Hydrolysis of inulin by inuliase from *A. niger*-245. Effect of reaction time.

Inulin hydrolysis of different origins. Fig. 6 summarizes the inulinase action on the inulin from several sources. It is possible to verify that the fructose liberation curves of the fructans from artichoke and dahlia are, extremely, similar. However, the inulinase showed larger activity on inulin from chicory, once 72% of the reaction medium sugars were identified as fructose at the 30 min of reaction, against only 27% of the dahlia and artichoke inulins. After 1 hour of reaction, these values arose, respectively, to 88% and 80% and after 3 h, total hydrolysis was obtained for all the fructans when the fructose reached to about 90%, characterizing the remainder one as glucose.

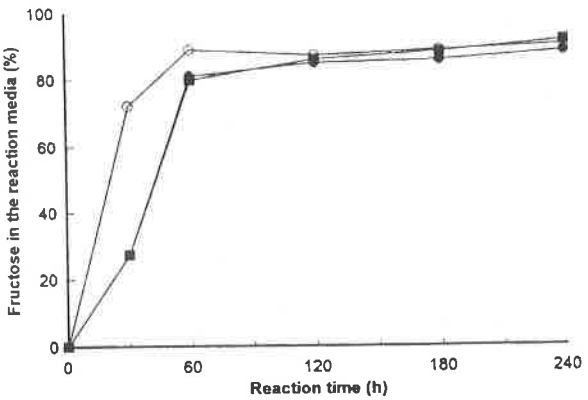


Figure 6: Action of the inulinase from *Aspergillus niger*-245 on inulin from several sources.
● Dahlia ○ Chicory
■ Artichoke.

The largest hydrolysis speed on the inulin from chicory leads to the supposition that this fructan presents a structure more susceptible to the enzymatic attack (perhaps less condensed or with different polimerization degree) than the other ones. Unfortunately, similar studies were not found in the literature to compare with them.

ACKNOWLEDGEMENT

The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the financial support

RESUMO

Produção e mecanismo de ação de inulinase de *Aspergillus niger*-245: Hidrólise de inulinas de diferentes origens

Uma linhagem de *Aspergillus niger* isolada de amostras de solo mostrou grande capacidade de produzir inulinase extracelular. Embora a enzima tenha sido sintetizada na presença de monossacarídeos, sacarose e melaço de cana, a produtividade foi significativamente maior ($p<0.01$) quando o microrganismo foi inoculado em meios formulados com extrato de dália e inulina pura, como fontes de carbono. Em relação à fonte de nitrogênio, os melhores resultados foram obtidos com caseína e outras fontes de nitrogênio proteico, comparativamente ao nitrogênio mineral. Entretanto, somente foi encontrada significância ($p<0.01$) entre a produtividade obtida nos meios preparados com caseína e sulfato de amônia. O pH ótimo da enzima purificada foi localizado entre 4.0 e 4.5 e a temperatura ótima a 60°C. Quando tratada por 30 minutos nesta temperatura nenhuma perda de atividade foi observada. A enzima mostrou capacidade de hidrolisar sacarose, rafinose e inulina, da qual liberou apenas unidades de frutose, mostrando, portanto, um mecanismo de exo-ação. Atuando sobre inulinas de diversas fontes, a enzima mostrou maior velocidade de hidrólise sobre o polissacarídeo da chicória, comparativamente, às inulinas de raízes de dalia e alcachofra.

Palavras chaves: inulinase, *Aspergillus niger*, inulina, xarope de frutose

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Revista de Microbiologia (1998) 29:307-310
ISSN 0001-3714

STRUCTURE-BIOLOGICAL ACTIVITY RELATIONSHIP OF SYNTHETIC TRIHYDROXILATED CHALCONES

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Submitted: December 02, 1997; Approved: September 17, 1998.

ABSTRACT

The bacteriostatic activity of 2',4',2-trihydroxychalcone; 2',4',3-trihydroxychalcone and 2',4',4-trihydroxychalcone, prepared by condensation of 2,4-dihydroxyacetophenone and benzaldehyde substituted, against *Staphylococcus aureus* ATCC 25923 was assayed by agar plate method. The three compounds presented important inhibition halos. In order to elucidate structure-activity relationships, the minimal inhibitory concentrations against *S. aureus* were determined by the broth dilution method and the results obtained were compared to that of 2',4'-dihydroxychalcone. The sequence observed was: MIC 2',4',3-$(OH)_3$ > MIC 2',4'-$(OH)_2$ > MIC 2',4',4-$(OH)_3$ > > MIC 2',4',2-$(OH)_3$. These results showed that the introduction of an electron donating group (-OH) in the aromatic B-ring causes an increase in bioactivity, and that the intensity of action depends on the position of the OH substitute.

Key Word: bacteriostatic activity, structure-activity relationship, flavonoids, trihydroxylated chalcones.

INTRODUCTION

A large number of natural flavonoids with biological activity have been identified in recent decades. One group of these products, the polihydroxylated chalcones, exhibit antimicrobial (8,15), antiviral (6,9), antitumoral (7,10,12) and antiinflamatory (17) activities, and applications of therapeutic effects (12) have been reported. The increase of the bacteriostatic action due to free hydroxyl groups on the aromatic A- and B- rings has been demonstrated in a previous work, which showed that the introduction of hydroxylic groups, specially in the 4- and 4'- positions, enhance the bioactivity of 2'-hydroxychalcone (15).

In order to elucidate structure-activity relationships of natural and synthetic polyhydroxylated chalcones, the minimal inhibitory concentrations (MICs) of 2',4',2-trihydroxychalcone **1**, 2',4',3-trihydroxychalcone **2** and 2',4',4-trihydroxychalcone **3** against *Staphylococcus aureus* ATCC 25923 were evaluated. These compounds were obtained by condensation of 2,4-dihydroxyacetophenone and benzaldehyde substituted under specific work conditions (2,16). The chalcone **3** (isoliquiritigenin) occurs free in barck and wood of several *Leguminosae* (4) as well as in *Compositae* (5).

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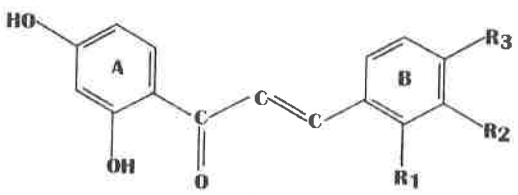
MATERIALS AND METHODS

General Experimental Procedure: Chalcones were prepared by adding KOH solution (35 g in 50 ml of water) to an equimolar aldehyde and ketone solution in ethyl alcohol (50 ml). The mixture was maintained in ice. Their structures and numbering system are shown in Fig. 1.



R_2=R_3=H; R_1=OH: 2',4',2-trihydroxychalcone
R_1=R_3=H; R_2=OH: 2',4',3-trihydroxychalcone
R_1=R_2=H; R_3=OH: 2',4',4-trihydroxychalcone

Figure 1. Structure of trihydroxychalcones.

2',4',2-Trihydroxychalcone 1: The reaction mixture was kept in the refrigerator at 4°C for 30 days. It was diluted with water and acidified with concentrated HCl. The KCl formed was separated by filtration. The total solution was treated with ethyl acetate for to obtain the desired product and dried with Na_2SO_4. This extract was concentrated and purified by silica gel and Sephadex LH 20 columns chromatography using benzene and methanol as eluents, respectively. All eluates collected were analyzed by thin layer chromatography (TLC) (polyamide 11 F_{254}, methanol-acetic acid-water, 90:5:5), and spots on the plate were visualized under UV light. After removal of methanol under reduced pressure at 50°C, an orange crystalline solid was obtained. The structure of **1** was determined by the chromatographic and spectroscopic data: Rf (TLC): 0.163; UV λmax (MeOH) nm: 370; 307; 251; 220. These data are in agreement with those reported by Dhar and Shing (3). ^{1}H NMR (200.13 Mhz, CD_3OD): δ 6.28 (d, J = 2.3 Hz, H3'); 6.4 (dd, J = 8.9, 2.3 Hz, H5'); 7.91 (d, J = 8.9 Hz, H6'); 7.85 (d, J = 15.6 Hz, H α); 8.14 (d, J = 15.6 Hz, Hß); 6.85 (dd, J = 7.6, 1.7 Hz, H3); 7.24 (td, J = 7.6, 7.6, 1.7 Hz, H4); 6.85 (td, J = 7.6, 7.6, 1.7 Hz, H5); 7.66 (dd, J = 7.6, 1.7 Hz, H6). ^{13}C NMR (50.33 Mhz, CD_3OD): δ 114.7, 162.5, 103.8, 166.4, 109.1, 133.3 (C1'-C6'); 193.9 (C=O, ß'); 121.2, 141.3 (Cα, Cß); 123.1, 158.7, 117.0, 132.9, 120.8, 130.0 (C1-C6).

2',4',3-Trihydroxychalcone 2: The reaction mixture, kept in the refrigerator at 4°C for 7 days was diluted with water and acidified with HCl, resulting in a yellow product. The precipitate was suction filtered, water washed, dried and recrystallized from ethanol-water to give 2',4',3-trihydroxychalcone, that exhibit the expected spectroscopic properties (3). Rf (TLC): 0.185; UV λmax (MeOH) nm: 358; 314; 253; 210. ^{1}H NMR (200.13 Mhz, CD_3OD): δ 6.28 (d, J = 2.3 Hz, H3'); 6.4 (dd, J = 9.0, 2.3 Hz, H5'); 7.95 (d, J = 9.0 Hz, H6'); 7.66 (d, J = 15.4 Hz, H α); 7.75 (d, J = 15.4 Hz, Hß); 7.12 (br.t, J = 2.1 Hz, H2); 6.84 (dt, J = 7.6, 2.1, 2.1 Hz, H4); 7.21 (dt, J = 7.6, 2.1, 2.1 Hz, H5); 7.25 (dd, J= 7.6, 2.1 Hz, H6). ^{13}C NMR (50.33Mhz, CD_3OD): δ 114.6, 167.6, 103.8, 166.7, 109.3, 133.5 (C1'-C6'); 193.3 (C=O, ß'); 121.6, 145.3 (Cα, Cß); 137.6, 115.8, 159.0, 118.8, 122.3, 131.0 (C1-C6).

2',4',4-Trihydroxychalcone 3: The compound was prepared as described for 2',4',2-trihydroxychalcone **1**, resulting in a yellow-orange crystalline solid, with Rf (TLC): 0.174; UV λmax (MeOH) nm: 368; 242; 205. ^{1}H NMR (200.13 Mhz, CD_3OD): δ 6.26 (d, J = 2.3 Hz, H3'); 6.39 (dd, J = 8.9, 2.3 Hz, H5') 7.96 (d, J = 8.9 Hz, H6'); 7.6 (d, J = 15.4 Hz, Hα); 7.78 (d, J = 15.6 Hz, Hβ); 7.61 (d, J = 8.6 Hz, H2); 6.83 (d, J = 8.6 Hz, H3); 6.83 (d, J = 8.6 Hz, H5); 7.61 (d, J =8.6 Hz, H6). ^{13}C NMR (50.33 Mhz, CD_3OD): δ 114.6, 167.5, 103.8, 166.4, 109.2, 133.3 (C1' - C6'); 193.5 (C=O, ß'); 118.3, 145.6 (Cα, Cß); 122.8, 131.8, 116.9, 161.5, 116.9, 131.8 (C1-C6).

For the assignment of ^{1}H NMR and ^{13}C NMR spectra, resonance double experiences, DD COSY, XHCORR and COLOC spectra were used.

Antibacterial assays: Antibacterial activity was examined by the method of agar plate and broth dilution:

1. Agar plate method: 0.2 ml of a 1:4 dilution of a 18 hours broth culture of *S.aureus* ATCC 25923 was plated on agar surface, where wells of 10 mm diameter were made. In each well was filled with 0.1 ml of the test substance containing 30 - 130 µg/0.1 ml. After incubation at 37°C, for 18h, the inhibition zones were measured with a vernier. Chalcones derivatives were diluted in ethanol-water mixture. No inhibitory effect was observed for ethanol in the concentration used in the tests.

2. Broth dilution method: 4 ml of 1/400 diluted inocula, prepared with a fresh culture of *S. aureus* ATCC 25923, were added to 100 ml of Mueller-Hinton broth (MHB). Aliquots of 7 ml were distributed in test tubes, followed by addition of chalcones at concentrations of 13-55 µg/ml. After

incubation for 24 hours at 35°C, the minimal inhibitory concentration (MIC, µg/ml) was determined by turbidimetry at 720 nm (13).

RESULTS AND DISCUSSION

The structures of the prepared compounds were confirmed by chromatographic and spectroscopic data.

Although agar plate method does not yield an absolute measurement of antimicrobial activity, it provides a rapid and preliminary screening determination of the relative activity of the tested compounds. Every chalcone derivatives analysed showed significant bacteriostatic activity (Table 1), with the best activity achieved by compound **1**.

Broth dilution method (14) was able to determine more accurate MIC values: 23.0µg/ml; 55.0 µg/ml and 33.5 µg/ml, for compounds 1, 2 and 3 respectively.

Results obtained in a previous work led us to propose inhibition mechanisms in which the molecular region responsible for the biological properties involves the carbonyl group (1).

The inhibition sequence presented by trihydroxylated chalcones when compared to 2',4'-dihydroxychalcone which presented a MIC of 36.5 µg/ml, was:

MIC 2 > MIC 2',4'-(OH)2-CHALCONE > MIC 3 > MIC 1

This sequence clearly shows that the introduction of an electron donating group (-OH) in the aromatic B-ring increased bioactivity, the intensity depending on the position of the substituent OH. The closes the active region (C=O) is to the hydroxyl group, the strongest the inhibition effect.

The lower efficacy of 2',4',3-trihydroxychalcone can be attributed to the occurrence of torsion at the C (carbonylic) Cα axis, which forms an angle between the plane that contains the B-ring and the double bond Cα = Cβ and the plane that includes the rest of the molecule in chalcone 3-R- substituted (16).

These results suggest that chalcone synthesis should be directed to select compounds with greater bioactivity, like the introduction of hydroxyl groups in the B-ring of the molecule.

ACKNOWLEDGEMENTS

The present work was supported by the San Luis National University.

RESUMO

Relação estrutura – atividade biológica de chalconas trihidroxiladas sintéticas

A atividade bacteriostática de 2',4',2-trihidroxichalcona, 2',4',3-trihidroxichalcona e 2',4',4-trihidroxichalcona, preparadas por condensação de 2,4-dihidroxiacetofenona e benzaldeido convenientemente substituído, contra *Staphylococcus aureus* ATCC 25923 foi avaliada pela técnica de difusão em placas. Importantes halos de inibição foram observados para os três compostos. Com o propósito de esclarecer a relação estrutura-atividade biológica, as concentrações inibitórias mínimas (CIM) frente a *S. aureus* foram determinadas, empregando o método da diluição em caldo. Os resultados obtidos foram semelhantes à 2',4'-dihidroxichalcona, observando-se a seguinte ordem: CIM 2',4',3-$(OH)_3$ > CIM 2',4'-$(OH)_2$ > CIM 2',4',4-$(OH)_3$ > CIM 2',4',2-(OH). A seqüência obtida mostra que a introdução de um grupo doador de eletrons (OH) no anel aromático B provoca um aumento da bioatividade, sendo a intensidade dependente da posição do substituinte -OH.

Table 1. Grown Inhibition of *S. aureus* ATCC 25923 by trihydroxylated chalcones

Chalcone 1	C	122.5	98.0	85.7	73.5	49.0	0
	D	21	20	19	19	19	0
Chalcone 2	C	116.7	105.0	81.7	70.0	46.7	0
	D	16	15	14	14	14	0
Chalcone 3	C	131.7	118.5	92.2	79.0	52.7	0
	D	20	19	18	18	18	0

C: Concentration (µg/0.1 ml); D: Inhibition (mm).
1 = 2',4',2-trihydroxychalcone 2 = 2',4',3-trihydroxychalcone 3 = 2',4',4-trihydroxychalcone

Palavras-chave: atividade bacteriostática, relação estrutura-atividade, flavonóides, chalconas trihidroxiladas.

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Revista de Microbiologia (1998) 29:311-313
ISSN 0001-3714

CERVICOVAGINAL AEROBIC MICROFLORA OF WOMEN WITH SPONTANEOUS ABORTION OR PRETERM DELIVERY IN ARARAQUARA-BRAZIL

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Submitted: June 02, 1997; Returned to authors for corrections: June 02, 1998; Approved: July 23, 1998

SHORT COMMUNICATION

ABSTRACT

Microbiological routine exams of endocervix and vaginal specimens of 22 women with clinical history of recent spontaneous abortion or premature rupture of membranes were accomplished. *Chlamydia trachomatis, Streptococcus pyogenes, Streptococcus agalactiae, Candida* sp and *Gardnerella vaginalis* were recovered from 54.5% (12) of the women. *Ureaplasma urealyticum* was frequently isolated (45.5%) but 5 out of 22 had *U. urealyticum* only. Our report stands for the importance of quantitative as well as qualitative investigation on genital microflora in pregnant women, since it is likely to influence on pregnancy outcome.

Key words: abortion, preterm birth, genital microflora

Most abortions occur during the first trimester, which is due to chromosomal abnormalities (7). Corioaminionitis is due the most usual factor for abortion in the second trimester. Causes of prematurity can be found in few cases and the frequency of preterm births has not significantly decreases over the past 30 years, despite the widespread use of potent tocolytic agents. The major causes for such a situation has been connected with lower socioeconomic status, antepartum hemorrhage, and a background of adverse pregnancy outcome (8). In addition different researches have shown that abnormal pregnancy outcomes, i.e., preterm labor (PTL), preterm delivery (PDT), and preterm membrane ruptures, may be related to ascending genital microflora. Specific organisms such as *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis* and *Streptococcus agalactiae* (group B streptococci), have all been responsible for one or more of such abnormal outcomes of pregnancy (1,5). *Mycoplasma hominis* and *Ureaplasma urealyticum* also have been suggested as causing agents for spontaneous abortion (15). The purpose of the present study was to examine the cervicovaginal microflora of women with abnormal pregnancy outcomes.

Laboratory routine of endocervix and vaginal specimens from 22 women with clinical history of recent abnormal pregnancy outcome were carried out. A specimen from the posterior fornix was collected with Ayre's spattle, direct into saline over a slide, for evaluation of yeast and protozoa. The diagnosis was

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made by wet-mount microscopy. A specimen of the vaginal mucous was collectted with cotton-tipped swab, rolled over a glass slide and Gram stained, for microscopic observation and evaluation of microorganisms and polymorfonuclear leucoytes (PMN), as described by Evangelista and Beilstein (6). A cotton-tipped swab was used to transfer vaginal fluid into 5% sheep's blood agar, 5% human's blood agar and chocolate agar for isolation of aerobic or facultative organisms. Each agar plate was streaked into four zones, and the growth of the different species was semiquantitated, as follows: 1+,≤10 colonies in the primary streak area; 2+, > 10 colonies in the primary streak area and > 10 colonies in the secondary streak area; 3+, > 10 colonies in the secondary streak area and < 10 colonies in the tertiary streak area; 4+, > 10 colonies in the tertiary streak area. Aerobic and facultative bacteria were identified by standard methods (8). A sample from endocervix was collected with dacron swab so as to isolate *Chlamydia trachomatis* onto cycloheximide-treated McCoy cells, and *Ureaplasma urealyticum* as described by Shepard (14).

Out of 22 patients 12 (54.5%) were found to have one or more organisms with recognized clinical significance, and 4 (33.4%) of these had *Ureaplasma urealyticum* associated. *Lactobacillus* sp, *Corynebacterium* sp, coagulase-negative staphylococci, viridans group streptococci were not tabulated. Among the 10 remaining women without any specific bacterial infection, 5 (50%) had *U. urealyticum*. The occurrence of the different microorganisms is shown in Table 1. These organisms were consistently present in, at least 3+, but mostly in 4+ quantities, except for *U. urealyticum*.

Table 1- Microorganisms recovered from 22 cervicovaginal specimens of women with spontaneous abortion or premature rupture of membranes and their relationship with inflamatory cells

Organism	PMN leucocytes		*U. urealyticum*
	With	Without	associated
C. trachomatis	2		2
S. pyogenes	1	1	
S. agalactiae	2	2	1
Candida sp		1	
G. vaginalis	2*	1	1
Organisms not tabulated (≤2+)		10	5

* BV associated with *Trichomonas vaginalis* in one case.

Preterm delivery has been associated with maternal genital infections, most commonly with *Neisseria gonorrhoeae* and group B streptococci (1). Martin *et al.* (9) were the first to report a relationship between cervical occurence of *C. trachomatis* and prematurity. The life cicle of *C. trachomatis*, which has been proved to replicate in human amnion cells, requires cellular death as the organism is released from the infected cell to spread to others. This cythopatic effect produces tissue injury. Symptoms as well as signs of clamydial infection are often either extremely mild or totally absent (4). Recently the presence of bacterial vaginosis in middle or late gestation has been related to PTL, PDT and preterm rupture of membranes (10).

U. urealyticum is an organism prevalent in sexually active women and its role as fetoplacental pathogenic agent is still discussed. We found it present in 40.9% of the women in this research. There is a great amount of literature dealing with compromised pregnancies and such microorganisms, including placental colonization, perinatal morbidity and mortality spontaneous abortion, amnionitis, and chorioamnionitis. All of these contribute significantly to female reprodution failure. *M. hominis* and *U. urealyticum* can colonize the endometrium with, or without evidence of inflammation. Both organisms can invade the amniotic sac in the first 16 or 20 weeks of gestation, in the presence of intact fetal membranes and in the absence of others microorganisms. The evidence of inflamatory cells and ureaplasmas in the amniotic fluid over two month period in the absence of other demonstrable microorganisms, provides a convincing argument to prove those organisms' capacity in actually producing chorioamnionitis by themselves (11). Indirect evidence of association between *U. urealyticum* setorype 4 and pregnancy loss was described by Quinn *et al.* (12), who found higher levels of *U. urealyticum* antibody in women with previous unsuccessful pregnancies compared with those found in normal pregnant women.

The high concentration of potencially pathogenic microorganisms in the vagina and cervix of pregnant woman may increase the possibility of an ascending infection via the cervix, decidua, maternal placenta, and amniotic fluid. Several cervicovaginal microorganisms produce proteases, neuraminidase and mucinase, with may facilitate their passage across the cervical barriers to the lower uterine segment. Collagenases, which may focally contribute to weakness of choroamnion, is also concurrent. One

theory whereby microorganisms may initiate preterm labor deals with the ability of some bacteria to produce enough protease so as to weaken the fetal membrane strength and cause rupture. Schwarez *et al.* (13) demonstrated that lysosomes within fetal membrane cells contain phospholipase A_2 in high concentrations. Phospholipase A_2 releases the arachidonic acid bound to fetal chorioamnion and maternal decidua tissue with the consequent rise in prostaglandin synthesis. That, in turn, stimulates uterine contractions. Benett *et al.* (3) demonstrated that bacterial products of group B streptococci, *Escherichia coli*, and *Bacteroides fragilis* increase the prostagladin synthesis in the membranes. Bejar *et al.* (2) found a high rate of phospholipase A_2 production by anaerobic streptococci, *Fusobacterium* sp, *Bacteroides* sp and *Gardnerella vaginalis*. It has been reported that pregnant women with vaginal colonization by facultative lactobacilli producing H_2O_2 were less likely to have bacterial vaginosis, symptomatic candidiasis, *Mycoplasma hominis*, or viridans streptococci.

In conclusion, the importance of investigate, quantitative as well as qualitative, genital microflora in pregnant women is fairly clear, since it appears to influence on preterm delivery or fetal loss.

ACKNOWLEDGMENT

We thank Antonio Fernando Longo Vidal for his expert medical assistance.

RESUMO

Microbiota acróbica cérvico-vaginal de mulheres com aborto espontâneo ou prematuridade fetal em Araraquara - Brasil

Rotina bacteriológica do conteúdo vaginal e cervical de 22 mulheres com histórico de aborto recente ou ruptura precoce das membranas foi realizada. *Chlamydia trachomatis, Streptococcus pyogenes, Streptococcus agalactiae, Candida* sp e *Gardnerella vaginalis* foram isolados em 54,5% (12) das pacientes. Apesar de *Ureaplasma urealyticum* ter sido frequentemente encontrado (45,5%), somente em 5 das 22 mulheres foi o único microrganismo presente nos materiais analisados. Esses resultados chamam a atenção para a importância de investigação quantitativa bem como qualitativa da microbiota genital em gestantes, tendo em vista ter consequências na gestação.

Palavras-chave: aborto, prematuridade fetal, microbiota vaginal

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Revista de Microbiologia (1998) 29:314-316
ISSN 0001-3714

AGAR DIFFUSION TESTS WITH CEFUROXIME DISKS FOR PREDICTING CEFTRIAXONE SUSCEPTIBILITY AMONG ISOLATES OF *STREPTOCOCCUS PNEUMONIAE*.

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Submitted: February 02, 1998; Returned to authors for corrections: May 25, 1998; Approved: July 23, 1998

SHORT COMMUNICATION

ABSTRACT

The performance of agar diffusion tests using disks of cefuroxime (30μg) for predicting ceftriaxone susceptibility in 33 isolates *of Streptococcus pneumoniae* was studied. All 7 resistant isolates to ceftriaxone (MIC ≥1.0 μg/ml) exhibited zones of inhibition <28mm. The procedure can be easily adapted to clinical laboratories.

Key words: Screening test, resistance, pneumococcus.

Infections due to *Streptococcus pneumoniae* with resistance to third generation cephalosporins are being detected worldwide (2,4,6,7,11,12). Isolates of *S. pneumoniae* showing this behavior limit treatment options, especially in infections located in the central nervous system. Very few information is available about resistance to third generation cephalosporins among *S. pneumoniae* isolated in Brazil. Recently, however, intermediate resistance to ceftriaxone was detected in 2.8% (5/175) strains of *S. pneumoniae* isolated in the south of Brazil (3). Lack of more information about resistance of *S. pneumoniae* against third generation cephalosporin in Brazil may be, at least in part, due to the fact that the determination of minimal inhibitory concentration (MIC) of each particular isolate is necessary to discriminate between resistance (intermediate or frank) and susceptibility. The National Committee for Clinical Laboratory Standards (NCCLS) (9) does not recommend agar diffusion tests for ceftriaxone and cefotaxime for *S. pneumoniae*. Some investigators have studied the performance of agar diffusion tests using different cephalosporins to predict ceftriaxone and cefotaxime resistance among *S. pneumoniae* (5,8). According to a more recent study by Barry and Fuchs (1), 30μg cefuroxime disks would be preferred to predict susceptibility of *S. pneumoniae* to both cefotaxime and ceftriaxone. Such authors proposed that strains showing inhibition zones ≥28 mm in diameter would be predictably susceptible to both drugs.

Our objective in the present study was to investigate

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the use of cefuroxime disks in agar diffusion tests for predicting ceftriaxone susceptibility among *S. pneumoniae* stains isolated in Porto Alegre, RS.

A total of 33 isolates of *S. pneumoniae* isolated in Porto Alegre, RS, was studied. All isolates showed intermediate (MIC between 0.12 and 1.0 μg/ml) or frank resistance (MIC>1.0 μ.g/ml) to penicillin. Agar diffusion tests using 30 μg cefuroxime disks were carried out on Mueller-Hinton agar with 5% defibrinated sheep blood, as recommended by the NCCLS (9). All isolated had theirs MICs to ceftriaxone determined by agar dilution tests on Mueller-Hinton agar with 5% defibrinated sheep blood containing different drug concentrations (in a range between 0.25 and 8.0 μg/ml) (10). Plates of both agar diffusion and agar dilution tests were read after 20-24 hours of incubation at 35°C in CO_2.

We observed six isolates with MICs of 1.0 μg/ml (intermediate resistance) and one with MIC of 4.0 μg/ml (frank resistance) to ceftriaxone. All isolates with resistance to ceftriaxone showed inhibition zones <28 mm around 30 μg cefuroxime disks. Among those isolates with MICs < 1.0 μg/ml (susceptible) to ceftriaxone, 23/26 presented zones of inhibition <28 mm in the agar diffusion test. In three susceptible isolates, however, an inhibition zone < 28 mm was observed (Table 1). So, applying Barry and Fuchs' (1) criteria to our results, we could say that all ceftriaxone resistant strains would have been detected by agar diffusion tests using 30 μg cefuroxime disks and would deserve MIC determination. On the other hand, only 3/26 (11.5%) susceptible strains were classified as "resistant" (zone < 28 mm). Although the study developed by Barry and Fuchs had tested a much larger number of isolates (n=199) containing a larger proportion of resistant strains than our study, the results are quite comparable. In that study, for instance, 18/143 (12.6%) ceftriaxone susceptible strains had inhibition zones < 28 (false "resistant"), as compared to 11.5% observed in our study.

The test using a surrogate disk of cefuroxime appears to be safe for predicting ceftriaxone susceptibilities of *S. pneumoniae*. This procedure can be adapted to the routine of clinical laboratories and the use of this test use would contribute to a better understanding about ceftriaxone resistance of *S. pneumoniae* in Brazil. However, we strongly suggest that strains with zones of inhibition <28mm must be submitted to a dilution procedure to determine MIC for confirmation of resistance and to discriminate between intermediate and frank resistance to ceftriaxone, in order to provide reliable susceptibility test results for the correct therapeutic assessment.

Table 1. Distribution of 33 isolates of *Streptococcus pneumoniae* tested by agar dilution and agar diffusion (30 μg cefuroxime disks)

	Minimal inhibitory concentration by agar dilution (μg/ml)			
zones of inhibition (mm) in agar diffusion	<1.0	1.0	4.0	total
= or > 28	23	0	0	23
<28	3	6	1	10
total	26	6	1	33

ACKNOWLEDGEMENTS

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

RESUMO

Teste de difusão em ágar usando discos de cefuroxima para predizer suscetibilidade à ceftriaxona em *Streptococcus pneumoniae*

O desempenho do teste de difusão em ágar usando-se discos de cefuroxima (30 μg) para predizer suscetibilidade à ceftriaxona foi estudado em 33 cepas de *Streptococcus pneumoniae*. As 7 amostras resistentes à ceftriaxona (CIM ≥1.0 μg/ml) apresentaram zonas de inibição < 28mm. O teste pode ser facilmente adaptado a laboratórios clínicos.

Palavras-chave: triagem, resistência, pneumococo.

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Revista de Microbiologia (1998) 29:317-320
ISSN 0001-3714

CINNABARIN SYNTHESIS BY *PYCNOPORUS SANGUINEUS* STRAINS AND ANTIMICROBIAL ACTIVITY AGAINST BACTERIA FROM FOOD PRODUCTS

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Submitted: March 31, 1998; Returned to authors for corrections: July 21, 1998; Approved: September 08, 1998.

ABSTRACT

Among three strains of *Pycnoporus sanguineus*, MIP 89007 produced more cinnabarin than MIP 95001 and MIP 95002. The antimicrobial activity of cinnabarin was tested against 11 species of bacteria isolated from food. *Bacillus cereus* and *Leuconostoc plantarum* were the most sensitive to cinnabarin, being inhibited by 0.0625 mg/ml. *Klebsiella pneumoniae* was the least sensitive (>4.0 mg/ml).

Key words: *Pycnoporus sanguineus*, cinnabarin, antimicrobial activity, fungus, bacteria.

INTRODUCTION

Cinnabarin is an antibiotic substance produced by *Pycnoporus sanguineus* (Polyporaceae). This compound is an orange pigment which has a basic phenoxazin-3-one structure, with a carbonyl group at C-1, an amino group at C-2 and an hydroxyl group at C-9 (1,3). Production of this substance *in vitro* occurs between the 18th and the 23rd day of incubation (7). Moreover, the synthesis of cinnabarin is increased significantly when the pH of the broth media is adjusted to 9.0, with incubation at 25°C, under light (8).

As this substance has a cromophore orange colour and can transfer this colour to food products, the substance was tested against bacteria obtained from foods.

In this study, the production of cinnabarin by three *P. sanguineus* strains was investigated, and the antimicrobial activity of this substance against bacterial species from food sources was tested.

MATERIALS AND METHODS

Fungal strains: Three isolates, MIP 89007, MIP 95001 and MIP 95002, were obtained from carpophores of *P. sanguineus* collected in Criciúma, Florianópolis and Imbituba, respectively, in the state of Santa Catarina, southern Brazil. The fungus was cultured on potato dextrose agar, and the isolates were maintained in the same medium (slant) and stored at 4°C.

Bacteria: *Staphylococcus aureus* ATCC 25923 strain was used as a bacteria indicator in the antimicrobial dosage. For determinations of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of cinnabarin, the following bacteria, isolated from foods, were used: *Bacillus cereus, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae, Leuconostoc mesenteroides, Leuconostoc plantarum, Pseudomonas aeruginosa, Salmonella* sp., *Salmonella typhimurium* and *Staphylococcus aureus*.

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***Production of cinnabarin by Pycnoporus sanguineus*:** In this experiment, cinnabarin synthesis by *P. sanguineus* MIP 89007, MIP 95001 and MIP 95002 was studied. Each isolate was inoculated into 6 Roux flasks containing 150 ml of potato dextrose broth. In 3 flasks, the pH of the media was adjusted to 7.0 and in the other 3, to 9.0. All cultures were incubated, without shaking, at 25°C, under light. After 20 days of incubation, the cultures were filtered in Whatman n° 1 paper, tared previously. The mycelial masses were weighed and an aliquot of each pellet was left at 105°C for approximately 20 h, for dry weight determination. The remains of the mycelial masses were homogenized in a blender and filtered, and the cinnabarin was extracted from supernatants with ethyl acetate. The solvent was evaporated and the residues were maintained *in vacuum* until the moment of use. The cinnabarin content of the extracts was determined, indirectly, by method described in Smânia *et al.*, 1997 (8). The experiments were carried out in triplicate.

***Cinnabarin quantification*:** The agar diffusion method was used to determine the calibration curve for cinnabarin as described by Smânia *et al.* (8), and to test the antimicrobial activity of the extracts. An overnight culture of *S. aureus* ATCC 25923 was diluted to a final concentration of approximately 10^6 CFU/ml, and the bacterial suspension was spread over the surface of Mueller-Hinton agar, containing five wells of 7 mm diameter. The wells were filled with each one of the ethyl acetate extracts (range 0.5 to 30 mg), in separate experiments. The plates were incubated at 36°C for 20 h. A zone of 9 mm or more in diameter of growth inhibition was defined as a positive result. The concentration of cinnabarin in each extract was estimated by the formula $y = 9.2949x + 12.3926$, where y = diameter of the inhibition zone, and x = amount of cinnabarin (8).

***Minimal inhibitory and minimal bactericidal concentrations*:** The cinnabarin used in this study was obtained from the culture of strain MIP 89007, which was extracted and purified according to the procedure of Smânia *et al* (6). The antimicrobial activity of this substance was tested against 11 bacterial species obtained from food, by determinations of minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) (6). Test strains were cultured overnight at 36°C in Mueller-Hinton broth, diluted to a final concentration of approximately 10^6 CFU/ml and inoculated into tubes containing serial dilutions of cinnabarin and incubated at 36°C for 20 h. The MIC was defined as the lowest concentration of antibiotic, expressed in mg/ml, able to completely inhibit the growth of each bacterial strain. For the MBCs, a subculture of each tube without apparent growth was made on Mueller-Hinton agar. The MBC was the lowest concentration of the antibiotic which yielded a >99.9% reduction in the number of colony forming units.

RESULTS

***Production of cinnabarin by P. sanguineus*:** The biomasses for the three isolates of *P. sanguineus* are shown in Fig. 1. In average, at pH 7.0, the MIP 89007 produced 4.6 g/l, MIP 95001 5.2 g/l and MIP 95002 5.1 g/l of mycelial mass. When the media had the pH adjusted to 9.0, the yields were 6.3, 5.7 and 5.7 g/l, respectively. Fungal growth was similar for the 3 isolates when the cultures were carried out at either pH 7.0 or at pH 9.0 ($p > 0.1$). With respect to cinnabarin synthesis (Fig. 2), the culture of MIP 89007 at pH 7.0 had a yield of 21.6 InU/l, MIP 95001 a yield of 4.2 InU/l, and MIP 95002 a yield of 2.0 InU/l, and at pH 9.0 these yields were 29.3 InU/l, 11.8 InU/l and 3.1 InU/l, respectively. Synthesis of cinnabarin by MIP 89007 and MIP 95001 was significantly increased ($p < 0.01$) when the pH of the broth medium was adjusted to 9.0. At both pH values, MIP 89007 was the best producer of cinnabarin.

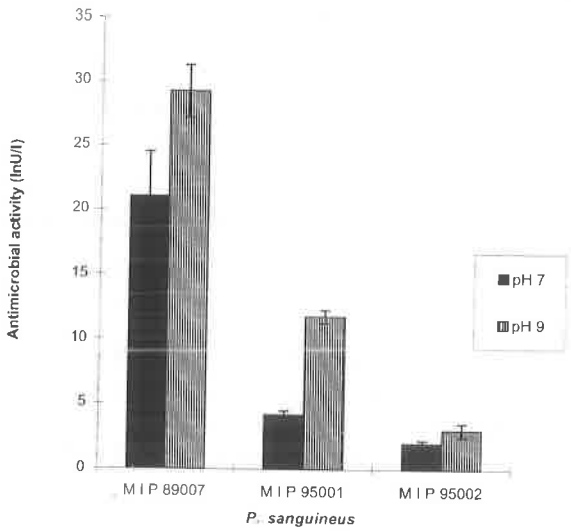

Figure 1 - Production of biomass by three strains of Pycnoporus sanguineus. Error bars indicate standard deviation (triplicate samples).

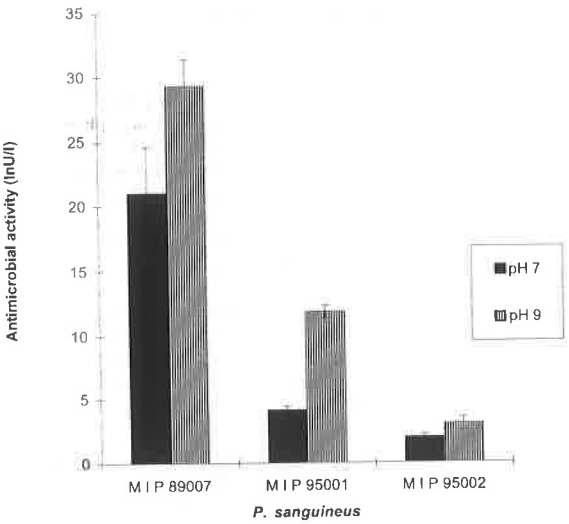

Figure 2- Cinnabarin synthesis by three strains of Pycnoporus sanguineus. Error bars indicate standard deviation (triplicate samples). 1 InU = 1 inhibitory unit = amount (mg) which gives an inhibition equivalent to 1 mg cinnabarin, by diffusion test.

***Antibacterial activity*:** The results obtained for the MICs and the MBCs are shown in Table 1. *B. cereus* and *L. plantarum* were inhibited by the lowest concentration of cinnabarin (0.0625 mg/ml). The Gram-negative bacteria presented the highest values for MIC and MBC. The same MBCs and MICs were detected in *E. faecalis, L. plantarum, Salmonella* sp., *S. typhimurium,* and *S. aureus*. For *K. pneumoniae* and *L. mesenteroides*, the CMB was above the highest concentration used (4 mg/ml). In five of the eleven species the MBC/MIC ratio was 1, in four it was 2, and in the remaining two, this ratio was not determined.

Table 1. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for cinnabarin against 11 bacteria species from food

Microorganisms	MIC[a]	MBC[a]
B. cereus	0.0625	0.125
E. faecalis	2.0	2.0
E. faecium	2.0	4.0
E. coli	2.0	4.0
K. pneumoniae	>4.0	>4.0
L. mesenteroides	2.0	>4.0
L. plantarum	0.0625	0.0625
P. aeruginosa	1.0	2.0
Salmonella sp.	4.0	4.0
S. typhimurium	4.0	4.0
S. aureus	4.0	4.0

[a]Expressed mg/ml

DISCUSSION

P. sanguineus MIP 89007 strain, when cultured at 25°C under light, for 20 days (7,8), is a poor cinnabarin producer. For this reason, new cultures were obtained from carpophores collected in several geographical regions of Santa Catarina State. Among the new isolates, MIP 95001 and MIP 95002 were chosen for this study because they produced strong orange-coloured cultures, suggesting good cinnabarin production. However, the new isolates produced less amounts of cinnabarin than MIP 89007. A better production by the new isolates was expected since these secondary metabolites are usually produced for self defence (9), and MIP 95001 and MIP 95002 were recently obtained from nature. Results previously reported for MIP 89007 indicated a better cinnabarin production at a non-neutral pH (9.0) (8), so the new isolates were also cultivated at two pHs. The influence of pH on secondary metabolite synthesis has also been reported by authors using other non-Basidiomycete fungi species. Shipanova *et al.* (5) evaluated the relationship between pH and biosynthesis of the antibiotic produced by *Fusidium coccineum* (Deuteromycetes) and observed that basic pH favoured the synthesis. *Monascus purpureus* (Ascomycetes) increased production of mycelial mass and pigment ankaflavina when cultivated in acidic pH and in darkness (2).

As described previously (6), cinnabarin is active on some bacteria species obtained from humans. The bacteria isolated from foods, were slightly more resistant than those from human sources. These results agree with those previously described, with the Gram-positives being more susceptible than the Gram-negatives. Antimicrobial activity of pigments has also been investigated by food industries and the same results were reported. *Monascus purpureus* (Ascomycetes) produce hexaketide pigments, and among these, red pigments are used by many Asiatic countries to colour and flavour foods (2). Another pigment used for this purpose is the ß-carotene synthesised by *Phycomyces blaskeleanus* (Zygomycetes) (4).

The results of this study demonstrate that, among the tested strains, the *P. sanguineus* MIP 89007 strain is the best producer of cinnabarin and presents antibacterial activity against bacteria from foods.

ACKNOWLEGMENTS

This study was supported by the Federal University of Santa Catarina (FUNPESQUISA/94).

RESUMO

Síntese de cinabarina por *Pycnoporus sanguineus* e sua atividade antimicrobiana sobre bactérias isoladas de alimentos

Entre três estirpes de *Pycnoporus sanguineus*, MIP 89007 produziu maior quantidade de cinabarina que MIP 95001 e MIP 95002. A atividade antimicrobiana dessa substância foi testada contra 11 espécies de bactérias associadas a fontes alimentares. *Bacillus cereus* e *Leuconostoc plantarum* foram os microrganismos inibidos com a menor concentração de cinabarina (0.0625 mg/ml), e *Klebsiella pneumoniae* foi a espécie menos sensível (>4.0 mg/ml).

Palavras-chave: *Pycnoporus sanguineus*, cinabarina, atividade antimicrobiana, fungo, bactéria.

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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.

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