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MOLECULAR MICROBIAL ECOLOGY: A MINIREVIEW

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ABSTRACT

Our knowledge regarding the ecology of microorganisms in natural environments has long been limited by an almost total reliance on classical cultivation based techniques. However, recent advances in molecular biology techniques, together with ongoing developments in information/computer technologies, have transformed studies on Microbial Ecology and opened a new field, often referred to as 'Molecular Microbial Ecology'. Currently, studies on natural microbial communities are possible even without the necessity of cultivation and new molecular strategies have been developed that allow improvements on gene expression studies as well as on understanding the interactions among microbial communities in ecosystems. Some of the main and/or newest approaches are discussed in this review.

Key words: Microbiology, molecular ecology, environment, biodiversity

INTRODUCTION

It is well known that only a minor fraction of the microbial world has been revealed by the conventional microbiological methods used in Microbial Ecology. Traditional detection techniques require isolation and cultivation of pure cultures in the laboratory and cannot detect the so-called 'nonculturable cells'. Viable microbial cells can only be partially recovered from complex environmental samples by conventional plating methods because we do not yet know the growth conditions for many of them or how to reverse the differentiation of cells into dormant forms, in order to turn them into a viable stage. Actually, a large fraction, often 90-99%, of microbial cells present in environmental samples is not culturable and will therefore escape detection if cultivation-based methods are applied. Other techniques, based on microscopy and fluorescently-labelled antibodies, allow the detection of nonculturable cells. However, they do not always distinguish living and dead cells and usually present problems of background fluorescence and autofluorescence. Since a large percentage of microbial cells in environmental samples may be viable, yet not culturable, their detection via molecular techniques becomes important.

Recently, genetically engineered microorganisms (GEMs) have been constructed by recombinant DNA techniques for environmental applications such as bioremediation, improvement of plant growth and biological control (56, 93). In order to assess the risk associated with the release (deliberate or accidental) of GEMs in the environment, sensitive and accurate detection techniques are required (69, 74).

The development of molecular biology techniques, as well as the technological developments in information/computer technology - Bioinformatics (7, 11) that allow characterization of microbes at the nucleic acid level, have improved the knowledge of

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the ecology of microorganisms and opened a new interdisciplinary field called 'Molecular Microbial Ecology'. During the last few years a number of review papers and books have focused on this subject (2, 3, 35, 79, 92).

In this paper we aim to give an overview of the molecular methods that are being used in Molecular Microbial Ecology, with an emphasis on the most current and promising ones. As we also list a collection of some of the more recent references as well as key articles concerning this subject, the intent is to guide the reader to more detailed papers relating to a particular field of interest. Fig. 1 shows a schematic overview of different molecular approaches that can be used to study microbial communities in the environment.

Isolation of nucleic acids from environmental samples. The extraction and analysis of nucleic acids, both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), from environmental samples such as soil, water and sediment are useful for several purposes (79, 80). Basically, the methodologies for DNA extraction can yield information on the genetic composition of the microbial community whereas methods for RNA extraction are used to determine the activity of the community. However, both approaches can also be used to provide insight in the prevalence and/or activity of specific genes in microbial communities in the environment.

The techniques for isolation of microbial nucleic acids, pioneered by Torsvik and Goksoyr (76) and Torsvik (77), provide an excellent basis for future methods of DNA extraction from complex environments such as soils and sediments (28, 50). Most of the protocols developed for extraction of environmental nucleic acids are based on the following sequential steps: (a) cell lysis or cell recovery and lysis, (b) nucleic acid extraction and (c) nucleic acid purification (Table 1).

Nucleic acids obtained from environmental samples consist of a mixture of molecules theoretically representative of the biota present. With these samples, it is necessary to assess both the quality and quantity of the nucleic acid obtained. The quality (e.g. degree of purity and shearing) as well as the quantity of environmental DNA can be checked on agarose gels by comparison with a molecular size marker of known concentration and by eventual scanning of band intensity (e.g. using a Pharmacia LKB UltroScan XL Scanner). Other methods for quantification of DNA obtained from environmental samples include the fluorometric determination of DNA using Hoechst 33258 dye or ethidium bromide (39).

Even though most of the research has focused on the isolation of DNA from environmental samples, RNA extraction and analysis is an equally important approach, since it analyses bacterial activity in the environment. There are three major classes of RNA: transfer RNA (tRNA) and ribosomal RNA (rRNA), which represent classes of stable DNA, and messenger RNA (mRNA), that is an unstable class (1). Several protocols have been developed in the past few years, which allow the extraction of RNA from marine environments, microbial mats, sediments and soil (3). In general, rRNA and tRNA can be used to provide information on growth rates and diversity of specific organisms or microbial populations, since their abundance is related to the ribosomal copy number per cell, and hence to metabolic activity. On the other hand, the analysis of the mRNA of particular genes (including marker genes) allows studies of gene expression in the environment.

Analysis of environmental nucleic acids. Analysis of environmental DNA and RNA with respect to detection and quantification of specific sequences is commonly done by using hybridization techniques in various ways, e.g. Southern or Northern blotting, dot or slot blotting, colony hybridization or even solution hybridization (28, 63, 64). The presence of target organisms can be detected by direct hybridization of colonies, cell material or DNA with a specific nucleic acid probe. Nucleic acid probes are

Table 1. Different steps in cell lysis, nucleic acid extraction and purification protocols (direct lysis and cell extraction lysis procedure). Adapted from Trevors and Van Elsas (79).

Purpose	Step	Reference
Cell lysis	Lysozyme/SDS treatment	(50)
	Bead beating	(50, 68)
	Ultrasonication	(53)
	Microwave oven	(53)
Extraction	Phenol/chloroform	(63)
Concentration	Precipitations with ethanol or isopropanol	(63)
Purification	CsCl/EtBr density gradient centrifugation	(28)
Precipitation of impurities	CsCl or KAc treatment	(68)
Purification	Gel electrophoresis	(84)
	Hydroxyapatite chromatography	(50)
	Elutip-d elution	(53)
	Spun column (resin)	(61, 85)
	CTAB treatment	(61)

SDS, sodium dodecyl sulphate; CTAB, cetyltrimethylammonium bromide.

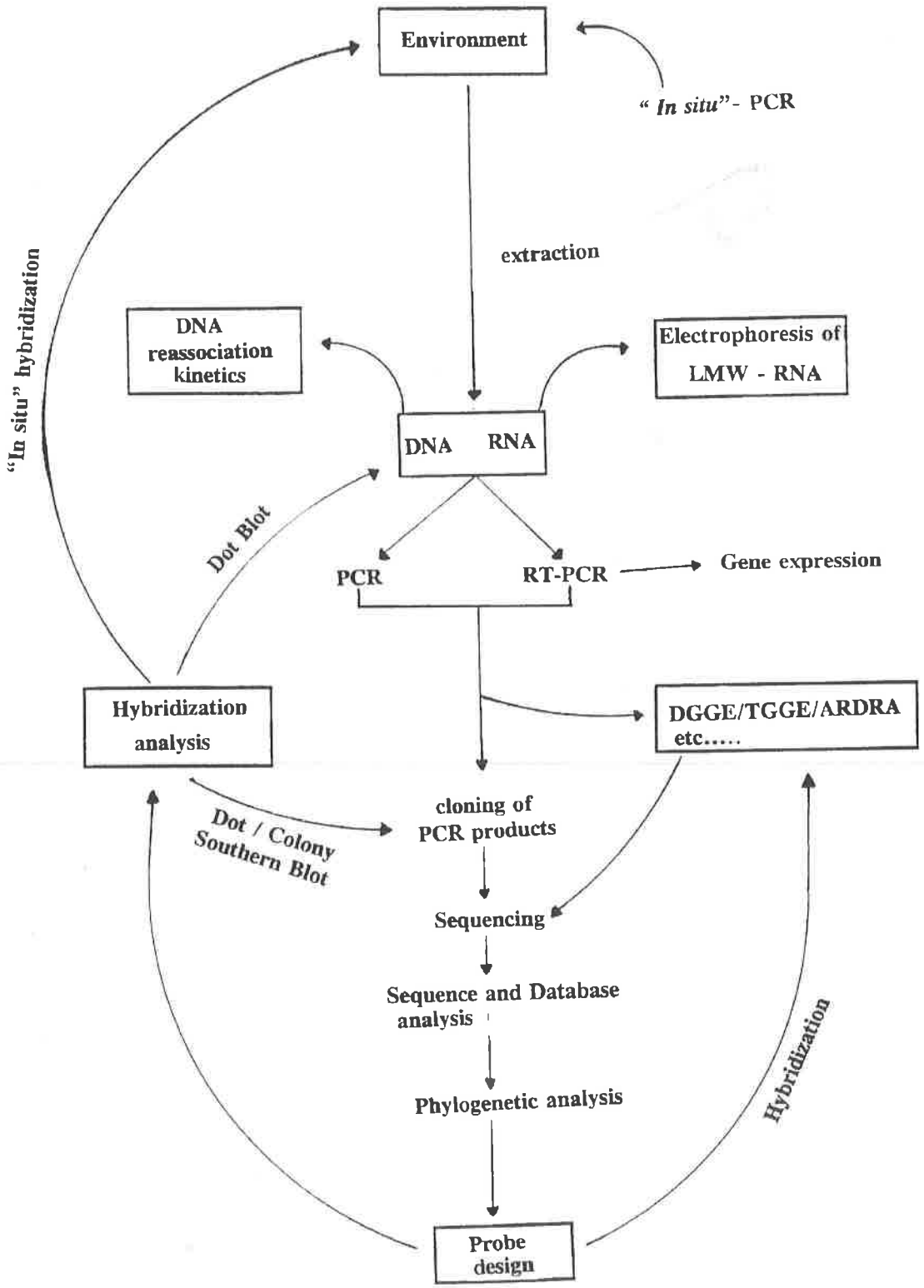


Figure 1. Schematic diagram showing an overview of different molecular techniques for characterizing microbes in environmental samples. See text for a full discussion.

nucleotide sequences complementary to sequences in the target nucleic acid. The probes can be either DNA or RNA and labelled with radioisotopes [e.g. α-P^{32} (65, 15)] or with non-radioactive reporter molecules [e.g. digoxigenin or biotin (16)]. Various parameters, including hybridization and wash temperatures, probe length, complexity and concentration, ionic strength of hybridization solution, and the presence of adjuvants (e.g. formamide) can be manipulated to affect the rate of nucleic acid hybrid formation and the stability of the resulting probe-target hybrids in hybridization experiments (47).

Hybridization of DNA extracted from whole communities against that obtained from individual species, or even other communities, allows the determination of the presence or absence of defined taxa, and may be useful in assessing the spatial and temporal distribution of defined and undefined community members (65).

Another powerful approach is the application of fluorescent phylogenetic oligonucleotide probes binding to intracellular rRNA within fixed whole cells taken directly from their natural environments, a process called phylogenetic staining (5, 18). Different probes can be labelled with different fluorescent dyes (57) and the methodology can be greatly improved by using scanning confocal laser microscopy (SCLM) to localize the bacteria *in situ* (5).

Torsvik *et al.* (78) used DNA reassociation analysis to determine the diversity of microbial communities in soil from Norway. Their results showed that the genetic diversity (measure of the number and frequency of genetically diverse microorganisms in a population or community) of soil microbial communities is ~200-fold higher than the diversity of the isolated bacteria, and therefore the number of species in a single soil sample may even exceed the number of all described prokaryotic species (4).

Although these hybridization techniques are very useful, a general lack of sensitivity is a major drawback, limiting the analysis to populations of cells or genes which occur in relatively high numbers in the environmental samples (84).

Polymerase chain reaction (PCR) in environmental microbiology. PCR amplification (62, 72) of nucleic acids (DNA/RNA) from environmental samples is a major step forward in the study of microbial ecology. PCR is based on repeated cyclic enzymatic extension of primers at two opposite ends of a DNA template. Generally, the amplification cycle involves repetitive cycling between a high temperature to melt the DNA (often 94°C), a relatively low temperature to allow the annealing of the primers (e.g 37-60°C) and an intermediate temperature for primer extension (often 70-72°C). Due to the high denaturing temperature, DNA polymerases used in PCR have to be thermostable (e.g. *Thermus aquaticus Taq* polymerase). In general, PCR amplification is at least 2-3 orders of magnitude more sensitive than DNA hybridization methods (20, 75). The specificity and sensitivity of the PCR reaction are dictated by a variety of factors such: the primers used, the cycling regime, reaction components and their concentrations used, the presence of additives (e.g. DMSO, formamide etc.), target DNA copy number, nature of the primer (nested or normal), and the purity of the DNA sample (58, 75). For enhanced sensitivity, PCR and DNA hybridization methods can be combined using a labelled probe to detect the PCR products (9, 21, 59). DNA extracted from the environment can be directly used as a template for PCR amplification, whereas RNA can be reverse transcribed into cDNA using reverse transcriptase (RT-PCR). Multiplication of target sequences can then ensue to facilitate subsequent detection or identification via restriction fragment length polymorphism (RFLP-PCR), hybridization to a specific probe, or cloning, sequencing and sequence analysis (40). Variations of the basic PCR technique, that permit typing of bacteria at the strain level, have been developed. One of these techniques that involves the use of oligonucleotide primer sets constructed in the absence of specific sequence information is the randomly amplified polymorphic DNA PCR (RAPD-PCR) also known as arbitrarily primed PCR [AP-PCR (10)]. We have been using a combination of molecular techniques such as RAPD-PCR and RFLP (restriction fragment length polymorphism) of *nif*H genes to compare *Paenibacillus azotofixans* strains isolated from different plant genera as well as from different soil types (Rosado *et al.*, submitted). A high degree of diversity was observed among the strains tested and the diversity was considered to be independent of the origin of the strains, since various different groups could be found in each plant studied. The results indicated that these methods yielded sensitive indicators of diversity among the strains tested and thus have great potential to assist in understanding microbial community structures and interactions in an ecosystem.

Another rapid and reproducible method for genomic fingerprinting is the repetitive extragenic

palindromic PCR (rep-PCR), which is based on the amplification of repetitive sequences (rep-elements) in a bacterial genome (87). Several rep (repeat) elements are known, such as REP (repetitive enterobacterial palindromic), ERIC (enterobacterial repetitive intergenic consensus) and BOX elements (87). When such a repeat element occurs within amplifiable distance, a PCR product of characteristic size is generated, and hence the bacterial genome can generate a characteristic banding pattern on gel ('fingerprint'). This method can be a very powerful tool to study the diversity within a species at strain level, by analysing the specific fingerprint generated from distinct genomes (17, 87). The specific patterns obtained from each strain, using rep-PCR, can be stored in a database and analysed using software such as 'GELCOMPAR' (66).

Quantification of DNA in environmental samples by PCR. Recently, various techniques have been developed to quantify the number of target sequences in environmental samples via PCR (33, 53, 58). In general, these methods fall in three major categories: (a) extrapolation from a standard curve during the linear range of amplification(QPCR); (b) competitive PCR (cPCR); and (c) most probable number PCR (MPN-PCR). In order to quantify DNA by reference to a standard curve (QPCR), it is necessary to determine the number of amplification cycles resulting in a linear range of amplification. Subsequent amplification cycles are then carried out in this range. Romanowski *et al.* (58) quantified a *nah*A (gene involved in naphthalene degradation) target sequence in soil by laser densitometry of the PCR amplified product and reference to a standard curve. The range of direct proportionality was found to be rather narrow, falling between $2x10^3$ to $2x10^5$ *nah*A targets.

Competitive PCR uses a dilution series of an internal standard, or competitor, which is simultaneously amplified in a series of sample tubes containing an unknown concentration of target DNA to be analysed. The competitor is a fragment of DNA containing the primer binding sites of the target, with a different internal sequence. Because the competitor DNA concentration is known, and the competitor and target templates each are subject to the same conditions during amplification, the original target DNA concentration can be calculated from the ratio of the final concentrations of the PCR amplification products of target and competitor DNA (33). One of the first examples of an environmental application was the use of cPCR for quantification of endomycorrhizal fungi on roots (73).

MPN-PCR is currently being used to quantitate DNA in soil and rhizosphere samples (53, 59). The method is based on the most probable number theory of Cochran (14), and often uses a triplicate three-fold dilution series, after an initial preliminary ten-fold dilution series to estimate the cut-off point (point where a positive PCR reaction turns negative). Then the target number is calculated using an MPN table (48). In a recent paper by our group (59), an MPN-PCR detection method based on amplification of 16S rDNA of *P. azotofixans* in soil and rhizosphere samples was developed. The method was able to assess the impact of stress conditions on *P. azotofixans* in soil and allowed the detection of a positive effect of the wheat rhizosphere on the introduced cells.

The ribosomal RNA approach: implications in molecular ecological studies. Prokaryotic ribosomes consist of three rRNA molecules (5S, 16S and 23S) of different size and about 50 ribosomal proteins. The 16S rRNA molecule consists of around 1540 nucleotides and forms, together with approximately 20 proteins, the 30S small-subunit; the 23S rRNA (2900 nucleotides) together with the 5S rRNA (120 nucleotides) and approximately 30 proteins forms the 50S large-subunit. These two subunits together form the 70S functional ribosome which catalyzes protein synthesis. The rRNA operons (rrn) have been studied extensively for several bacteria. The number of operons varies between organisms, from 1 copy (e.g. mycoplasmas) to up to twelve copies (bacilli) per chromosome (6, 34).

The rRNA molecule species are currently important for studies in microbial ecology. They can be considered the most useful molecular Achronometers@ of evolution (phylogeny) as they meet all characteristics that define a phylogenetic marker: (i) rRNAs are present and have the same function in all organisms; (ii) they originated from a common ancestor, hence they are homologous; (iii) their nucleotide sequences are highly conserved in some regions and contain variable regions; the mutation rate during evolution is relatively low as compared to other organisms; (iv) 16S and 23S rDNA are reasonably large molecules, hence they contain sufficient sequence information to perform statistically significant comparisons; (v) the primary structures of these marker molecules contain independently evolving domains, thus they contain enough variable regions (Fig. 2) to discriminate

between the different molecules; (vi) a great number of sequences are accessible via databases, and sequence alignments are possible, thus facilitating the identification of regions with unique sequences (51, 70, 94). Several papers have shown the usefulness of the application of 16S rDNA sequence information (see Fig. 2) to design primers and/or probes which are genus-, species- or even strain-specific (e.g. 9, 23, 59). Moreover, the intergenic spacers (IGS) of variable lengths that are present between genes arranged in clusters (e.g. the ribosomal operon), are good candidate sequences for use as molecular tools to discriminate between closely related strains (49). For instance, Orso *et al.* (52) used a PCR/RFLP approach to study the IGS regions of two *Nitrobacter* genomic species, that were shown to be taxonomically closely related by 16S sequence analysis, and could successfully differentiate both genomic species (*N. winogradsky* and *Nitrobacter* sp. LL).

More recently, studies based on SSU rRNA (SSU is the small ribosomal subunit RNA, i.e. 16S rRNA in prokaryotes and 18S rRNA in eukaryotes) are being used to study the phylogeny (and the ecology) of fungi and/or yeasts (e.g. 82) and great progress is expected in the understanding of their interactions in the natural environment.

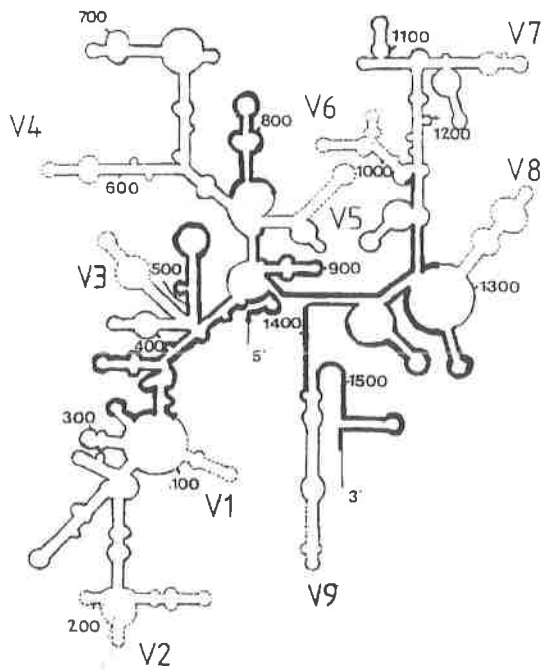


Figure. 2. Secondary structure of 16S rRNA of *Escherichia coli*. Adapted from Ward *et al.* (89), with variable region-numbering according to Van de Peer *et al.* (83). In this scheme, adapted from Harmsen (23), the secondary structure highlights sequence domains that are of nearly universal conservation (thick lines), intermediate conservation (normal lines), or hypervariable domains (dashed lines).

Cloning and sequencing as a tool in molecular microbial ecology. In 1985, Stahl and coworkers started to use the sequences of ribosomal molecules as markers to study microbial communities. 5S rRNA molecules were isolated from relatively simple microbial communities, like those of hot springs and hydrothermal vents (71). However, as discussed above, the 5S molecule (with only 120 nucleotides) contains limited information.

In 1990, an approach based on the extraction of 16S rRNA from microbial mats of hot springs followed by its transcription into cDNA using reverse transcriptase, and subsequent cloning and sequencing (22, 88) revealed that a lot of sequences retrieved from this ecosystem were until then unknown. This finding was in agreement with the concern about the impossibility of culturing the vast majority (90-99%) of microbes occurring in many natural ecosystems using classical techniques.

The introduction of PCR in molecular biology has simplified the methods used for cloning and sequencing of DNA molecules retrieved from the environment. Studies using this technique allow the identification of unculturable and hence so far unknown organisms. The gene commonly used for this purpose encodes the 16S rRNA, since this molecule offers the advantages discussed above (8, 40, 94).

Besides molecular phylogenetic studies of the bacteria present using the 16S rRNA molecule, other studies extended this approach by applying it to functionally important genes, e.g. nitrogenase genes (*nif*H) and ribulose bisphosphate carboxylase (*rbc*L)gene (54, 81, 95). These studies gave new insights in the genetic diversity of the organisms that perform such functions.

Retrieving RNA/DNA sequences directly from the environment has an important advantage over other methods, because it allows the identification and detection of organisms without culturing (so it includes the 90-99% of unculturable bacteria). The advantages and drawbacks of using sequence information obtained from ecosystems have been reviewed (90).

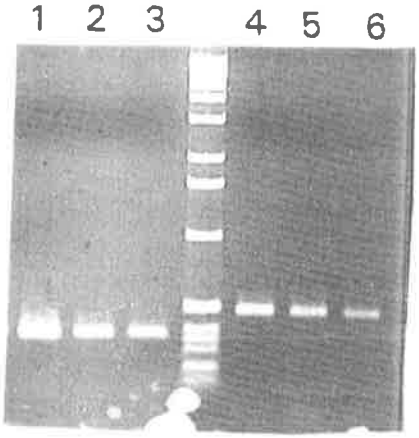
Selected new techniques in molecular microbial ecology. Besides direct analysis of genomic DNA and cloning strategies, additional techniques available

now extend the toolbox of molecular microbial ecology. One strategy for diversity analysis is based on electrophoresis of low-molecular weight (LMW) RNA (5S and tRNA) in high-resolution polyacrylamide gels and has been applied to study the structure and dynamics of a freshwater bacterioplankton community (27).

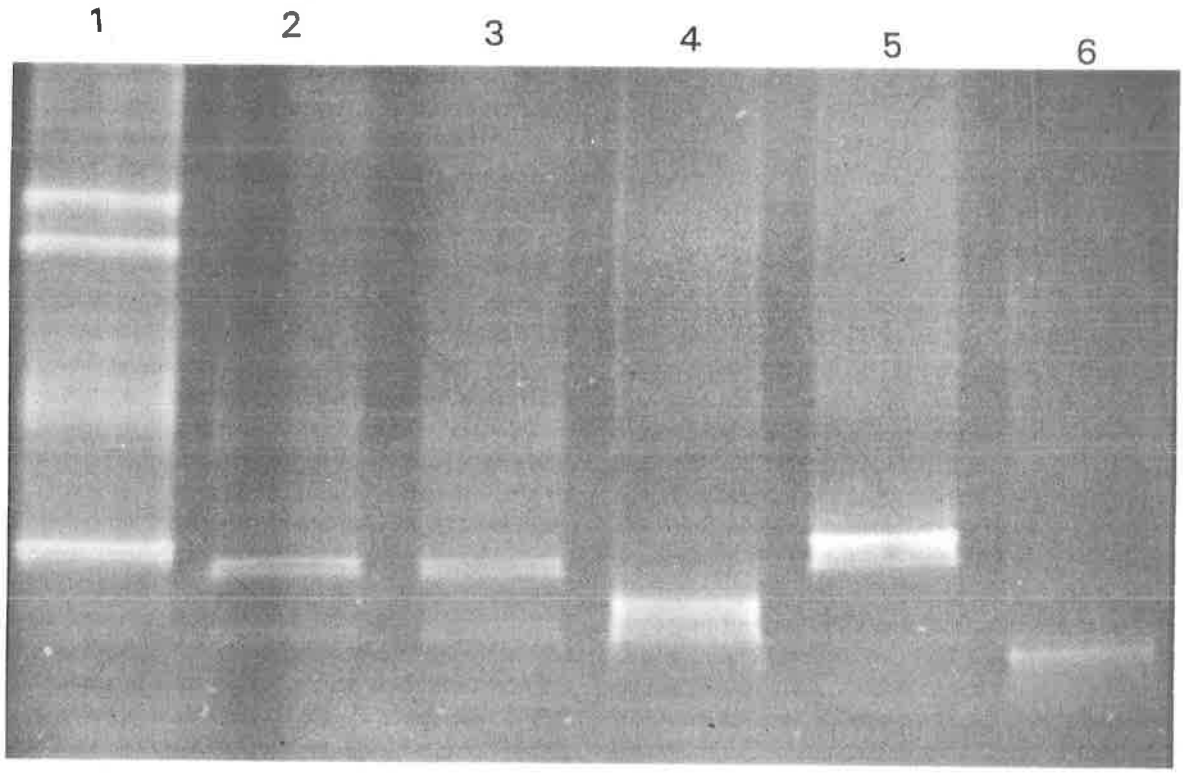
A method termed ARDRA (Amplified Ribosomal DNA Restriction Analysis) is also being used for analyzing mixed bacterial populations (43). This method involves PCR amplification of ribosomal genes (16S or 23S rDNA) followed by restriction endonuclease digestions. The resulting restriction patterns are then used as fingerprints for the identification of bacterial genomes. This method is based on the principle that the restriction sites on the RNA operon are conserved according to phylogenetic patterns (43). Other powerful methods that have been recently introduced in molecular microbial ecology are Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) (25, 45, e.g. Fig. 3). In DGGE, DNA fragments of the same length but with different base-pair sequences can be separated in polyacrylamide gels containing a linear gradient of DNA denaturants (urea and formamide). This separation is based on the decreased difference in mobility of partially melted molecules compared with that of the completely helical form of the molecule (see 46). TGGE works in a way very similar to DGGE, but the denaturing conditions are formed in the polyacrylamide gel by superimposing a temperature gradient on the electric field (25). With these techniques, PCR-amplified 16S rDNA fragments have been used to study the genetic diversity of microbial communities from a range of different environments (19, 25, 45). Separated 16S rDNA fragments can be hybridized with group-specific probes or extracted from the gel, reamplified and sequenced (46). By this method, information about the diversity of bacterial or fungal populations in the ecosystem can be obtained without constructing clone libraries, and the phylogeny of uncultured (or unculturable) forms can be studied. Recently, DGGE has also been used to study the genetic diversity of PCR amplified fragments of important functional genes such as the [NiFe] hydrogenase gene from *Desulfovibrio* spp from experimental bioreactors samples (91), as well as the nitrogenase Fe protein (*nif*H) gene from *Paenibacillus azotofixans* populations in the rhizosphere of crop plants (see reference 60 and Fig. 3) and the desulfurization genes (operon *Sox)* from *Rhodococcus* sp in oil-contamined soil samples (19).

DGGE/TGGE fingerprinting of microbial communities has allowed a rapid determination of the complexity of a microbial community within an environmental sample as well as of the temporal and spatial changes of some of its members. Depending on the primers, either the dominant or rare bacteria can be specifically detected (25). Also very promising is the application of a two-dimensional DGGE system (2D-DGGE). This method involves separation of DNA fragments on the basis of both size and basepair sequence, in polyacrylamide gels containing a gradient of denaturants. This offers the advantage of a higher resolution, making it possible to subject a large number of fragments simultaneously to the mutation detection capability of DGGE.

Another new approach to study diversity of microbial communities is based on PCR and single-strand-conformation polymorphism (SSCP). Under nondenaturing conditions, single stranded DNA has a folded structure which is determined by intramolecular interactions and its nucleotide sequence. The eletrophoretic mobility of the DNA in a gel is dependent not only on its length and molecular weight but also on its shape. Therefore, in PCR-SSCP analysis, DNA fragments with the same size but different sequences can be separated into different bands in polyacryamide gel electrophoresis because of the different mobilities of their folded structure. Using a combination of PCR amplification of 16S rRNA genes and SSCP pattern analysis, Dong-Hun Lee *et al.* (37) were able to distinguish differences among complex bacterial populations in an oligotrophic lake and a eutrophic pond. Most of the techniques currently available allow the measurement of the distribution of various microbial communities and processes at macroscale levels in natural systems. However, at a microscale level (individual cells in a natural bacterial assemblage), the only available methods are immunofluorescence or *in situ* (whole cell) hybridizations with rRNA-targeted nucleic acid probes (5). The latter method relies on the presence of naturally-occurring multiple targets within the bacterial cell to provide a detectable signal and thus is limited in utility by the small number of rRNA molecules in many slow-growing or dormant bacteria from environmental samples. A recently developed technique uses a combination of phylogenetic staining by *in situ* hybridization with the isolation of microbes with 'optical tweezers', consisting of laserbeams that



(A)



(B)

Figure. 3. An example of DGGE analysis of PCR-amplified *nifH* gene fragments (lanes 1-3) and 16S rDNA fragments (lanes 4-6) from different *Paenibacillus* sp. (A) PCR products analyzed by agarose gel electrophoresis. Lanes: 1, *P. azotofixans* C31.4; 2, *P. azotofixans* P3F20; 3, *P. azotofixans* F102; 4, *P. azotofixans* ATCC 35681; 5, *P. polymyxa* DSM 356; 6, *P. macerans* LMD24.3. (B) DGGE analysis of the PCR products shown in panel (A), using a gradient of 25-75% UF (urea/formamide), 60EC, 200V, 4 hours.

are used to separate specific individual cells from the rest of the population in a complex ecosystem (29).

Another potential approach to characterizing the microscale genetic and taxonomic properties of a natural bacterial community is *in situ* PCR (30). This technique, denominated PI-PCR (prokaryotic *in situ* PCR) involves the amplification of specific target nucleic acid sequences (DNA or mRNA) inside intact

prokaryotic cells followed by color or fluorescent detection of the localized PCR product via bright-field or epifluorescence microscopy (30).

Gene expression. Most of the molecular methods discussed above offer new opportunities for characterizing the structures of complex microbial communities. However, for a better understanding of the behaviour and function of natural microorganisms and the fate of released bacteria in different environments, both their presence and activity should be studied.

Microbial gene expression in the environment can be studied at the level of proteins by measuring specific enzyme activities or by analyzing protein synthesis (67). Only recently, data have become available on the detection of mRNA in environmental samples (e.g. 44, 67). This approach is preferable, because the enzyme content of the cell is regulated not only at the transcriptional, but also at the translational level. In many cases, reverse transcriptase dependent (RT)-PCR amplification of total RNA (mRNA/rRNA) recovered by direct lysis from environmental samples can be very useful (67). By analyzing the mRNA of particular genes, including marker genes, studies of the gene expression in microbial cells under environmental conditions can be performed. A possible pitfall in this approach is the fact that the half-life of prokaryotic mRNA is very short compared to eukaryotic mRNA.

Reporter genes. The environmental application of genetically engineered microorganisms (GEMs) is now a reality. This has raised concerns regarding potential risks of such applications. In many countries that have established regulations, the risk of release of each GEM is currently evaluated on a case-by-case basis. Monitoring of GEMs is, therefore, required to evaluate risk factors as well as to obtain information on survival and expression of the heterologous gene (32, 86).

To track GEMs in nature, molecular techniques based on the detection of specific DNA sequences (e.g. probing) of e.g. marker genes or heterologous gene inserts can be used. However, in most cases, tracking the phenotype of the marker gene is more advantageous. A number of useful marker genes have recently been proposed (32, 55). There are a number of general requirements for marker gene systems. The phenotype should not or only slightly be exhibited by the indigenous population, the genes should be stably inserted into the chromosome in order to reduce marker instability and the risk of horizontal gene transfer (32, 55), and the marker gene should, if possible, be selectable or elective (i.e. detectable by some unique feature).

Antibiotic resistance markers (e.g. resistance to kanamycin: *npt*II gene) were the first genes to be used as selectable markers. However, there is a general concern over the use of antibiotic resistance genes and their contribution to the resistance gene pool in nature. Genes encoding metabolic enzymes have therefore been suggested to serve as reporter genes (elective markers). These include *xyl*E (catechol 2,3 dioxygenase), *lac*ZY (β-galactosidase and lactose permease) and *gus*A (β-glucuronidase [GUS]) (32, 55). Recently Tn5-B20 (*lac*Z as a reporter gene) transcriptional fusion mutants of *Pseudomonas fluorescens* R2f were screened for their response to wheat root exudate (86). A mutant showing specific gene expression induced by the proline present in root exudates, but not in bulk soil, was obtained. This approach offers opportunities for ecologically safe and efficient use of genetically modified inoculant bacteria in areas of application, like the biocontrol of soil-borne plant pathogens (86).

Another marker system currently in use employs genes encoding luciferase proteins. These proteins enable organisms to emit visible light, which allows for their detection in environments where this phenotype is rare (e.g. soil). Luciferase proteins and corresponding marker genes fall into two distinct classes (32): (i) the prokaryotic luciferase encoded by *lux*AB (genes originally cloned from the naturally luminescent marine bacteria *Vibrio fischeri* or *V. harveyi*), and (ii) eucaryotic luciferase encoded by the *luc* gene. Bacteria tagged with luciferase marker genes can be monitored in environmental samples by a variety of methods (32) and the *luc* gene system has the versatility of utilization of *luc* gene derivatives, each luminescing at a characteristic wavelength. It can hence be used as a second phenotypic tag (12); the two tags would allow the distinction of two populations on the basis of their different color or luminescence.

The *gfp* gene, that encodes a naturally fluorescent protein, GFP (green fluorescent protein) from jellyfish, *Aequorea victoria* (13), is the most recent reporter gene for use in environmental research. The GFP, unlike the light-emitting proteins, fluoresces under illumination by long-wave ultraviolet light, without exogenous substrate or energy requirements. Recently, this approach was used to monitor the survival of a genetically engineered *Escherichia coli* in aquatic ecosystems (38).

CONCLUDING REMARKS

Molecular Microbial Ecology, as a new field of Ecology, has an important impact on the biological sciences in general, in particular the biotechnological employment of microbes in the environment. The combination of molecular biology techniques and the recent advances in computer technology are leading to new approaches in this area and studies on the exploitation of microbial interactions *in situ* are becoming possible. As from an ecological point of view studies with pure cultures are not very relevant, the molecular techniques that allow *in situ* studies of microbial fate, interactions and diversity (culturable and unculturable/uncultured microbes) are a necessity. Furthermore, the commercial exploitation of microorganisms, genetically modified or not, in the environment for bioremediation or improvement in agricultural biotechnology, has raised concerns about their potential risks. Molecular Microbial Ecology offers tools for a better understanding of the implications of the release (intentional or accidental) of these microorganisms in the environment.

The traditional field of Microbial Ecology has evolved rapidly and significantly, mainly in the light of the exciting insights originating from Molecular Microbial Ecology approaches.

These emerging techniques help to answer some questions raised from studies on microbial communities, about their structure and activity, which cannot be elucidated by traditional microbiological techniques and conventional microscopy. Molecular strategies as cloning and sequencing of DNA from the environment or less time consuming methods as the genetic fingerprinting of microbial communities using DGGE/TGGE analysis of PCR-amplified 16S rDNA or 18S rDNA fragments allow a direct visualization of the phylogenetic diversity of bacteria or fungi in nature, far greater than had ever been thought. Such improved knowledge is important both from a fundamental and an applied perspective.

It should be emphasized, however, that no single technique can provide all the information required to have a fair picture of what is going on in the environment, and hence a combination of molecular techniques and traditional methods remains essential.

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RESUMO

Ecologia Molecular Microbiana: uma minirevisão

Nossos conhecimentos sobre ecologia de microrganismos em ambientes naturais têm sido limitados por se basearem em técnicas clássicas de Microbiologia. Entretanto, os recentes avanços em técnicas de biologia molecular, juntamente com os constantes desenvolvimentos em tecnologias de informação e computadores, têm transformado os estudos de Ecologia Microbiana e abriram uma nova área da Microbiologia denominada Ecologia Molecular Microbiana. Atualmente, estudos sobre comunidades microbianas naturais são possíveis mesmo sem a necesssidade de técnicas baseadas em cultivo, e novas estratégias moleculares têm sido desenvolvidas permitindo a realização de estudos sobre expressão gênica, assim como um melhor entendimento sobre as interações entre comunidades microbianas no ecossistema. Algumas das principais e/ou mais recentes técnicas foram revisadas.

Palavras-chave: Microbiologia, ecologia molecular, meio ambiente, biodiversidade

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XANTHAN PRODUCTION BY *XANTHOMONAS CAMPESTRIS* IN A WHEY-BASED MEDIUM

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SHORT COMMUNICATION

ABSTRACT

Xanthomonas campestris isolates were selected for their ability to use lactose for xanthan production. A minimal whey-based medium (unhydrolyzed and unfiltered whey) was developed containing 0,5% K_2HPO_4; 0,01% $MgSO_4$ and 4% whey giving 14 g Kg^{-1} xanthan. Solutions viscosities of gums obtained from isolates were also evaluated.

Key words: whey, *Xanthomonas campestris*, xanthan gum.

Xanthan gum, produced by *Xanthomonas campestris*, is a bacterial polysaccharide of great commercial significance used as a thickener, stabilizer and suspending agent in the food industry and in other industrial processes (3, 13). While glucose and sucrose are normally used as carbon sources, some alternative substrates, e.g. molasses, glucose syrups, jaggery, have been suggested as cheaper feedstocks for xanthan gum production (4, 12).

Whey from cheese-making has about 4 to 5% lactose, 0.8 to 1.0% protein and small quantities of organic acids, mineral salts and vitamins (1) and it could be used as a substrate for xanthan gum production if lactose-utilizing *Xanthomonas* strains were available.

Stauffer and Leeder (16) reported that *Xanthomonas campestris* strain NRRL B-1459 (Northern Regional Research Laboratories - USA) did not produce viscous broth from unhydrolyzed whey or lactose. Charles and Radjai (1), using hydrolyzed and ultrafiltered whey, showed xanthan conversion levels around 85%. A possible reason for the non-utilization of lactose by *Xanthomonas campestris* may be that the ß-galactosidase of this organism has a low affinity for lactose (5). Schwartz and Bodie (14) isolated a spontaneous derivative of *Xanthomonas campestris* NRRL B-1459 capable of converting whey to xanthan, but the isolate lost productivity when maintained under non-selective conditions. Work has also been done to develop *Xanthomonas* strains with a high capacity for lactose utilization by cloning techniques (6, 7, 17).

In the present work, strains derived from wild isolates of *Xanthomonas campestris* were selected for their ability to utilize lactose and produce xanthan in media containing unhydrolyzed whey and mineral salts. The rheology of gums produced in whey medium was also evaluated.

Isolates from the wild type *Xanthomonas campestris* were obtained in previous work (11). The nine isolates tested and the standard strain NRRL B-1459 were maintained on Difco Yeast-Malt (YM) agar slants at 4°C, and transferred to fresh media every 14 days (8).

Lactose minimal medium (14) contained (g l^{-1}): lactose, 15.0; K_2HPO_4, 5.0; NH_4Cl, 2.0; NaCl, 1.0;

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$MgSO_4$ 0.1; yeast extract 1.0 (pH 7.3). Whey powder provided by CCGL Co. (Teutônia-RS, Brazil) had the following composition (% w/w): lactose 76.81; protein 12.03; mineral salts 8.67; lactic acid 0.065 and fat 1.0.

Whey media were prepared as water solutions to give final whey concentrations of 2%, 3%, 4%, 5% and 6% (w/v) plus 0.01% $MgSO_4$ (w/v) and 0.1% (w/v) yeast extract. A 45% (w/v) K_2HPO_4 solution was autoclaved separately and added asseptically to give a final concentration of 0.5% (w/v) and the pH was adjusted to 7.2 with HCl.

Whey media with different salt compositions were prepared as follows; medium A: 4% whey + 0.5% K_2HPO_4 + 0.01% $MgSO_4$; medium B: 4% whey + 0.1% yeast extract; medium C: 4% whey and medium D: medium A + 0.1% yeast extract.

Wild-type *Xanthomonas campestris* isolates were screened for lactose utilization by serial transfer in a lactose medium, growth being followed by turbidity measurements (610 nm) until viscosity became visible. A loop of this culture was spread on an agar plate containing Lac-min agar and incubated at 28°C. The largest, most viscous, colonies were selected and maintained on Lac-min slants at 4°C.

Isolates were grown on Lac-min agar slants for 24 h at 28°C and a loop of culture inoculated into a tube containing 5 ml of Lac-min broth and shaken at 160 rev min^{-1}, 28°C, 24 h. This starter culture was added to 45 ml of whey broth in a 250 ml Erlenmeyer flask and shaken at 180 rev min^{-1}, 28°C, 72 h.

Lactose was assayed by the DNS method (9). Xanthan dry weight was determined after cell removal as described by Moraine and Rogovin (10) using 10% (w/v) NaCl and three volumes of 95% ethanol. Polysaccharide rheology was evaluated using 1.0% (w/v) aqueous solutions of the extracted xanthan gum plus 0.1% (w/v) KCl. Solutions of commercial xanthan gum (Gelodan X-Grindsted do Brasil, SP) were used as standards for the rheological evaluation. Initial selection of isolates and whey media formulation was assessed by visual inspection of the broth for observable increase in viscosity. Apparent viscosities were measured in a Brookfield LVTDV-II viscosimeter using spindle number 4 at 25°C and different shear rates.

After screening, four isolates (R_1L, R_3L, C_7L and Cv_2C_1L) were able to produce viscous broths using lactose as carbon source. The NRRL B-1459 L selected from NRRL B-1459 by serial transfers in Lac-min medium, did not produce any increase in viscosity, grew slowly and showed smaller colonies than wild isolates.

In whey medium D two isolates, C_7L and R_3L, produced the highest broth viscosity and xanthan concentration. Isolate C_7L, from cabbage, was selected for subsequent experiments.

Table 1 shows that whey concentrations of 4% and 5% (w/v) were better for xanthan production; in medium with 2%, 3% and 6% whey little or no visible viscosity was observed. The rheological behavior of gums obtained from 4% whey medium (data not shown) was better than 5% whey medium; and the medium with 4% whey was chosen for later work.

The pure whey medium (C) and whey plus yeast extract (medium B) did not produce any visual viscosity or xanthan. Medium A (whey plus salts) and D (whey, salts and yeast extract) showed similar xanthan production but medium A showed higher viscosity (data not shown). These data suggest that the addition of yeast extract is not necessary while the phosphate and magnesium salts proved to be important for xanthan production.

Gums obtained from the different isolates had typical pseudoplastic behavior (Fig. 1) and showed lower viscosities than commercial xanthan, with practically no difference between isolates.

Table 1. The effect of whey concentration on xanthan production by *Xanthomonas campestris* C_7L in liquid medium incubated at 28°C for 72 h.

% whey (w/v)	viscosity		xanthan* ($g\ Kg^{-1}$)	lactose used ($g\ l^{-1}$)	yield** (%)
	visible	cP			
2	-	nd	6.5 ± 0.4	14.1	46.0
3	+	3060	10.0 ± 0.4	20.9	47.8
4	+	5110	14.0 ± 0.2	27.7	50.5
5	+++	6250	15.7 ± 0.6	33.8	46.4
6	-	nd	12.0 ± 0.8	40.3	30.0

All data are means (n=3) SD. Whey media contained $MgSO_4$ 0.01%, K_2HPO_4 0.5% and yeast extract 0.1%. * g xanthan/ Kg broth; ** g xanthan/g lactose x 100. cP = centipoise.

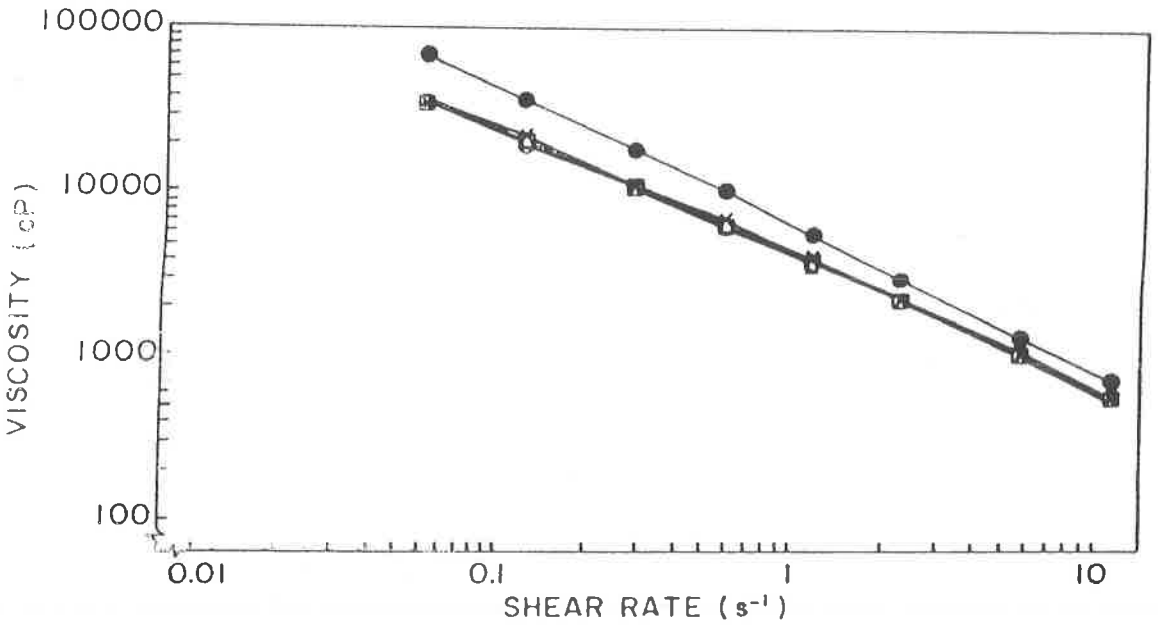

Figure 1. Viscosities of 1.0% aqueous solutions (+0.1% KCl) of gums obtained from the isolates (grown in medium A) and commercial xanthan at different shear rates (25°C). ○, R_1L; △, R_3L; □, C_7L; x, Cv_2C_1L; ●, commercial xanthan

The wild-type isolates were capable of lactose and whey utilization, both for increase in cell number and xanthan production, while isolate NRRL B-1459L used lactose and whey only for growth without any increase in viscosity and no recoverable xanthan. Frank and Somkuti (5) reported that the ß-galactosidase activity was low for some *Xanthomonas campestris* strains, and this may be a factor in the apparent inability of NRRL B-1459 to use lactose or whey as a substrate for xanthan production. The ability of some of the isolates to utilize the alternative substrate could be due to the selection of a new population of bacterial cells which have suitable enzymatic profile.

Broth viscosities were variable depending on whey concentration in the medium; in 2% and 6% whey no viscosity was observed despite the quantity of polysaccharide extracted. This could be due to changes in gum structure and composition resulting in poor quality gums. Souw and Demain (15), using glucose as the carbon source, showed that gum production is affected by sugar concentration, which could be the case in our study.

The magnesium and phosphate salts added were important for xanthan production in whey medium, which discords with the observations of previous study (14). These authors suggested that yeast extract addition and not K_2HPO_4 were essential for producing broths with maximum viscosity while in our study the addition of yeast extract resulted in the production of a lower viscosity gum. These differences between studies reflect the variable composition of whey and underline the need to assess each whey source for its potential as a fermentation substrate.

Although C_7L was chosen for our work based on broth viscosity and final xanthan concentration, Fig. 1 shows that the gums obtained from R_1L, R_3L and Cv_2C_1L have practically the same rheological behavior, in conclusion, the gums qualities are similar. These findings are in agreement with previous work (2,11) that pointed out the importance of final solution viscosities assessment when screening for quality xanthan producers and discussed the value of broth viscosity and colony diameter which could lead to erroneous conclusions. In our case, any of the isolates could be chosen and these strains are currently being investigated for their potential as xanthan producers.

The final xanthan solutions viscosities were obtained using a semi-purified xanthan which may account for the differences seen between these

samples and sample of commercial xanthan which may have been highly purified. In addition, the gums produced in the whey medium could be different from those produced with other substrates, as suggested by Thorne *et al.* (17), who reported the presence of an unknown component in whey which decreased the viscosity of polysaccharide produced.

The yield from whey lactose to xanthan was about 50% and was comparable to similar work (14) that reported an yield of 44% and a xanthan concentration about 11 g Kg^{-1}. The production of xanthan in unhydrolyzed and unfiltered whey showed modest yields and xanthan concentrations when compared to other carbon sources; Souw and Demain (15) using sucrose in synthetic medium reached about 30 g Kg^{-1} in shake flasks experiment. Preliminary work in a fermenter using C7L isolate in whey medium by a batch system shown xanthan concentrations of 14-15 g Kg^{-1}, nevertheless a proposed fed-batch strategy showed a xanthan concentration around 30 g Kg^{-1}.

This work indicates that some isolates of *Xanthomonas campestris* have the ability to use whey as a substrate for xanthan production and that unhydrolyzed and unfiltered whey can be used as an alternative fermentation substrate, but further investigation must be done to evaluate the gum chemical characteristics and industrial viability of the process.

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RESUMO

Produção de goma xantana por *Xanthomonas campestris* em meio de cultura à base de soro de leite

Isodados de *Xanthomonas campestris* foram adaptados à utilização de lactose para a produção de xantana. Um meio mínimo de soro de leite (sem desproteinização e hidrólise) foi desenvolvido contendo 0,5% K_2HPO_4; 0,01% $MgSO_4$ e 4% soro leite gerando 14 g Kg^{-1} de goma. A viscosidade de soluções das gomas obtidas pelos isolados também foi avaliada.

Palavra-chave: goma xantana, soro de leite, *Xanthomonas campestris*.

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CARBOHYDRATE-HYDROLYSING ENZYME ACTIVITY PRODUCTION BY SOLID-STATE CULTURES OF *TRICHODERMA HARZIANUM* STRAINS

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ABSTRACT

Screening of nine *Trichoderma harzianum* strains grown by solid-state fermentation on media containing wheat bran as the carbon source demonstrated that different carbohydrase activities were produced by most of the strains investigated. β-Glucosidase, β-xylosidase, pectinase and α-amylase activities were produced by all strains. α-Amylase, β-xylanase and β-glucosidase activities were the best yields obtained. β-Mannosidase, α-glucuronidase, carboxymethyl-cellulase and FPase activities were poorly produced. The best balance of enzyme activities was obtained from cultures of *T. harzianum* strains 4 and 8. The procedure for enzyme extraction (25 mM sodium acetate buffer, pH 5.0) of *T. harzianum* strain C was compared with a second method using the above buffer solution containing 0.1% tween 80 plus blending in a homogenizer. The results indicated that the best yield of specific activity was obtained from the former extraction procedure. Furthermore, the protein content obtained by using this extraction procedure was very low. Carboxymethyl-cellulase activity was only detected when the second extraction procedure was used.

Key words: biomass, *Trichoderma harzianum*, carbohydrate-hydrolysing enzyme activity

INTRODUCTION

The cell wall of plants is a complex structure composed of polysaccharides associated with non-polysaccharide components (15). Cellulose, hemicellulose, lignin and pectin are in close association in the cell wall (8). Polysaccharides from plant biomass are a valuable source for the production of industrial chemicals, liquid fuels, chemical feedstocks and nutrition for herbivores (12). Starchy materials are also considered part of the biomass (10). Each component may exhibit, depending on the source, considerable variation in composition and structure. The enzyme systems responsible for the breakdown of these complex polysaccharides are essential in recycling carbon fixed by photosynthesis in natural environments and in promoting digestion of plants by rumen microbes (5). The structural complexity of these polymers requires a group of specific enzymes which interact synergistically to carry out their hydrolysis (6). Carbohydrase activities are not only of academic interest since they have potential applications in saccharification of biomass; in pulp, paper, textile and food industries (15). The majority of the microorganisms growing in plant residues are known to produce more than one form of carbohydrate-hydrolysing enzyme activity (14). *Trichoderma* species are reported to produce enzymes involved in the degradation of cellulose, xylan and pectin to fermentable sugars (1, 13). We investigated

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the capacity of *T. harzianum* strains to produce carbohydrate-hydrolysing activities when grown by solid-state cultivation on media containing wheat bran as the carbon source.

MATERIALS AND METHODS

Organism and Enzyme Production. The aerobic fungus *T. harzianum* strain C was kindly provided by C. J. Ulhoa (Universidade de Brasília - Brasil). The following isolates and recombinants of this fungus have been maintained in culture collection at the National Research Center for Monitoring and Environmental Impact Assessment - CNPMA/EMBRAPA: 2 (I3/523 x 609 W5); 3 (2/523 x 609 W5); 4 (CNP 17); 5 (29/523 x 609 W5); 6 (T25); 7 (8/523 x 609 W5); 8 (SS13); and 9 (SS14). *Trichoderma harzianum* strains C, 4 and 6 are wild types, while strains 2, 3, 5, 7, 8, 9 are mutants. The strains 8 (SS13) and 9 (SS14) were isolated from sclerotia of the fungus *Sclerotinia* sclerotiorum. The strains 4 and 6 were kindly provided by the National Research Center of Soybean (Paraná - Brasil) and Samir Michereff (Universidade Federal de Pernambuco - Brasil), respectively. The hybrid recombinants (I3, 2, 29 and 8) came from the cross of the auxotrophic mutants W5 523 and W5 609. These mutants carry distinct auxotrophic markers W5 523 (riboflavina, biotina) and benomyl resistance (500 µg/ml) and W5 609 (arginina). The fungi were maintained at 4°C after growing for 7 days in MYG medium (0.2% malt extract, 0.2% yeast extract, 2% glucose and 2% agar) at 28°C. A 125 ml flask containing agar was inoculated with 30 µl of a spore suspension (1 x 10^7) from a 4 days routine subculture and incubated at 28°C for 7 days. Then, pre-sterilized supplemented substrate, 7 g of wheat bran plus 7 ml of supplemented medium (0.7%KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_47H_2O$, 0.1% $(NH_4)_2SO_4$, 0.06% yeast extract), was added to the flasks and incubation was continued for 7 more days. The contents (other than the agar) were transferred to a 1-liter flask containing pre-sterilized supplemented substrate (7 g of wheat bran plus 7 ml of supplemented medium). Growth was continued for another 7 days (15). Two enzyme extraction procedures were used. The routine procedure was carried out as follows: the contents of the flasks were extracted with 150 ml of 25 mM sodium acetate buffer, pH 5.0. The extracts were incubated with shaking at room temperature for 2 h and centrifuged for 30 min at 5000 rpm. The second extraction procedure used 150 ml of 25 mM sodium acetate buffer, pH 5.0 containing 0.1% (v/v) tween 80, by blending for 20 sec in a homogenizer. The extracts were further incubated and centrifuged as described above. The supernatants obtained from both extraction procedures were stored at 4°C for subsequent use as enzyme assay solutions (16).

Enzyme Assays. In experiments involving p-nitrophenylglycosides, enzyme activities were determined by measuring the amount of p-nitrophenol released from the substrates. The assays were carried out at 50°C in 100 mM sodium acetate buffer, pH 5.0, with p-nitrophenyl-β-D-xyloside, p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-β-D-mannopyranoside, p-nitrophenyl-α-D-glucuronoside and p-nitrophenyl-β-D-glucopyranoside as substrates. The final concentration of these substrates in the reaction mixtures was 0.25 mM. An appropriately diluted enzyme solution (10 - 100 µl) was mixed with each substrate in a total volume of 1.0 ml. The p-nitrophenol released was measured by monitoring the increase in A_{410} nm after 10 min (β-glucosidase and β-xylosidase activities) and 15 min (α-arabinofuranosidase, α-glucuronidase and β-mannosidase activities) of incubation time. The reaction was stopped by the addition of 1.0 ml of 1.0 M sodium carbonate. One unit of activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of p-nitrophenol per min at 50°C (IU). Enzymatic activity was expressed per mg of total protein (IU mg^{-1}). The hydrolysis of polysaccharides (oat spelts xylan, carboxymethyl-cellulose and pectin, 1%, w/v) and filter paper (Whatman No 1) was determined by measuring the release of reducing sugars in 100 mM sodium acetate buffer, pH 5.0, at 50°C for 30 min and 2 h, respectively. The assay volume was 600 µl. Reducing sugars were measured using the dinitrosalicylic reagent (11). Xylose, glucose and galacturonic acid were used as standards. α-Amylase activity was determined as described by Fuwa (7). Enzyme activities were expressed as µmol reducing sugar formed min^{-1} mg^{-1} protein, i.e., as IU mg^{-1}.

Protein Determination. Protein concentration was measured by the method of Bradford (3), using bovine serum albumin as the standard.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate (non-denaturing conditions)

were carried out as described by Laemmli (9) using 12% gels. After electrophoresis, the protein bands were silver stained by the method of Blum *et al.* (4). Samples were electrophoresed on polyacrylamide gel under non-denaturing conditions and containing oat spelts xylan (2). After electrophoresis, the polyacrylamide gel was stained in a Congo red solution (1 mg/ml) for 1 h at room temperature. It was destained with 1 M NaCl and fixed with 0.5% acetic acid. Clear areas in a dark blue background indicated xylanase activity.

Chemicals. Oat spelts xylan, carboxymethyl-cellulose, xylose, p-nitrophenyl-β-D-xyloside, p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-mannopyranoside, p-nitrophenol, pectin, starch, SDS-PAGE calibration kit, non-denaturing electrophoresis calibration kit, sodium dodecyl sulfate (SDS) and Congo red were from Sigma Chemical Co., USA. Wheat bran was obtained from a local source, p-Nitrophenyl-α-D-glucuronoside was a gift from Karin Bronnenmeier (Lehrstuhl für Mikrobiologie, Technische Universität, München, Germany).

RESULTS AND DISCUSSION

The production of several carbohydrate-hydrolysing enzyme activities by solid-state cultivation on wheat bran was studied in strains of *T. harzianum*. From the point of view of economical carbohydrate-hydrolysing activity production, it is desirable to use an inexpensive lignocellulose biomass, like wheat bran (12). Biomass-degrading enzymes are of several types that act synergistically in the complete hydrolysis of lignocellulosic materials (10). Nine strains were screened for β-xylanase, ß-xylosidase, α-arabinofuranosidase, ß-glucosidase,

Table 1 A. Enzyme activity and protein production by solid-state cultures of *Trichoderma harzianum* strains grown on wheat bran.

Strain	Protein mg/ml	Xylanase IU.mg^{-1}	ß-Xylosidase IU. mg^{-1}	α-Arabinofuranosidase IU. mg^{-1}	ß-Mannosidase IU. mg^{-1}	α-Glucuronidase IU. mg^{-1}
C	0.448 ± 0.004	2.315 ± 0.004	0.447 ± 0.036	0.024 ± 0.005	0.018 ± 0.001	0.0016 ± 0.0003
2	0.597 ± 0.004	2.652 ± 0.025	0.336 ± 0.018	0.049 ± 0.003	0.021 ± 0.002	0.0017 ± 0.0008
3	0.529 ± 0.014	2.803 ± 0.017	0.418 ± 0. 013	0.035 ± 0.002	0.012 ± 0. 002	0.0017 ± 0.0010
4	0.322 ± 0.001	4.019 ± 0.056	1.342 ± 0.038	0.091 ± 0.005	ND[a]	0.0002 ± 0.0000
5	0.557 ± 0.003	2.765 ± 0.014	0.204 ± 0.005	0.034 ± 0.003	0.011 ± 0.001	0.0016 ± 0.0006
6	0.521 ± 0.001	2.965 ± 0.042	0.297 ± 0.002	0.048 ± 0.000	0.012 ± 0.003	0.0020 ± 0.0003
7	0.586 ± 0.002	2.606 ± 0.046	0.915 ± 0.017	0.096 ± 0.001	0.002 ± 0.001	0.0005 ± 0.003
8	0.370 ± 0.012	4.124 ± 0.008	0.346 ± 0.002	0.300 ± 0.009	0.013 ± 0.002	ND[a]
9	0.513 ± 0.005	2.770 ± 0.027	0.475 ± 0.003	0.037 ± 0.001	0.002 ± 0.002	ND[a]

Each value represents the mean ± SD for triplicate assays;
[a] not detected.

Table 1 B. Enzyme activity and protein production by solid-state cultures of *Trichoderma harzianum* strains grown on wheat bran.

Strain[1]	Protein mg/ml	Pectinase IU. mg^{-1}	CMCase IU. mg^{-1}	FPase IU. mg^{-1}	ß-Glucosidase IU. mg^{-1}	α-Amylase IU. mg^{-1}
C	0.448 ± 0.004	0.183 ± 0.026	ND[a]	0.001 ± 0.001	2.645 ± 0.029	1.331 ± 0.595
2	0.597 ± 0.004	0.135 ± 0.018	ND[a]	0.002 ± 0.001	1.101 ± 0.214	7.894 ± 0.242
3	0.529 ± 0.014	0.069 ± 0.003	ND[a]	0.002 ± 0.001	1.230 ± 0.040	3.887 ± 0.063
4	0.322 ± 0.001	0.123 ± 0.000	ND[a]	0.009 ± 0.001	4.968 ± 0.666	0.817 ± 0.207
5	0.557 ± 0.003	0.105 ± 0.007	ND[a]	0.001 ± 0.000	0.897 ± 0.003	3.752 ± 0.093
6	0.521 ± 0.001	0.064 ± 0.006	ND[a]	0.005 ± 0.000	0.929 ± 0.266	4.117 ± 0.021
7	0.586 ± 0.002	0.078 ± 0.009	ND[a]	ND[a]	1.763 ± 0.184	5.273 ± 0.493
8	0.370 ± 0.012	0.096 ± 0.031	0.033 ± 0.000	0.012 ± 0.001	1.709 ± 0.030	12.947 ± 1.172
9	0.513 ± 0.005	0.080 ± 0.011	ND[a]	ND[a]	0.131 ± 0.032	9.620 ± 0.650

Each value represents the mean ± SD for triplicate assays;
[a] not detected.

α-glucuronidase, β-mannosidase, pectinase, carboxymethyl-cellulase, FPase and α-amylase activities (Tables 1A, 1B). All strains produced carbohydrate-hydrolysing enzyme activity with variations in the levels of enzyme activity. Strains 4 and 8 were efficient producers of xylan-degrading activities. Screening results showed that all strains were good producers of xylanase activity. The best yields of specific activity were obtained from strains 4 and 8. Most of the cultures showed a weak but detectable β-mannosidase and α-glucuronidase activities. All cultures produced β-xylosidase (15) and ß-glucosidase activities. The highest specific activity was observed in the extracts of the strain 4. FPase activity was generally very low. Pectinase activity was detected at low level in all strains. All cultures, with the exception of strain 8, were not active against carboxymethyl-cellulose. Furthermore, strain 8 gave the best yields of α-arabinofuranosidase and α-amylase activities.

Two enzyme extraction procedures were used on the cultures of strain C. In comparing the two procedures, we observed that the best yields of β-xylanase and α-amylase activities were obtained by extracting without tween 80 (Table 2 - Procedure One). It is noteworthy that the protein content found for this former procedure was very low. Carboxymethyl-cellulase activity was only detected in cultures of strain C extracted with tween 80.

The influence of wheat bran on the synthesis of carbohydate-hydrolysing enzyme activity was examined by electrophoresis. Analysis of the crude extract preparations by SDS-PAGE revealed protein bands with a molecular weight range from 14 to 66 kDa (Fig. 1). It should be noted that a pronounced protein band of 55 kDa was detected in most of the samples. Each crude extract produced some protein bands coincident with those staining for β-xylanase activity after non-denaturing electrophoresis (result not shown). A clear hydrolysis zone was formed against a dark background.

In conclusion, a number of *T. harzianum* strains were found to contain a group of enzyme activities able to breakdown of cellulose, hemicellulose, starch and pectin when grown on wheat bran as the carbon source. Some points have to be considered in the use of enzymes in the processing of agricultural and forestry materials and upgrading of the products. These complex structures have materials that interact with one another in ways that are not yet fully understood (6, 10). The biological conversion of these structures must require several enzyme systems which interact synergistically to effect the release of products, such as single-cell protein, fuel and other chemicals (6, 14).

Table 2. Effect of the extraction procedures in enzyme activities of *Trichoderma harzianum* strain C.

Activity[a]	Extraction Procedure One[b]	Extraction Procedure Two[c]
	IU. mg^{-1}	IU. mg^{-1}
Xylanase	2.315 ± 0.004	1.864 ± 0.028
ß-xylosidase	0.447 ± 0.036	0.334 ± 0.007
α-arabinofuranosidase	0.024 ± 0.005	0.041 ± 0.001
ß-mannosidase	0.018 ± 0.001	0.018 ± 0.001
α-glucuronidase	0.002 ± 0.0003	0.001 ± 0.0001
ß-glicosidase	2.645 ± 0.029	2.041 ± 0.124
Pectinase	0.059 ± 0.008	0.040 ± 0.018
CMCase	ND[d]	0.002 ± 0.000
Fpase	0.001 ± 0.001	0.001 ± 0.000
α-amylase	7.894 ± 0.242	2.452 ± 0.005

Protein contents 0.448 ± 0.004 and 0.677 ± 0.0005 for extraction procedure one and two, respectively.
[a] Each value represents the mean ± SD for triplicate assays;
[b] 25 mM sodium acetate buffer pH 5.0;
[c] 25 mM sodium acetate buffer pH 5.0 containing 0.1% tween 80 plus blending in a homogeneizer;
[d] not detected.

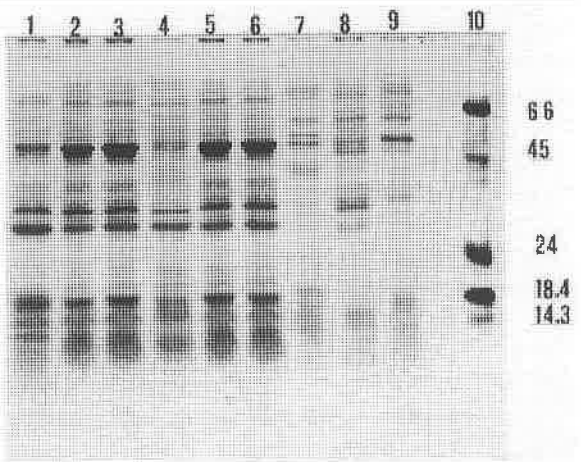

Figure 1. SDS-PAGE banding pattern of proteins from *Trichoderma harzianum* strains in a 12% slab gel stained with silver nitrate. The amount of protein applied to lanes 1-9 was 20 µg. Lane 1-9 crude extracts from *Trichoderma harzianum* strains C, 2, 3, 4, 5, 6, 7, 8 and 9, respectively. Lane 10, molecular mass standards (from the top): bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa).

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RESUMO

Hidrólise de Carboidratos por Enzimas Produzidas por Linhagens de *Trichoderma harzianum* Crescidas em Meio Sólido

Linhagens de ***Trichoderma harzianum*** produzem, quando crescidas em meio sólido contendo farelo de trigo como fonte de carbono, atividades enzimáticas que hidrolisam diferentes tipos de carboidratos. As maiores atividades enzimáticas foram de β-glicosidase, β-xilosidase e α-amilase, enquanto que foram observados níveis muitos baixos de atividades de β-manosidase, α-glucuronidase, carboximetil celulase e FPase. As Linhagens 4 (CNP 17) e 8 (SS 13) foram os melhores produtores de atividade enzimática. O método de extração enzimática na presença de Tween 80, tampão acetato de sódio, 25 mM, pH 5.0 e homogenização em liquidificador foi menos eficiente que o procedimento que utiliza apenas a solução tampão descrita acima. O conteúdo de proteína obtido pelo método de extração na ausência de Tween 80 foi muito baixo. A atividade de carboximetil celulase foi detectada somente através da extração com Tween 80.

Palavras-chave: biomassa, *Trichoderma harzianum*, hidrólise enzimática de carboidrato.

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SCREENING OF ALKALOPHILIC BACTERIA FOR CYCLODEXTRIN GLYCOSYLTRANSFERASE PRODUCTION

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ABSTRACT

Sixty eight cyclodextrin glycosyltransferase-producing alkalophilic bacteria were selected from 400 soil bacteria colonies. Based on the high dextrinizing and CGTase activities of the cell free culture broth, the strain codified as 76 was selected as the source of the enzyme to be studied. The enzyme was partially purified by starch adsorption and some of its properties investigated. Its optimum pH was around 5.4 at 50°C. At pH 8.2 the enzyme activity was 93.3% of its maximum showing high activity in a wide range of pH values. The maximum enzyme activity at pH 5.4 ocurred at 60-65°C and at pH 8.2 it was at 60-70°C. Although the maximum enzyme activity was observed at pH 5.4 this CGTase was less stable at this pH than in buffer solution at pH 8.2. The enzyme in buffer solution at pH 5.4 was significantly activated when maintained for 15 and 30 minutes at 60°C. $CaCl_2$ in concentrations of 10 mM and 20 mM in buffer solutions stabilized the enzyme at both pH 5.4 and 8.2.

Key Words: cyclodextrin glycosyltransferase, *Bacillus circulans*, cyclodextrin.

INTRODUCTION

Cyclodextrins (CDs) are cyclic homogeneous oligosaccharides of six to twelve α-1,4-D linked glucose residues. The most stable CDs are composed of 6, 7 and 8 glucose units named α-, β- and γ-cyclodextrins, respectively. In these compounds glucose molecules are bound together in a hollow toroidal-shaped structure where polar hydroxyl groups of the glucose units are oriented to the outside of the structure conferring a hydrophilic character to the CD molecule. The relatively high electron density in the internal cavity due to the orientation of the hydrogen and oxygen glycosidic atoms confers hydrophobic properties to the inner surface of the molecule (10). These structural features enable the cyclodextrin molecules to form inclusion complexes with many organic substances changing the physical and chemical characteristics of the guest molecules. Due to these particular properties cyclodextrins have been used in food, pharmaceutical, agrochemical, cosmetic, chemical and plastic industries as solubilizers, emulsifiers, masking colors, masking odors and tastes and controlling flavor and hormone release (14).

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a class of microbial enzymes which degrade starch and other α-1,4 glucans to cyclodextrins catalyzing cyclization, coupling and disproportionation reactions (1). As result of the enzyme action α-, β-, and γ-CD are formed in the reaction medium in proportions that vary depending on the selected starch, enzyme properties, medium composition and processing conditions. Since Tilden and Hudson first discovered CGTase in the culture

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filtrate of *Aeromonas macerans* (17), enzymes with different properties have been found in many microorganisms. There are CGTases with optimum pH varying from 4.5-5.0, as for one of the optimum pH of the enzyme produced by alkalophilic *Bacillus* sp 21783 (7), up to 10, as for the enzyme from *Micrococcus* sp ATCC 31606 (16). High values for optimum pH of the enzyme are particularly important when the process for cyclodextrin production includes alkaline treatment of the starch. Most of CGTases have their maximum activity at relatively low temperature around 50-60°C, excepting the enzyme produced by *Thermoanaerobacter* ATCC 53627 which optimum is at 95°C (18). Thermal resistant enzymes have some advantages like a faster reaction, less water cooling consumption and less microbial contamination.

Lately we have dealt with CGTase-producing bacteria isolated from Brazilian soil and in this paper we report on the screening of alkalophilic strains and on the properties of the partially purified enzyme from an selected *Bacillus circulans* which will be further used in the cyclodextrin preparation in alkaline medium.

MATERIALS AND METHODS

Strain isolation. Approximately 1 g of ground soil samples of potato, sweet potato, cassava, peanut, corn and bean crops was suspended in 10 ml of sterilized water. After soil setting the liquid supernatant was properly diluted and one drop of the solution was spread on a plate containing alkaline medium composed of soluble starch 2.0%, peptone 0.5%, yeast extract 0.5%, $MgSO_4.7H_2O$ 0.02%, K_2HPO_4 0.1%, Na_2CO_3 1.0%, phenolphthalein 0.03%, methyl orange 0.01% and agar 1.5% (w/v) (9). After incubation until growth at 37°C colonies surrounded by a yellowish halo due to phenolphtalein-cyclodextrin complex were considered CGTase producers (9). Colonies were first purified on the same medium and later on agar-nutrient plates. Slants of purified CGTase-producing bacteria were prepared in the same isolation medium with neither phenolphthalein nor methyl orange, covered with mineral oil and maintained at 5°C. Microorganisms from the maintenance medium incubated for 5 days at 37°C were employed in all experiments. *Bacillus circulans* var. alkalophilus ATCC 21783, purchased from the American Type Culture Collection was employed as the reference microorganism.

CGTase production. In order to evaluate the ability of the isolated bacteria to produce CGTase in liquid medium, 25 ml of a broth composed as the maintenance medium supplied with $CaCl_2$ 0.05% were inoculated with a loopful of the purified strains. The media contained in 250 ml erlenmeyer flasks were incubated for 5 and 7 days at 150 rpm and 37°C. After centrifugation dextrinizing and CGTase activities were measured in the supernatant of the cultivated broth.

Crude enzyme preparation. Enzyme solution employed in the purification steps were obtained from a inoculum prepared from a loopful of the selected strain inoculated in 25 ml of the enzyme production medium and incubated at 37°C and 150 rpm for 24 hours. One liter Erlenmeyer flasks containing 200 ml of the same medium were inoculated with the whole 25 ml of the inoculum and incubated at 37°C and 150 rpm for 5 days. After cultivation cells were removed by centrifugation at 13,000xg and the enzyme in the supernatant was purified by starch adsorption.

Dextrinizing activity. Dextrinizing activity was assayed using soluble starch as substrate by measuring the decrease in iodine-staining power according to Salva and Moraes (12). One unit of dextrinizing activity (DU) was defined as that which brings about the hydrolysis of 1 mg of starch in 30 minutes in the presence of 5 mg of substrate at the temperature and pH of the experiment.

CGTase activity. Assayed by a slight modification of the Nakamura and Horikoshi (7) method and also by the method presented by Nomoto *et al.* (8), both of which based on the amount of the cyclodextrin synthesized. According to the first method 0.5 ml of the enzyme solution was added to 2 ml of a 3% soluble potato starch solution in borate buffer (50 mM) pH 8.5, at 50°C. After 30 minutes 0.5 ml of trichloroethylene (TCE) was added to the mixture with vigorous stirring and the relative amount of precipitated CD-TCE complex visually estimated. According to Nomoto *et al.* (8) the enzyme solution was successively diluted twice with 25 mM borate buffer (50 mM) pH 8.5 to bring the enzyme concentration from 1:2 up to $1:2^n$. One milliliter of each enzyme dilution was mixed with 5 ml of a 2% soluble potato starch solution in the same buffer. After incubation at 50°C for 48 hours 2.5 ml of TCE were added to the mixture with vigorous stirring. Samples from 5 or 7 days cultivated media were chosen basically considering the results from Nakamura and

Horikoshi (7) method. The enzyme activity was expressed as the maximum enzyme dilution rate in which CD-TCE complex was observed after resting for 24 hours.

Enzyme purification. Enzyme was partially purified by starch adsorption as follows: 500 ml of the enzyme solution was mixed with 3.3 liters of a 4.6% (w/v) corn starch suspension containing 20% (w/v) of ammonium sulfate. The mixture was kept at 5°C for 60 minutes with mild stirring. The starch which adsorbed CGTase was collected by filtration on filter paper Whatman n° 1 under vacuum and washed with 1 liter of 10 mM phosphate buffer pH 7.0 containing 20% (w/v) ammonium sulfate and 1 M NaCl. The enzyme was eluted from the starch with 300 ml of the same phosphate buffer containing 3M NaCl and 0.1 M maltose under constant stirring for 60 minutes at 40°C (3, 7). The enzyme solution was dialyzed 20 hours against 10 mM phosphate buffer pH 7.0. During purification the protein concentration in enzyme solutions was assayed according to Lowry *et al.* (5) using bovine serum albumin as standard protein.

Effect of the pH and temperature on enzyme activity. The effect of the pH and temperature on CGTase activity was followed by the dextrinizing activity. The pH effect was monitored in the pH range from 4.4 up to 9.7. Based on the results of these experiments the effect of the temperature on the enzyme activity was determined at both pH 5.4 and pH 8.2.

In all experiments soluble starch solutions were prepared in the same buffers used for enzyme activity measurements.

Thermal stability of the enzyme. Samples of enzyme solutions diluted 1:5 in buffer solutions at pH 5.4 and pH 8.2 containing 0, 10 and 20 mM $CaCl_2$ were kept for 60 minutes at 60°C and 65°C. The remaining dextrinizing activity was measured at each 15 minutes.

RESULTS AND DISCUSSION

Four hundred colonies were isolated from 115 soil samples. CGTase-producing bacteria were found in the following crop soil samples: 14 in cassava, 9 in corn, 3 in potato, 2 in sweet potato, 3 in bean and 1 in peanut as showed in Table 1. Several soil samples presented more than one CGTase-producing colony completing 68 isolated colonies.

All cultures around which the clear halo was formed in the medium of Park *et al.* (9) showed dextrinizing and CGTase activity in liquid medium. Fifty percent of the strains showed highest dextrinizing activity after 5 days of incubation and the other 50% after 7 days.

Results for CGTase activity of the crude enzyme assayed by the modified Nakamura and Horikoshi (7) method revealed a tendency for a direct relationship between dextrinizing activity and the amount of the CD-TCE complex formed, that is, with the CGTase activity. However some strains did not present such a behavior and even showing low dextrinizing activity presented very high CGTase activity when assayed according to the both methods of CGTase activitiy measurement. These results suggested that these strains produce a CGTase less contaminated by other starch hydrolyzing enzymes.

A CGTase-producing strain isolated from cassava crop soil was selected based on its high dextrinizing and CGTase activities. Cells from the selected strain was rod shaped, Gram positive, strict aerobe and sporeforming. The microorganism grew at pH 7, grew in nutrient broth and in media with 2% NaCl, 3% NaCl and 5% NaCl. Acid was produced from glucose, arabinose, xylose and manitol. The bacteria was inert for O-F test in medium for Gram positive bacteria and did not grow in open or closed tube. The strain did not ferment glucose but could reduce nitrate. It was indol negative, did not use citrate as the only carbon source but hydrolysed starch and casein. The strain was catalase positive and could not hydrolyse gelatin. It grew at 55°C and reduced methylene blue. The strain was initially codified as n° 76 and later identified as *Bacillus circulans* n° 76 in accordance with the description by Sneath *et al.* (15) and similarities with the reference microorganism. When the microorganism was cultivated in liquid medium for 5 days the culture broth showed 36.9 DU/ml and CGTase activity 5+ and 2^9 for Nakamura and Horikoshi (7) and Nomoto *et al.* (8) methods, respectively (Table 1). Enzyme adsorption in starch resulted in high enzyme purification and recovery as shown in Table 2. This method seemed faster and more suitable for the enzyme purification than its precipitation with acetone or 30% $(NH_4)_2SO_4$ followed by precipitation with 70% $(NH_4)_2SO_4$, which resulted in only 3% recovery (results not showed).

The pH-activity curve for the CGTase from the alkalophilic *Bacillus circulans* n° 76 (Fig. 1) was similar to that of crude enzyme from *Bacillus* sp ATCC 21783 (7, 6), and also to those of *Bacillus* sp ATCC 21595 and *Bacillus* sp ATCC 21594 (4) that

Table 1: Enzyme activities in liquid media

Soil origin	Code culture number	Dextrinizing activity (DU/ml)		CGTase activity **		***	
		5 days	7 days	5 days	7 days	5 days	7 days
cassava	11	28.7	8.2	2^7	-	5+	4+
cassava	11p	17.8	5.4	2^4	-	3+	3+
cassava	11.1	41.6	9.6	2^8	-	5+	5+
cassava	14	29.3	9.0	2^8	-	5+	3+
cassava	17	20.1	13.7	2^9	-	1+	2+
corn	19	24.2	6.4	2^6	-	3+	1+
corn	21	0	2.6	2^2	-	1+	-
corn	23	18.6	5.5	2^4	-	3+	1+
corn	26	12.1	4.1	-	2^2	1+	2+
corn	27	33.9	9.2	-	2^3	5+	5+
corn	29	14.2	12.5	2^9	-	6+	1+
potato	43.1	7.0	10.7	-	2^2	1+	2+
potato	44	13.5	11.5	2^2	-	-	2+
potato	51.1	17.0	16.0	2^5	-	5+	2+
potato	51.2	7.9	37.7	2^3	-	1+	1+
potato	51.4	38.3	23.5	2^7	2^7	5+	4+
cassava	76	36.9	32.3	-	2^9	5+	5+
cassava	77	22.4	5.4	2^4	-	3+	2+
cassava	77.1	11.7	14.7	-	2^3	3+	3+
cassava	77.2	3.4	18.2	-	2^2	3+	3+
cassava	77.3	6.0	10.5	2^6	-	3+	2+
cassava	78	5.4	26.8	-	2^2	1+	1+
cassava	78.1	21.0	13.4	-	2^8	4+	3+
cassava	78.2	24.3	10.2	-	2^4	1+	3+
cassava	79.1	20.6	13.4	-	2^9	4+	4+
cassava	79.2	9.2	30.7	-	2^6	3+	3+
cassava	81	13.6	13.2	-	2^5	3+	4+
cassava	82	13.3	7.8	-	2^2	2+	2+
cassava	82.1	10.5	13.2	2^2	2^2	1+	2+
cassava	83	10.3	13.1	2^2	-	3+	2+
cassava	83.1	9.5	30.0	-	2^9	2+	6+
cassava	83.2	17.0	14.8	2^6	-	4+	2+
cassava	83.3	30.6	20.6	-	2^6	3+	4+
cassava	83.4	20.5	8.5	-	2^2	3+	4+
cassava	84	12.9	5.3	-	2^3	4+	5+
cassava	84.1	19.5	18.8	-	2^3	3+	4+
cassava	85	17.6	28.9	2^9	-	5+	5+
cassava	85.1	22.5	17.7	2^9	-	4+	4+
bean	88	16.8	23.2	-	2^7	2+	5+
bean	88.1	18.8	21.8	-	2^4	2+	3+
bean	88.2	18.4	29.4	2^5	2^5	-	2+
bean	88.3	10.2	27.2	-	2^4	3+	4+
bean	88.4	21.0	14.4	2^3	-	3+	4+
bean	88.6	17.5	22.9	2^6	-	4+	3+
bean	89	9.9	10.1	-	2^2	1+	1+
bean	89.1	14.9	12.5	2^6	-	1+	1+

Table 1: Enzyme activities in liquid media (continued)

Soil origin	Code culture number	Dextrinizing activity (DU/ml)		CGTase activity **		***	
		5 days	7 days	5 days	7 days	5 days	7 days
bean	89.2	14.6	19.9	-	2^9	1+	1+
sweet potato	91	7.3	33.8	-	2^8	1+	1+
corn	93	15.6	14.9	-	2^5	2+	5+
corn	93.1	10.4	34.3	-	2^8	2+	3+
peanut	94	9.0	42.9	-	2^7	4+	4+
peanut	94.1	18.6	22.9	2^5	-	2+	3+
corn	99	11.0	16.2	-	2^3	2+	4+
sweet potato	100	16.2	23.0	-	2^6	3+	5+
sweet potato	100.1	19.8	12.2	-	2^5	4+	4+
sweet potato	100.3	8.7	14.2	-	2^4	3+	4+
sweet potato	100.4	9.9	11.9	-	2^2	2+	3+
corn	101	21.7	25.9	-	2^7	5+	5+
corn	101.1	6.8	2.8	-	2^2	3+	3+
corn	101.2	8.5	25.1	2^2	2^3	1+	1+
corn	101.3	1.3	0	2^2	-	1+	1+
bean	103	21.1	30.0	-	2^9	2+	6+
bean	103.1	14.4	10.9	-	2^6	3+	5+
bean	103.2	21.6	31.8	2^7	2^9	5+	5+
bean	103.3	12.6	18.5	-	2^9	3+	4+
bean	103.4	15.8	10.3	2^4	-	2+	2+
cassava	105	12.8	13.7	-	2^4	3+	5+
casssava	106	17.7	24.3	2^5	2$	5+	1+
	B. circulans var. alkalophilus ATCC 21783	34.2	30.8	2^7	2^7	2+	3+

**Nomoto *et al.* (8) method. Highest number preceding + corresponding to highest CD-TCE complex quantity
***Nakamura and Horikoshi (7) method. Highest number preceding + corresponding to highest CD-TCE complex quantity

showed two pH values at which the enzyme had high activity. The maximum activity (17.9 DU/ml) of the enzyme studied in this work occurred at pH 5.4, but at pH 8.2 the enzyme showed an activity of about 93% of that value. Even considering the pH range from 6.1 to 8.9 the minimum value was only 23% of the maximum revealing a broad pH activity profile. Since CGTase was only partially purified by starch adsorption and the enzyme activity was measured by dextrinizing activity it would be possible that one of these optimum pH values was due to contamination of the CGTase by another amylolytic enzymes. Nevertheless, when using the amount of CD-TCE complex formed to determine the enzyme activity it was also high at both pH values (results not showed).

According to Horikoshi and Nakamura (4) acid reaction conditions cause retrogradation of gelatinized starch, particularly at temperatures higher than 40-50°C causing a remarkable reduction of CD production in processes that employ acid or neutral amylases or CGTases. To overcome this problem starch may be hydrolysed with α-amylase (13) prior the action of CGTase and CD formation, but in this process low molecular weight saccharides in the reaction medium make difficult CD recovery. On the contrary an alkaline CGTase, as the present enzyme,

Table 2: Partial purification of the CGTase from *Bacillus circulans* nº 76

Step	Dextrinizing activity (DU)	Protein (mg)	Specific activity (DU/mg)	Purification	Recovery (%)
crude enzyme	12280	1974	6.2	1	100
starch adsorption and dialysis	6240	10.5	594	96	51

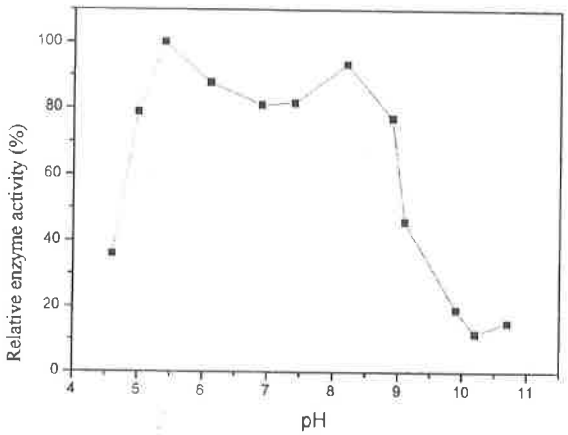

Figure 1. Effect of pH on CGTase activity. Reactions were carried out at 50°C in the following buffer solutions (100mM): acetate-acetic acid (ph 4.4-5.5); phosphate (pH 5.6-8.0); KCl-H_3BO_3-NaOH (pH 8.2-9.1) and Na_3BO_3-NaOH (pH 9.5-10.7)

could be used directely on gelatinized starch dissolved in aqueous NaOH without liquefying.

Moreover, the wide range of the enzyme pH working is interesting for cyclodextrin production because the control of the pH of the enzymatic reaction may be less severe and less reagent may be used to pH adjustments if some α-amylase or glucoamylase is used during the process.

CGTases from *Bacillus* genera have optimum temperatures which vary between 45°C and 70°C (16, 11) and the optimum temperature for the enzyme from *Bacillus circulans* n° 76 was between 60-70°C (Fig. 2). At pH 5.4 the optimum temperature of the CGTase was at 60-65°C, while at pH 8.2 it was at 60-70°C. At temperatures above 60°C the enzyme was more active in alkaline than in acid solution. In these experiments the maximum enzyme activity was detected at pH 8.2 and 70°C. Comparing this maximum enzyme activity with those measured at 75°C, for example, the enzyme showed 65% of activity at pH 5.4 and 92% at pH 8.2.

The enzyme was less sensitive to heat at pH 8.2 than at pH 5.4 (Fig. 3). At pH 8.2 when maintained for 30 minutes at 60°C the variation of the enzyme activity was around 10%. When heated under the same conditions but at pH 5.4, there was an increase of about 40% of the enzyme activity compared to the original activity (Fig. 3). The increase in the enzyme activity due to the heat was also observed in the enzyme heating treatment for 15 minutes and in other studies not reported here.

Loss of CGTase activity was lower in 10 mM and 20 mM $CaCl_2$ buffer solutions than in solutions lacking the salt in both acid and alkaline conditions (Figs. 4 and 5). While the enzyme at pH 5.4 incubated for 60 minutes at 65°C lost 100% of its initial activity in the calcium free solution, when the salt concentration was 20 mM the loss of activity was only 33%. Similarly to the enzyme treated at 60°C at pH 5.4 (Fig. 3), the enzyme activity in buffer solution at pH 8.2 containing 20 mM $CaCl_2$ held for 15, 30 and 60 minutes at 65°C was higher than that not submitted to thermal treatment.

ACKNOWLEDGMENTS

We thank RHAE/CNPq for granting the scholarships in the development of this research. We also thank Neusely Silva for microorganism identification.

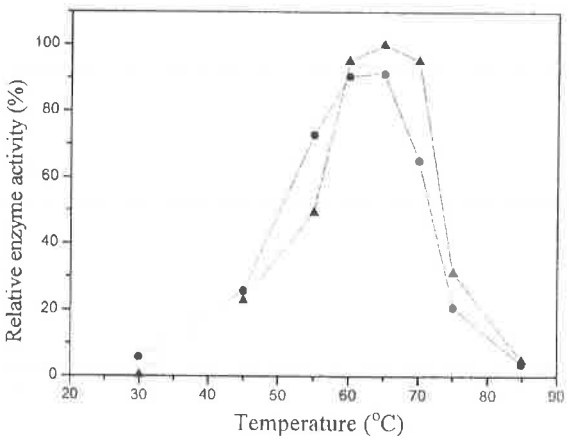

Figure 2. Effect of temperature on CGTase activity. Reactions were carried out at pH 5.4 (●) and pH 8.2 (▲).

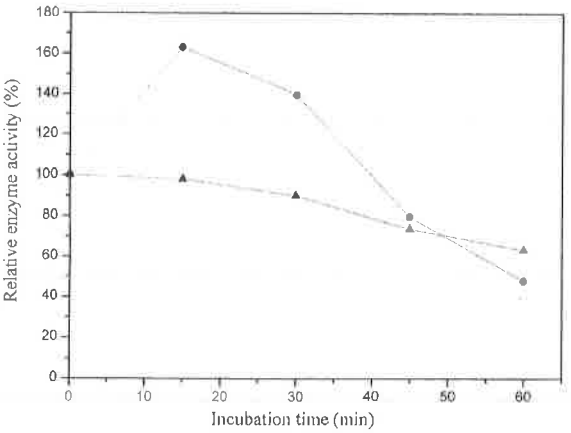

Figure 3. Effect of temperature on CGTase stability. Enzyme in buffer solutions were maintained at 60°C. (●) pH 5.4; (▲) pH 8.2.

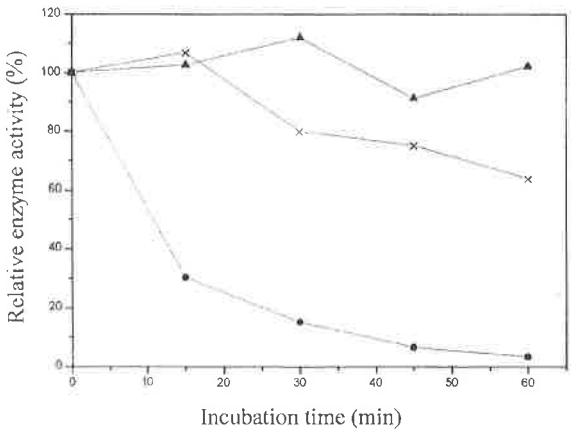

Figure 5. Effect of $CaCl_2$ on thermal stability of CGTase at pH 8.2. Enzyme solutions were maintained at 65°C. (●) 0mN CaCl2; (×) 10 mN $CaCl_2$; (▲) 20 mN CaCl2.

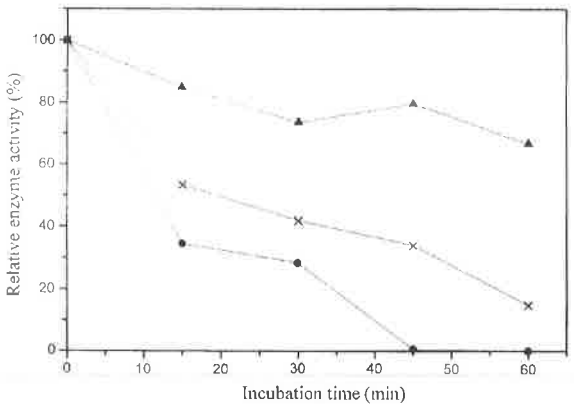

Figure 4. Effect of $CaCl_2$ on thermal stability of CGTase at pH 5.4. Enzyme solutions were maintained at 65°C. (●) 0mN $CaCl_2$; (×) 10mN $CaCl_2$; (▲) 20mN $CaCl_2$.

RESUMO

Isolamento e seleção de bactérias alcalofílicas produtoras de ciclodextrina glicosiltransferase

De 400 colônias de bactérias isoladas de solos cultivados com produtos amiláceos, 68 apresentaram capacidade de produzir ciclodextrina glicosiltransferase (CGTase) em meio alcalino. A enzima produzida pela linhagem codificada como 76, selecionada com base nas elevadas atividades dextrinizante e CGTase do caldo de cultura isento de células, foi parcialmente purificada por adsorção em amido e foram estudadas algumas de suas propriedades. Seu pH ótimo a 50°C esteve ao redor de 5,4, sendo que em pH 8,2 foi observada uma atividade igual a 93,3% da máxima. Sem grande variação, a enzima mostrou elevada atividade na faixa de pH compreendida entre 5,0 e 8,9. Quando em pH 5,4 a atividade enzimática máxima ocorreu entre 60 e 65°C e quando em pH 8,2 na faixa de 60 a 70°C. Embora o pico de atividade enzimática tenha sido observado em pH 5,4 essa CGTase se mostrou menos estável em solução tampão nesse pH do que em solução tampão em pH 8,2. Na solução tampão com pH ácido a enzima foi significativamente ativada quando mantida por 15 e 30 minutos a 60°C. $CaCl_2$ adicionado às soluções tampões em concentrações de 10 mM e 20 mM estabilizou a enzima tanto em pH 5,4 quanto em pH 8,2.

Palavras-chave: ciclodextrina glicosiltransferase, *Bacillus circulans*, ciclodextrina.

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BIOTRANSFORMATION OF LAPACHOL BY *PENICILLIUM CITREONIGRUM* DIERCKX: CHARACTERIZATION OF LOMATIOL

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ABSTRACT

Transformation of lapachol [2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone] by 63 strains of microorganisms was tested. Incubation of this substrate with a filamentous fungus isolated from soil and identified as *Penicillium citreonigrum* Dierckx gave one major metabolite that was characterized as lomatiol [2-hydroxy-3-(4-hydroxy-3-methyl-2-butenyl)-1,4-naphthoquinone] by spectrometric analyses (UV, IR, ^{1}HNMR and MS).

Key words: lapachol, lomatiol, microbiological transformation, *Penicillium citreonigrum,* naphthoquinones

INTRODUCTION

Microorganisms have been shown to possess the ability to transform a wide variety of organic compounds such as hydrocarbons, terpenoids, steroids, alkaloids, antibiotics and aminoacids (1,7,8,18,19). Some compounds of industrial or therapeutic interest are obtained by microbiological transformations (9,10).

Among natural substances of pharmacological importance, arising from Brazilian flora, lapachol(1), a 1,4-naphthoquinone derivative, is of special interest. This compound occurs in abundance, in several species of Bignoniaceae, and has disclosed antibiotic (4,5), antivirotic (11), antitumoral (2,17) and trypanocidal (12) activities.

Previous studies on microbiological transformations of lapachol were performed using *Penicillium notatum* (13), *Curvularia lunata* (14) and *Cunninghamella echinulata* (15). The interest in the microbial transformations of lapachol was due to the fact that these transformations could afford products with hitherto unknown biological activities and thus amplify the potential therapeutic use of this compound.

This report describes the microbial transformation of lapachol(1) to lomatiol(2) by *Penicillium citreonigrum* Dierckx, a filamentous fungus isolated from soil.

MATERIALS AND METHODS

Lapachol. Part of lapachol used in the experiments was extracted from the wood of *Tabebuia serratifolia* (Bignoniaceae). Alternatively, lapachol was purchased from PVP S/A - Parnaíba, Piauí, Brazil.

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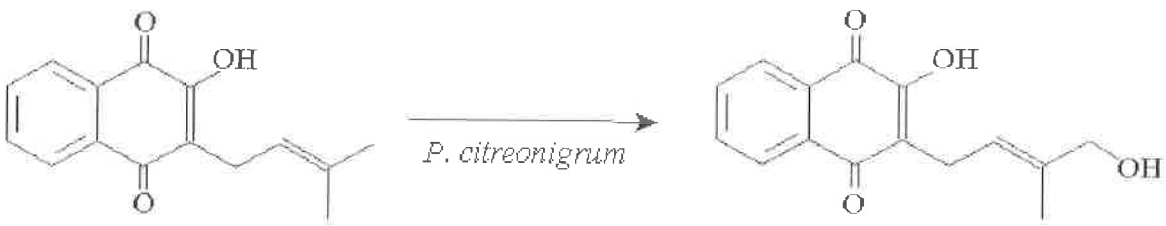

(1) (2)

Microorganisms. Six strains of filamentous bacteria (series GS), 03 non filamentous bacteria and 30 filamentous fungi (series F) isolated from soil and deposited in the Culture Collection of the Laboratory of Industrial Microbiology, Faculty of Pharmacy, Federal University of Minas Gerais were tested. Twenty strains of molds (series M) isolated from the surface of historical monuments of cities in the vicinity of Belo Horizonte were tested, too. Furthermore, the following strains, from the International Culture Collections, were also used: *Cunninghamella elegans* ATCC 10028, *Aspergillus ochraceus* ATCC 1009, *Penicillium notatum* KBR 830 and *Rhizopus nigricans* KBR 1478. All these strains were used in the screening experiments. Stock cultures were maintained on potato dextrose agar (fungi) and nutrient agar (bacteria), at 4°C.

Media. YPBG medium containing 1.0 g yeast extract, 1.0 g beef extract, 1.0 g peptone, and 5.0 g glucose at pH 5.6, in 1.0 l distilled water, was used for screening. SDB medium, composed of 10.0 g peptone and 40.0 g glucose, at pH 5.6, in 1.0 l distilled water, was used for transformation experiments. The components of YPBG and SDB, as well as the maintenance media, were purchased from Biobrás, Montes Claros, MG, Brazil.

Screening. Microorganisms were grown by a two stage fermentation procedure to detect those strains able to metabolize lapachol in YPBG medium. Incubations were carried out in cotton-plugged 125 ml Erlenmeyer flasks containing about one-fifth of their volumes of medium.

Inoculum was prepared by washing the freshly subculture slant of each strain with 10.0 ml sterilized distilled water. To each flask, 1.0 ml of the inoculum was added and incubated for 48 to 72 hours, at 28°C, in a rotary shaker, operating at 150 rpm (Tecnal-Marconi, Piracicaba, SP, Brazil). An aliquot (3.0 ml) was used to inoculate similar flasks with 30.0 ml of fresh YPBG medium and incubated under the same condition. After 24 hours of incubation, lapachol was added as a solution in N,N-dimethylformamide (DMF) to a final concentration of 0.1 g/l.

Two sets of controls were included, one flask contained cells of each microorganism and sterile YPBG broth and another contained sterile YPBG broth and lapachol (controls of metabolic products and lapachol stability, respectively).

Samples (2.0 ml) were withdrawn at various time intervals, acidified to approximately pH 2.0 using 6N HCl, and extracted with 1.0 ml of ethylic ether. The organic phase was separated, treated with anhydrous $NaSO_4$ and filtrated. These extracts were concentrated by evaporation of the solvent with hot air flow. The residues were dissolved in 1.0 ml of ethyl acetate and these solutions were spotted on silica gel thin-layer chromatography (TLC) plates with fluorescent indicator. The chromatography was developed in 3 different systems: chloroform-ethanol (13:1), dichloromethane-ethyl acetate (8:2) and n-hexane-dichloromethane (3:7). The chromatograms were visualized by fluorescence detection under ultraviolet light and by iodine vapor. The reactions were accompanied during 7 days.

Biotransformation by *P. citreonigrum*. The biotransformation with the F4 strain, identified as *P. citreonigrum* according to Pitt (16), was studied in more detail. Its ability to transform lapachol was examined under different conditions such as varying pH (5.6 - 7.0 - 9.0), substrate diluents (N,N-dimethylformamide, ethanol and dimethylsulfoxide), composition of the reaction medium (YPBG medium, SDB medium and citrate buffer solution) and physiological condition of the cells (continuous culture, replacement culture and resting cells) aiming to achieve the most complete transformation. The resting cells were obtained with a culture of 3 days in SDB medium. After vacuum filtration the cells (mycelium) were washed with

pH 5.0 citrate buffer solution and resuspended in the same solution for the reaction.

The biotransformation of lapachol in preparative scale by the strain F4 was carried out as follows: one loop from a slant of *P. citreonigrum* was inoculated into 30.0 ml of SDB-broth and incubated at 30°C for 48 hours, on a rotary shaker at 150 rpm; 3.0 ml of this culture was used as inoculum for 250.0 ml Erlenmeyer flasks containing 50.0 ml of the same medium which was incubated as described above. After 72 hours of incubation, 0.5 g of the substrate dissolved in 5.0 ml of dimethylformamide was added to the culture medium. The incubation was continued for 5 days. Samples (2.0 ml) of cultures broth were withdrawn at various time intervals and submitted to the same extraction procedures used in the screening. Chromatography (TLC) was developed in ethanol-chloroform (13:1) and the chromatograms were visualized under UV light and by iodine vapor.

Extraction and analysis of biotransformation products. After 5 days of incubation, the mycelium was removed by filtration and the culture filtrate was extracted with 3 equal volumes of ethyl acetate. The extracts were combined and the organic solution was dried over anhydrous sodium sulfate, the solvent being removed under reduced pressure, in a rotary evaporator.

A Hitachi model L-4250 HPLC apparatus equipped with a UV detector, at 254 nm, was employed for analytical separations. A sample of the residue was introduced off-column by a AS-2000. A sample injection fitted with a 10 µl sample loop. Reversed-phase separations were achieved with a LiChrospher 100 RP-18 column eluted with mixtures of methanol (MeOH) and water. Solvent program: 0-5 min., 10-30% MeOH; 5-20 min., 30-90% MeOH; 20-40 min., 90% MeOH and 40-60 min., 90-10% MeOH, at a flow rate of 0.8 ml/min..

The residue (328 mg) was applied to the top of a silica gel column chromatography (60; 30x1,5 cm) treated with a solution of 4% KH_2PO_4 using n-hexane, dichloromethane, ethyl acetate and methanol as eluents. Eighteen fractions of 2.0 ml were collected and combined in groups after TLC analyses. TLC was performed with silica gel plates (Kieselgel 60G 7731, 5-40 µm, Merck) which were activated for 50 min. at 110°C, prior to use, and visualized under UV light (254 nm) and by iodine vapor. Fractions 6-7 (8.0 mg), eluted with a mixture of n-hexane and dichloromethane (1:1), were combined and the final purification was carried in a Shimadzu model 6AV HPLC apparatus equipped with a UV detector, at 254 nm. A solution in n-hexane (1.0 ml) of the group of fractions 6-7 was introduced in the HPLC apparatus and separation was carried in a normal-phase Shim-pack Prep SIL(H) column eluted with mixtures of n-hexane and dichloromethane. Solvent program: 0-10 min., 30-20% n-hexane; 10-20 min., 20-10% n-hexane; 20-35 min., 10-5% n-hexane and 35-60 min., 5% n-hexane, at a flow rate of 8.0 ml/min. Fractions were collected according to peaks seen on the monitor read-out and, after evaporation of eluent, lomatiol (5.0 mg) was obtained.

UV and visible spectra were determined on a Perkin-Elmer 202 and 237 recording spectrophotometers. Melting points were determined on a Metler FPS apparatus and are uncorrect. Infrared (IR) spectra were recorded on a Shimadzu/IR - 408 spectrophotometer; crystalline samples were measured in KBr discs (1.0 mg%) and noncrystalline samples were analyzed on neat liquid films between NaCl plates. Proton nuclear magnetic resonance spectra (^{1}HNMR) were determined with a Bruker 80 and a Gemini 200 Varian spectrometer, using tetramethylsilane (TMS) as internal standard. Mass spectra (MS) were determined on a autospec model VG spectrometer.

RESULTS

Preliminary screening was carried out with 63 cultures to identify those capable of metabolizing lapachol. Of these, 11 cultures produced significant amounts of one or more products (Table 1).

Table 1 - Lapachol transformation products detected by TLC.

Fungal strains	Number of products	Rf*
F4	3	0.70 - 0.40 - 0.20
M3	1	0.80
M4	3	0.40 - 0,20 - 0.09
M5	1	0.41
M9	2	0.35 - 0.16
M11	2	0,40 - 0.20
GS2	4	0.68 - 0.63 - 0.23 - 0.11
Cunninghanella elegans	4	0.60 - 0.54 - 0.20 - 0.13
Aspergillus ochraceus	1	0,30
Penicillium notatum	1	0.65
Rhizopus nigricans	4	0.60 - 0.40 - 0.27 - 0.13

*: Rf values between 0.35 - 0.41 correspond to the major product formed in lapachol transformation (Rf lapachol: 0.57-0.60), silica gel TLC plates, eluent: ethanol/chloroform (13:1).

TLC-analyses, eluting with ethanol-chloroform (13:1), indicated that the most frequent product of biotransformation of lapachol showed a red color spot and a lower Rf value than the starting compound. The highest yield of this compound was produced by strain F4, identified as *P. citreonigrum*. This microorganism was selected for the preparative scale transformation aiming to obtain sufficient amount of the crude product for isolation, purification and structural elucidation of the components.

The best culture condition for lapachol transformation by *P. citreonigrum* was found to be continuous culture in SDB medium, at pH 5.6, using DMF as substrate diluent. In such conditions the reaction, in a preparative scale, was first monitored by sampling at regular intervals and analysis by TLC and HPLC (Fig. 1). It was observed that the first transformation product was produced continuously during the reaction and was partially metabolized by the cells. Lapachol was consumed progressively and disappeared completely after 6 to 7 days of reaction. The concentrations of the transformation products were maximum after 72 hours of incubation when two products could be visualized by TLC. Further

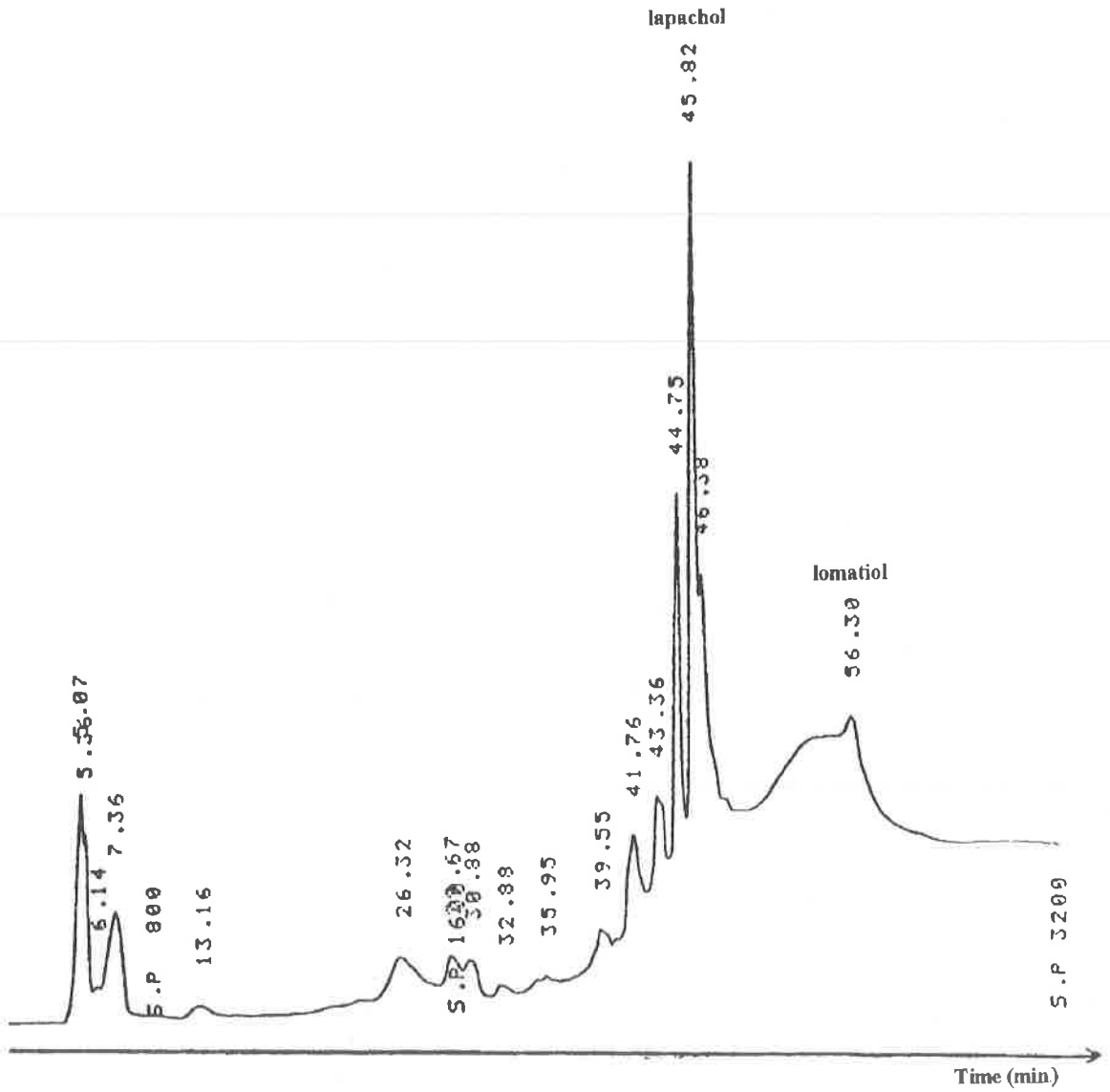

Figure 1 - High performance liquid chromatogram of the crude product from biotransformation of lapachol by *P. citreonigrum*. A 10 µl sample was analyzed with a reversed-phase LiChrospher 100 RP-18 under the conditions described in the text.

Table 2 - ^{1}HNMR spectral data (δ, multiplicity, J) for lomatiol obtained from biotransformation of lapachol by *P. citreonigrum* in comparison with lapachol and literature data[3] ($CDCl_3$, TMS, 200 MHz).

Atributions	Lapachol (δ, J)	Lomatiol (δ, J)	Lomatiol (δ)[3]
$-CH_3$	1.70s and 1.80s	1.85s	1.79s
$-CH_2-$	3.30d (10.0 Hz)	3.30m	3.32d
$-CH_2O-$	-	3.90s	3.91s
-CH=	5.20t (6.8 Hz)	5.50t (6.6 Hz)	5.48m
2H aromatics	7.70m	7.70m	7.82m
2H aromatics	8.00m	8.00m	8.03m
-OH	-	-	9.45s*

*: disappears after D_2O addition.

incubation led to the above mentioned consumption of the primary products and the formation of highly polar compounds. Without the addition of the biocatalyst, lapachol was stable under the conditions and reaction time tested.

The crude product of a 72 hours reaction culture was submitted to silica gel column chromatography and, then, purified by preparative HPLC as described under Materials and Methods. The major transformation product was identified by UV, IR, MS and ^{1}HNMR spectral data.

The UV spectrum of the product, in methanol, revealed absorption maximum at 204; 252 and 275 nm, which are characteristic of naphthoquinones. In the presence of potassium hydroxide and sodium acetate, an intensification of the coloration was observed besides modifications of the spectral curve, that, in comparison with data of literature for quinones (19), indicate the structure of a hydroxynaphthoquinone.

The IR spectrum revealed absorptions at 3300 cm^{-1}, corresponding to hydroxyl group stretching, and at 1670 and 1650 cm^{-1} characteristic of CO groups.

The mass spectrum (MS) showed the molecular ion peak [M+] at m/z 258, indicating one more oxygen atom mass in relation to lapachol (MW 242) and a molecular formula $C_{15}H_{14}O_4$ was deduced. A peak at m/z 227 indicated a loss of CH_2OH from the molecular ion. Peaks at m/z 240, 212 and 197 can be explained by initial loss of H_2O, followed by successive elimination of CO and CH_3. Peaks at m/z 104, 76 and 50 are consistent with naphthoquinones unsubstituted in the benzenoid ring (19).

The ^{1}HNMR spectrum of the product showed two multiplets at δ8.00 and δ7.70 corresponding each one to two hydrogenous of an AA'BB' system confirming the absence of substituents in the benzenoid ring. The spectrum showed also signals of hydrogenous of a phenyl group characterized by a multiplet at δ3.30, corresponding to the two allylic hydrogenous, and a triplet at δ5.50, due to an olefinic hydrogen. Comparison of the ^{1}HNMR data of this product with those for lapachol confirm that the naphthoquinone ring was unaltered, but that the side chain had been modified. The signals for the geminal methyl groups of lapachol were absent and were replaced by singlets at δ1.85 and δ3.90 which correspond to a methyl group and a hydroxymethyl group (CH_2OH), respectively (Table 2). These data are consistent with the structure of lomatiol [(2)].

Biological tests previously performed with lapachol showed antimicrobial activity against different Gram positive and Gram negative microorganisms, including the genera *Bacillus* and *Staphylococcus* (2,4,5). Lomatiol has been tested for antibiotic activity against standard bacteria but no activity was observed (20).

DISCUSSION

Previous studies on the microbial metabolism of lapachol by microorganisms were described by Otten and Rosazza (15). In small-scale screening experiments with 48 microorganisms, 20 cultures which actively metabolized lapachol to one or more metabolites were identified (13, 14, 15). We have carried out a screening to investigate the ability of 63 microorganisms to metabolize lapachol and we have observed that many genera of these microorganisms, particularly fungi, do show this ability. Among the tested microorganisms, many have converted lapachol into different products. The most frequent product showed a red spot on TLC and a Rf smaller than that

of lapachol (0.35 - 0.41, silica gel TLC, ethanol/chloroform 13:1 eluant). The major transformation product of *P. citreonigrum* was identified as lomatiol [2-hydroxy-3-(4-hydroxy-3-methyl-2-butenyl)-1,4-naphthoquinone]. Some strains, mainly bacteria, were able to degrade lapachol completely. Thus, the ability to attack this naphthoquinone seems to be more widespread than originally supposed.

The lomatiol degradation proceeds, probably, via oxidation to carboxylic acids and among the products formed after 5 days of reaction one could be a carboxylic acid or a derivative. Indeed, there are previous evidence on the further oxidation of lomatiol to the corresponding carboxylic acid, known as lomatic acid. Otten and Rosazza (15) and David and cols. (3) described the conversion of lapachol first to lomatiol, followed by lomatiol lactate or acetate and lomatic acid. Further transformation products observed during our work are actually being investigated with respect to the structural elucidation.

The sensitivity of microorganisms to antibiotics and the specific activity of antibiotics are interdependent. Many synthetic and semi-synthetic antibiotics have been used without discrimination, determining the appearance of resistant pathogenic strains to most of them. Much attention has been recently paid to multiresistant strains, particularly to methicillin-resistant *Staphylococcus aureus* (MRSA) which are responsible for the dramatically increasing incidence of infections in hospitals (12). As a consequence of these facts, it has become necessary to develop a continuous research for new antimicrobial drugs. Plants are important sources of many pharmaceutical agents (6). Lapachol, an abundant compound with antimicrobial activity, should be better explored as a therapeutic agent. Search for new antibiotics with broad host-ranges should, therefore, be continued. Hopefully, biocatalysts will be detected that will be able to transform lapachol into a more effective antibiotic.

ACKNOWLEDGMENTS

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RESUMO

Biotransformação do lapachol por *Penicillium citreonigrum* Dierckx: Caracterização do Lomatiol

Testou-se a biotransformação do lapachol [2-hidroxi-3-(3-metil-2-butenil)-1,4-naftoquinona] por 63 cepas de microorganismos. Com um fungo filamentoso, isolado do solo e identificado como *Penicillium citreonigrum* Dierckx, observou-se a formação de um metabólito majoritário. Este fungo foi avaliado mais intensamente como biocatalisador para a obtenção de quantidades suficientes de produtos transformados para elucidação estrutural. Um produto foi isolado e caracterizado como lomatiol [2-hidroxi-3-(-4-hidroxi-3-metil-2-butenil)-1,4-naftoquinona], por análises espectrométricas (UV, IV, RMN ^{1}H e massa).

Palavras-chave: lapachol, lomatiol, biotransformação, *Penicillium citreonigrum*, naftoquinonas.

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KINETICS OF A RECOMBINANT PROTEIN PRODUCTION BY *E.COLI* BL21

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ABSTRACT

Troponin C, TnC, a protein of the chicken skeletal muscle, was produced in cells of *Escherichia coli* BL21 (DE3) pLysS with the cDNA cloned in a pET vector under the control of the *lac UV5 promoter*. The repressed feature of this gene allows a growth phase clearly independent of the production phase. The gas balance data during induction to TnC synthesis in bioreactor runs, showed a cell yield on oxygen, $Y_{X/O}$, between 6.5 and 142 mg/mmol and a TnC fraction (relative to the cell protein content) whithin the cells, between 11.7 and 26.1%, being the higher the $Y_{X/O}$, the lower TnC content. Moreover, the higher the μ value in the beginning of the induction phase, the higher the TnC content. The present data indicate that in the production of TnC under the control of *lac UV5* promoter and T7 promoter by *E.coli*, the energy from aerobic metabolism is mainly directed to recombinant protein synthesis with deleterious effect upon the growth.

Key words: *Escherichia coli*, troponin C, *lac UV5*, T7 promoter, IPTG.

INTRODUCTION

The production level of intracellular recombinant protein is markedly dependent on cell concentration. Consequently, considerable effort has been spent over the past 15 years to develop high cell density cultures of recombinant microorganisms. This is particularly true for *E.coli*, the most common host for heterologous proteins. In the last few years however, plasmid stability and other variables affecting the efficiency of heterologous protein synthesis have received increasing attention (1, 3, 8, 16). The influence of the specific growth rate on the induction must also be considered as it reflects the physiological state of the cells. The reported results, however, are not in agreement. Protein production levels enhanced by high μ values (2), by low μ values (13) and, independent of the μ value (7, 17) are reported in the literature.

The present work deals with cultures of *E.coli* BL21 (DE3) pLysS for the production of troponin C (TnC). This protein takes part of the troponin-tropomyosin complex present in the actin filament of skeletal muscle. TnC has been used in the study of the molecular mechanism of this regulatory protein and the calcium induced conformational change (11, 12). The cDNA of TnC was cloned in a pET vector where the expression is repressed unless the inducer, lactose or IPTG (isopropyl-β-D-thiogalactopyranoside), is present in the culture medium (15).

By means of a shake flask experiment the specific concentration of IPTG relative to cell mass, IPTG/X (w/w), which maximizes the recombinant protein expression in this particular expression system was determined. The determined value of the specific IPTG concentration was employed in three bioreactor experiments in order to achieve the kinetics of

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intracellular accumulation of TnC and respiration data during the induction. The growth phase prior to induction was conducted as either a batch or a fed-batch type process. In this way, different μ values were established at the beginning of the induction phase.

MATERIALS AND METHODS

Microorganism and Inoculum. Lysogenic *Escherichia coli* BL21 (DE3) pLysS pET was used as a host to a pET vector containing the cDNA for TnC (14). The plasmids pLysS and pET contain chloramphenicol and carbenicillin resistance genes respectively. The culture media described had 50 mg/l of chloramphenicol and carbenicillin. For each run, the pET vector was transfected into the bacteria as previously described (11). The transformed microorganism was inoculated on a Petri dish with TYE solid medium (per liter: 10 g bacto-tryptone, 5 g yeast extract, 8 g NaCl, 15 g bacteriological agar; pH 7.5) and incubated overnight at 37°C. Six well grown and isolated colonies were cultured again on TYE solid medium to ensure that only the ones with the pET vector would be selected. The bioreactor inoculum was precultivated in two phases. First, one colony from the second Petri dish was cultivated in 250 ml shake flasks containing 50 ml of 2xTY medium (per liter: 16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl; pH 7.0) for 10 h at 37°C and 200 rpm. Second, 0.5 ml of this preculture was used to inoculate 50 ml medium containing per liter: 10 g glucose, 13 g KH_2PO_4, 10 g K_2HPO_4, 3 g $(NH_4)_2HPO_4$, 4.6 g $MgSO_4.7H_2O$, 1 g yeast extract, and 3 ml micronutrient solution (4). This second preculture was incubated in 500 ml shake flasks for 6 h at 37°C and 200 rpm. The inoculum preparation in two phases ensures a satisfactory reproducibility of the initial cell concentration in the bioreactor as the second phase of the inoculum preparation and the medium employed in the bioreactor, were the same except for the glucose concentration.

Shake Flask Experiment. In an exponentially growing culture of the second phase of the liquid culture inoculum preparation above described, variable volumes of IPTG solution (100 mg/ml) were added to 6 flasks in order to obtain IPTG/X (w/w) values from 2 to 125 mg/g. A cellular concentration as low as 0.6 g/l at the beginning of induction prevented dissolved oxygen limitation.

Bioreactor Experiments. All of the runs were performed in a 4 l Bioflo III system (New Brunswick Scientific, Edison, NJ). The inoculum were added at a ratio of 1% of the initial working volume. While run 1 was a batch type with 40 g/l of glucose, runs 2 and 3 had two growth phases: first, 10 g/l as a batch mode (hours 0 - 8) and second, 10 g/l as a fed-batch (hours 8 - 10). During the fed-batch phase, a glucose solution was fed by means of a peristaltic pump. The feed rate was manually adjusted every 30 min in order to support a μ value of 0.4 h^{-1} for run 2 and 0.3 h^{-1} for run 3 according to equation 1, determined through a mass balance on glucose. Values of $Y_{X/S}$ and X_o were determined in previous runs.

$$F = \frac{Xo.V.\mu.\exp(\mu.t)}{So.Yx/s} \quad (1)$$

F = flow rate of the feeding solution (l/h); X_O = cell concentration (g/l) in the beginning of the fed-batch phase; V = volume of the culture medium (l); μ = predicted specific growth rate (h^{-1}); t = time (h); S_O = concentration of glucose in the feeding solution (g/l); and $Y_{X/S}$ = cell yield on glucose (g/g). Values of the parameters: X_O = 3.3 g/l; V = 4 l; μ = 0.4 h^{-1}; S_O = 500 g/l; $Y_{X/S}$ = 0.35 g/g. The small volume of the glucose solution fed, 250 ml, relative to the culture volume, 4 L, besides the periodical sampling allowed to assume a constant volume of reaction.

After the consumption of 17 g/l in run 1 and 20 g/l in runs 2 and 3, the induction phase was started with the addition of 100 mg/ml IPTG solution, in order to obtain a specific inducer concentration, IPTG/X (w/w), of 100 mg/g. Runs 2 and 3 were also replenished with 20 g/l of glucose while run 1 had a remainder glucose concentration of 23 g/l relative to the 40 g/l of the culture beginning. Other culture conditions: temperature, pH and air rate controlled at 37°C, 7.0 and 1 l/l.min respectively; agitation rate was automatically varied from 700 to 950 rpm in order to maintain the dissolved oxygen level above 20% of the concentration of saturation.

Analytical methods. Samples were periodically taken from the bioreactor cultures in order to analyze dry cell weight, X (DCW), glucose (S), intracellular level of troponin C (%), O_2 and CO_2 molar fraction in the outlet gas. Samples were first chilled in an ice bath and subsequently vacuum filtered through a 0.22 μm membrane during the first 5 hours of cultivation to determine X. During the remainder of the experiment, centrifugation at 9500 g for 5 min was used instead of

filtration. In both cases, the mass of the pellet was determined after drying at 85°C for 6 h (filtered samples), or 32 h (centrifuged samples). Glucose concentration was measured in the clarified medium by means of the enzymatic glucose-oxidase method (Merck, Darmstadt, FRG). Molar fractions of O_2 and CO_2 in the outlet gas were measured by means of a paramagnetic analyzer (Beckmann model 755, Rosemunt Analytical Inc, La Habra, CA, USA) and an infrared spectrophotometer (Automated Custom System Inc. Model 3300, Orange, CA, USA), respectively. Intracellular level of TnC was determined by electrophoresis on SDS-polyacrilamide gel followed by staining with Coomassie blue (9). The TnC fraction was quantified through scanning of the electrophoresis gel bands by a densitometer Schimadzu CS-9000 (JAPAN) at λ=550 nm. The amount of heterologous protein as a percentage of total cell protein (TnC(%)) could then be estimated as the relative area of its correspondent peak on the densitogram minus the background proteins of *E.coli* with molecular weight equivalent to TnC.

RESULTS AND DISCUSSION

Influence of specific concentration of IPTG on protein production. Fig. 1 shows the values of TnC (%) as a function of the specific IPTG concentration, after two hours of induction in the shake flask culture above described. The highest TnC production, 19%, was verified for specific IPTG concentrations higher than 100 mg/g, which was the value adopted for the bioreactor runs.

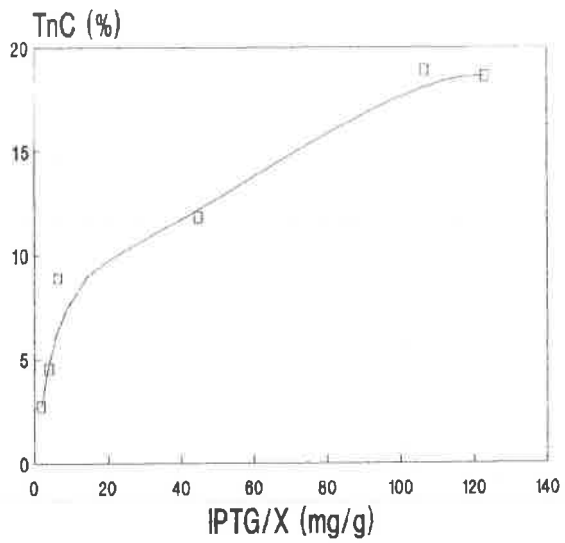

Figure 1: Percentage of total cell protein as Troponin C under variable specific IPTG concentrations.

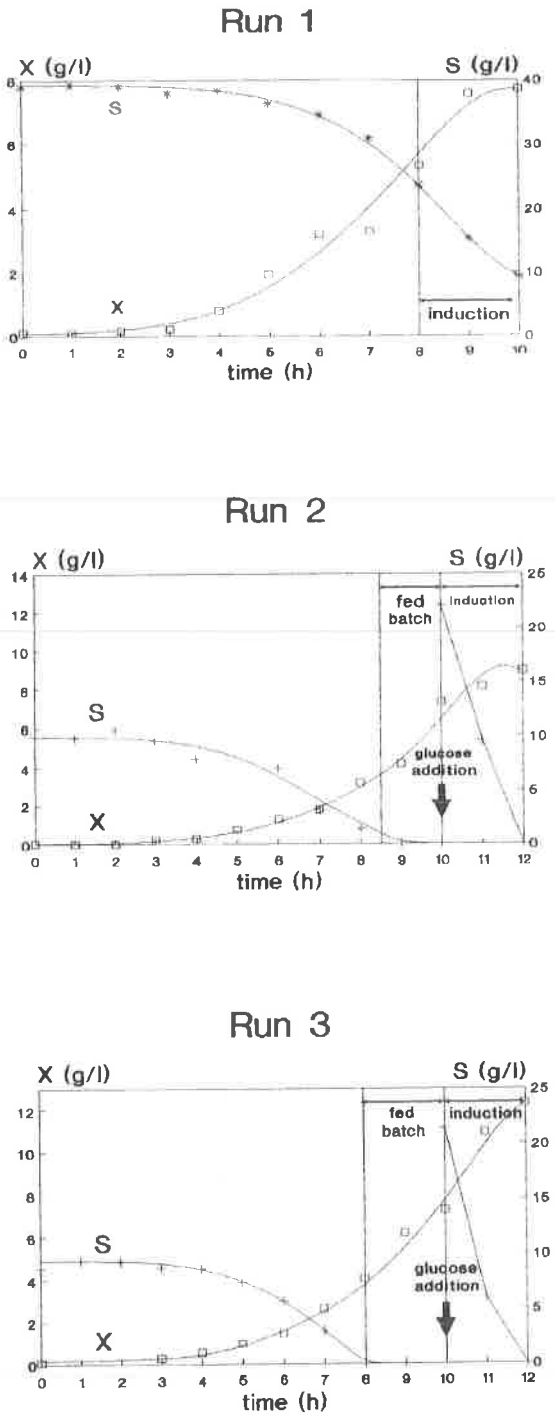

Figure 2: Cell (X) and residual glucose (S) concentrations as time functions for runs 1, 2 and 3.

It is interesting to note that for the same induction system and host cell, and other heterologous proteins, the literature reports different values of the specific concentration of IPTG for the maximum protein production: 9.4, 18.6 and 6.4 mg/g (4, 5, 16). Therefore, the adequate specific concentration for the inducer depends on the particular protein being expressed.

Kinetics of TnC production and respiration. Fig. 2 shows the curves of cell and glucose concentrations for runs 1, 2 and 3. The different ways of glucose supply resulted different time intervals for almost the same glucose consumption, 8 h for run 1 and 10 h for runs 2 and 3. The specific growth rate, μ, is depicted in Fig. 3. For all runs, the maximum specific growth rate were around 0.7 h^{-1} during the batch growth phase. The strategy employed in the subsequent fed-batch phase, had succeeded in supporting the desired μ value between hours 8 - 10 of runs 2 and 3. By the end of the growth phase, μ was higher for run 2, followed for run 3 and run 1. Finally, during the induction phase, μ sharply decreases for all runs until zero. Table 1 summarizes the conditions of the runs at the end of the growth phase.

The kinetics of TnC production was analyzed taking the derivatives from curves of TnC (%) relative to the time, dTnC/dt (%/h), and the results are depicted in Fig. 4. During the first hour of the induction phase, all of the runs had values of dTnC/dt around zero and 10%/h. However, from the first hour of the induction phase, run 2 exhibited values around 30%/h, which are extremely higher than that for the other runs, around 10%/h for run 1 and less than 5%/h for run 3. Considering that glucose concentration was almost the same at the end of the growth phase and beginning of the induction phase of the three runs (Table 1), and specific IPTG concentration was also equivalent, the higher values of dTnC/dt in run 2 can be a result of the

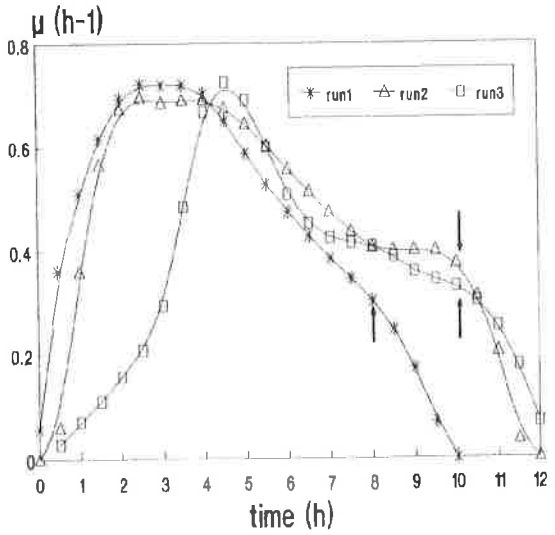

Figure 3: Specific growth rate (μ) of run 1 (batch, 0 – 8 h and induction, 8 – 10 h) and runs 2 and 3 (batch, 0 – 8 h, fed batch, 8 – 10 h and induction, 10 – 12 h) as time functions. The arrows shows the induction time beginning.

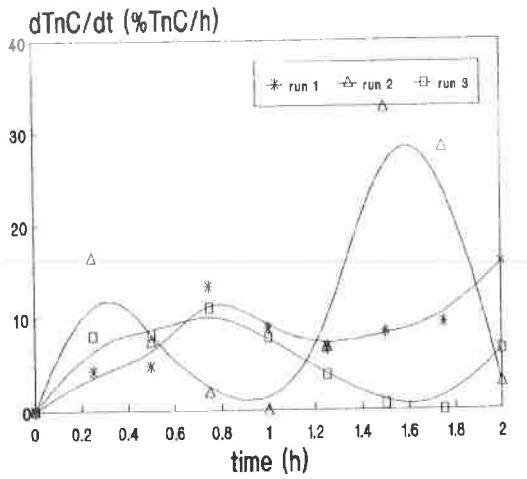

Figure 4: Rate of TnC accumulation within the cells relative to the total cell protein content during induction phase of runs 1, 2 and 3.

Table 1: Conditions at the end of the growth phase of runs 1, 2 and 3.

RUN	1	2	3
Elapsed time (h)	8	10	10
X (g/l)	5.7	6.5	7.7
μ (h^{-1})	0.30	0.40	0.33
Yx/s (g/g)	0.36	0.36	0.41
S (g/l)	23.5	0 __ 22.1*	0 __ 21.3*

* at this time the glucose was instantaneously replenished and its concentration raised from zero to 22.1g/l and 21.3 g/l for runs 2 and 3 respectively

X: cell concentration

μ: specific growth rate

$Y_{x/s}$: cell yield on glucose

S: glucose concentration

higher μ value in this run relative to runs 1 and 3, before the induction beginning. The higher the μ value, the higher the rate of energy production, resulting high production rate of the heterologous protein. This observation is particularly important in the case of the TnC production under the control of the *lac UV5* and T7 promoter, because the rate of transcription by means of the T7 RNA polymerase, is around 5 times faster than that for the other proteins of the cell (14). Curless et al. (2), using the same system in *E.coli* for the production of human α-interferon, also described the process as hardly influenced by the μ value.

The highest dTnC/dt values for run 2, which means higher synthesis capacity for cells of run 2 relative to the others, resulted from high TnC (%) values for run 2, according with the summary of the results showed in Table 2. The lowest production level was that for run 3, according to the lower values of dTnC/dt.

Cells of all runs stopped growing after 2 h of induction being the growth yield on glucose, $Y_{X/S}$ in the induction phase, significantly lower than that for the growth phase. It is interesting to note that cell yield on glucose, $Y_{X/S}$, for the induction phase, was higher for run 3, 0.22, relative to run 2, 0.11, indicating a deleterious effect of the protein production activity upon growth, mainly for run 2.

This aid in TnC production activity with concomitant reduction of the growth activity can be also well visualized through the respiration data.

Fig. 5 shows the specific rate of respiration, q_{O2}, for all runs. During the growth phase q_{O2} varies according to the μ values. On the other hand, for the induction phase, q_{O2} exhibits values completely independent of μ. Although μ sharply decreases to zero in all runs, q_{O2} is almost constant during the induction phase of runs 1 and 2, being the higher values those for run 2, around 25 mmol/g.h, followed by the values of run 1, around 20 mmol/g.h, and run 3, 5 mmol/g.h. Therefore, regarding the oxygen consumption, the production of the heterologous protein is an aerobic process. Moreover, by the end of the culture, when μ exhibited null values, q_{O2} is significant, indicating that cells are viable after the accumulation of such high levels of TnC.

The overall cell yield on oxygen, $Y_{X/O}$ (mg/mmol), and the maintenance coefficient, m_o (mmol/g.h) for the growth phase, were determined through the correlation between q_{O2} and μ accordingly to equation (2) using the average of five runs (data not shown)

$$q_{O2} = m_o + (1/Y_{X/O})\,\mu \quad (2)$$

$m_o = 4.5$ mmol/g.h; $Y_{X/O} = 20$ mg/mmol

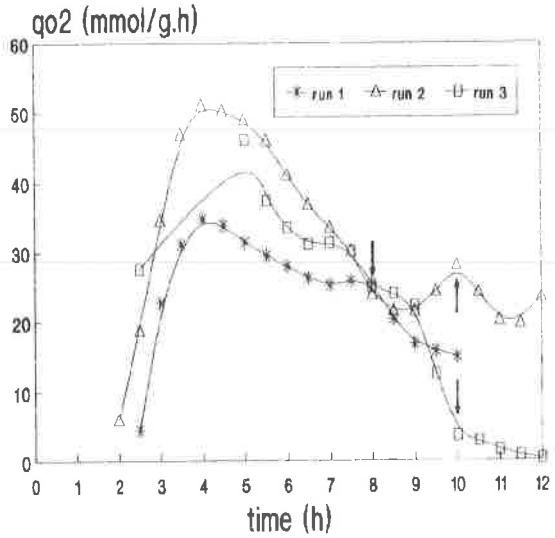

Figure 5: Specific respiration rate of run 1 (batch, 0 – 8 h and induction, 8 – 10 h) and runs 2 and 3 (batch, 0 – 8 h, fed batch, 8 – 10 h and induction, 10 – 12 h). The arrows shows the induction time beginning.

Table 2: Conditions at the end of the induction phase of runs 1, 2 and 3.

RUN	1	2	3
Elapsed time (h)	2	2	2
X (g/L)	7.7	8.9	12.4
μ (h^{-1})	0.0	0.0	0.07
Yx/s (g/g)	0.14	0.11	0.22
S (g/L)	9.3	0.0	0.0
TnC (%)	16.5	26.5	11.7
Yx/o (g/mmol)	0.03	0.024	0.373

X: cell concentration
μ: specific growth rate
$Y_{x/s}$: cell yield on glucose
S: glucose concentration
Tnc: percentage of total cell protein as Troponin C
$Y_{x/o}$: cell yield on oxygen

Through the integration of the oxygen uptake rate data, OUR (mmol/h), of the induction phase, it was possible to determine the cell yield on oxygen for this phase. The determined values of $Y_{X/O}$ are 8, 6.5 and 142 mg/mmol respectively for runs 1, 2 and 3. The higher cell yield on oxygen for run 3 relative to other runs, is in agreement with higher cell yield on glucose, that is, the growth activity during the induction was the highest in run 3.

According to Table 2 the final TnC (%) values are 16.5, 26.5 and 11.7% for runs 1 to 3 respectively. The values of $Y_{X/O}$ for growth and induction phases, and the final TnC (%) values, clearly shows that once the heterologous protein synthesis is started, the generated energy is strongly directed to the production activity followed by reduced growth activity. Moreover, the lower the efficiency for energy utilization for growth, the higher the production of the recombinant protein, for this fast expression system analyzed.

Considering a cell protein content of 55% (10), and the experimental values of TnC (%) and DCW of biomass, a specific TnC production relative to cell mass of 110 mg/g was estimated. The deleterious effect upon the growth is reasonable considering that such a high level production was obtained in 2 h only. To date, Zabriskie (17), showed a specific intracellular production of a recombinant antigen of only 15-20 mg/g, and a kinetics of production independent of specific growth rates between 0.1 and 0.5 h^{-1}.

For a specific IPTG concentration of 100 mg/g, no limiting glucose and oxygen concentrations, the troponin C production in *E. coli* under the control of the *lac UV5* promoter and the T7 RNA polymerase is fast and significative. Around 10% of the cell dry weight is the recombinant protein after only 2 h of induction. As a consequence, the specific respiration is high, around 25 mmol/g.h, and the growth activity stops.

Further development of this process with cell protein content analyzes during the TnC production will allow a better evaluation of the process through values of TnC yield on oxygen. The condition of glucose limitation must be also verified, in order to analyze its influence on q_{O2} and consequently, on the oxygen demand, which could be a constraint in high cell density cultures.

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RESUMO

Cinética de produção de uma proteína recombinante por *E.coli* BL21

Troponina C, TnC, uma proteína do músculo esquelético de galinha, foi produzida em células de *Escherichia coli* BL21 (DE3) pLysS contendo o cDNA no plasmídio pET sob controle do promotor *lac UV5*. Neste sistema, a expressão do gene da TnC depende da presença de uma molécula indutora, o que permitiu a realização de ensaios com uma fase de crescimento independente da fase de produção. O balanço gasoso aplicado à fase de produção da TnC em cultivos em biorreator, resultou em um fator de conversão de oxigênio a células, $Y_{X/O}$, entre 6,5 e 142 mg/mmol, e porcentagem de TnC na célula com relação ao teor de proteína celular, TnC (%), entre 11,7 e 26,1%, sendo que para os maiores valores de TnC (%) correspondem os menores valores de $Y_{X/O}$. Além disso, para o maior o valor de μ no início da fase de indução, obteve-se maior o valor de TnC (%). Os dados apresentados indicam que na produção de TnC sob controle do gene *lac UV5* e promotor de T7 em *E.coli*, a energia do metabolismo aeróbio é direcionada principalmente à síntese da proteína recombinante com consequente redução da atividade de crescimento.

Palavras-chave: *Escherichia coli*, troponina C, *lac UV5*, promotor da T7 RNA polimerase, IPTG.

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CHARACTERIZATION OF A CELLULASE-FREE XYLANASE PRODUCING *BACILLUS* SP FOR BIOBLEACHING OF KRAFT PULP

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ABSTRACT

Application of the xylanase in the pulp bleaching process has been shown to be effective in decreasing the amount of chlorinating agents in the process and improving the brightness of the pulp. The use of thermostable cellulase-free xylanase might enhance both the technical and economic feasibility of the process. In this work an alkalophylic strain of *Bacillus* sp 77-2, was isolated which showed a high production of xylanase and free cellulases. The xylanase of *Bacillus* sp displayed an optimum pH of 6.0 (with 70% activity at pH 9.0), an optimum temperature of 60°C, pH stability in the range 5-10 and thermal stability of 50°C. These characteristics are important to the kraft pulp bleaching because they are similar to those found in the industrial paper environment.

Key words: xylanase, kraft pulp, biobleaching

Introduction

In the manufacture of paper, kraft pulp is traditionally prepared using chlorine bleach to oxidize and remove lignin to achieve better product brightness. This process releases chlorine compounds that damage the environment. Consequently attempts have been made to remove or reduce the chlorine levels that are used in this process (2). One of the approaches for reducing environmental pollution is to use enzymes in the bleaching. Application of xylanase in the pulp bleaching process has been shown to be effective in decreasing the amount of chlorinating agents needed and improving the brightness of pulp in the bleaching by totally chlorine free (TCF) process (7). The xylanase applied to the pulp needs be free of cellulases, which degrade the cellulose, decrease the viscosity leading to reduced yields. Xylanases produced by mesophilic fungi or bacteria, however are not thermostable, resulting in low hydrolytic efficiencies. The use of thermostable xylanases at high temperatures over prolonged periods of time might enhance both the technical and economic feasibility of the hydrolysis process (4), leading to a cost effective and a more environmentally acceptable process.

Materials and methods

Culture medium. The screening of microorganisms was made in nutrient medium, pH 9.0, as described by Horikoshi (3): 1% beef extract powder, 1% peptone, 1% NaCl, 0.1% KH_2PO_4, 0.5% Na_2CO_3. Corn straw was used as a carbon source, replacing the filter paper (5). The production medium (3) was utilized having as carbon source 1% xylan (Birchwood - Sigma).

Screening of microorganism. Approximately 1 g of compound, culture soil or forest decayed wood was inoculated into test tubes containing 10 ml of

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screening medium and incubated at 50°C for 120 hours. The culture was then inoculated on plates with same culture medium containing 1.5% bacteriological agar, and incubated at the same temperature. The colonies were subsequently streaked out and transferred to slant cultures. Isolated alkalophilic and thermophilic microorganisms from the slant cultures were inoculated into 20 ml of the same medium without agar in 125 ml Erlenmeyer flasks and incubated at 50°C with shaking at 150 rpm. After 48 hours the bacterium was harvested by centrifugation at 6000 g and 4°C for 20 min. The free cell solution was assayed for enzymatic activity.

Microorganism. The taxonomic study of strain isolated was made until the genus (*Bacillus* sp 77-2) according to the Bergey's manual of determinative bacteriology (8).

Enzyme production by submerged culture. To determine the growth curve and maximal enzymatic production, the *Bacillus* sp 77-2 was inoculated in five 125 ml Erlenmeyer flasks containing 20 ml of production medium and incubated at 50°C with shaking at 150 rpm. Each day the contents at one flask was centrifuged, as noted above, and the enzymatic activities (xylanase, carboximethyl cellulase and avicelase) were determined in the cell free supernatant.

Enzymatic activity determination. The activities of xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), carboxymethyl cellulase or CMCase (endo-1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) and avicelase (exo-1,4-β-D-cellobiohydrolase, EC 3.2.1.91) were assayed by incubating 0.1 ml of appropriately diluted enzyme with 0.9 ml of solution containing the respective substrate, xylan (Birchwood - Sigma), carboxymethyl cellulose (Sigma-C5678) and or avicel (Merck), prepared in 0.1 M acetate buffer, pH 5.0. After incubation at 60°C for 10 min, the reducing sugars released were assayed colorimetrically by the addition of 1 ml of 3-5-dinitrosalicylic acid reagent (6). One unit of xylanase activity was defined as 1 µmole of xylose or glucose equivalent released per minute.

Optimum pH. The optimum pH was determined by incubating 0.1 ml of enzyme and 0.9 ml of buffers, adjusted to initial pH of 3.0 to 11.0, containing Birchwood xylan (0.5%). The buffers used were: sodium acetate buffer, pH 3.5 - 5.5; Tris-HCl buffer, pH 6.0 - 9.0; Gly-NaOH buffer, pH 9.5 - 10.5. After incubation at 60°C for 10 min. and the released reducing sugars were assayed as described above.

pH stability. The effect of pH on the stability of the xylanase was studied by incubating the enzyme, without substrate, with buffers covering a pH range of 3.5 - 10.5 (as used for optimum pH determinations). After treatment for 24 hours at room temperature, at a given pH, the residual enzymatic activity was determined.

Optimum temperature. The optimum temperature was determined by performing the standard reaction for 10 min. over a temperature range of 45 to 80°C.

Thermal stability. The effect of elevated temperature on the enzyme was accomplished by incubating 0.1 ml of the enzyme at 45 to 80°C for 1 hour. After treatment the residual activity was assayed.

Xylan hydrolysis. The hydrolysis of xylan (Birchwood Sigma) by crude xylanase was assessed using 0.1 ml of crude enzyme and 0.9ml of xylan in 0.1 M acetate buffer, pH 5.0. This mixture was incubated at 60°C for 5 to 180 min., and the reaction stopped by boiling for 10 min. The products of this reaction were identified by paper chromatography using xylose, xylobiose, xylotriose, xylotetralose and glucose as standards.

Hydrolysis of pulp. The crude xylanase was used to hydrolyze kraft pulp derived from eucalyptus (Champion Paper and Cellulose Ltd., Mogi Guaçu, Brazil). A 1% pulp was prepared in 0.1 M acetate buffer and 9.0 ml of this was incubated with 1 ml of the enzyme at 60°C for 1 to 4 hours. The enzyme was inactivated by boiling for 10 min. and the sugars released were identified by paper chromatography as above.

Hydrolysates identification. The hydrolysates identification was realized by descendent chromatography (9). Samples (20 µl) were spotted onto Whatman nº1 filter paper developed with ethyl acetate/isopropanol/water (6:3:1). The hydrolysis products were visualized with silver nitrate.

RESULTS AND DISCUSSION

Screening of microorganism. Out of 117 samples collected, fifteen microbial cultures were isolated by the above techniques. Besides this, was selected one strain (77-2) of alkalophilic and thermophilic bacterium with growth at 45°C and pH 9.0 that produced high levels of xylanase activity with negligible CMCase and avicelase activity. The isolated bacteria was a Gram positive, rod, formed subterminal spores in a distended vegetative cell, grew in aerobic conditions and produced catalase. On the

basis of these characteristics the bacterium was classified in the genus *Bacillus* (8), and denominated *Bacillus* sp 77-2.

Enzymatic production. Measurements of the enzyme activity and cell growth at a number of time intervals are shown in Fig. 1. The maximum level of cell growth was observed at 48 hours, coinciding with the maximum level of production of xylanase, which then decreased. The CMCase and avicelase activities were low and oscillated.

Enzymatic characteristics. The optimum pH and optimum reaction temperature of the xylanase was 6.0 and 70°C, as shown in Fig. 2 and 3. Fig. 2 shows that the enzyme lost stability below pH 5.0, but maintained stability at alkaline pH. This xylanase was inactive at above 50°C, as shown in Fig. 3. Activity and stability of the xylanase at higher pH and temperature is expected to increase its suitability for application in the paper and pulp industry (1).

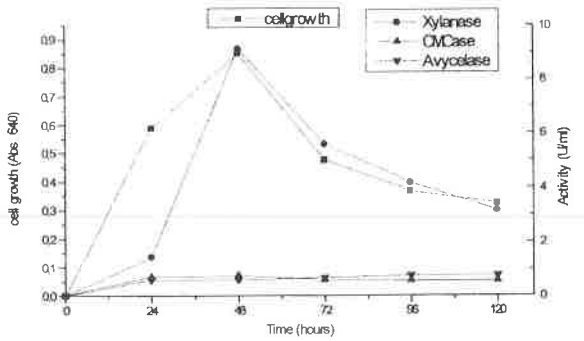

Figure 1 - Cell growth and enzyme production by *Bacillus* sp 77-2

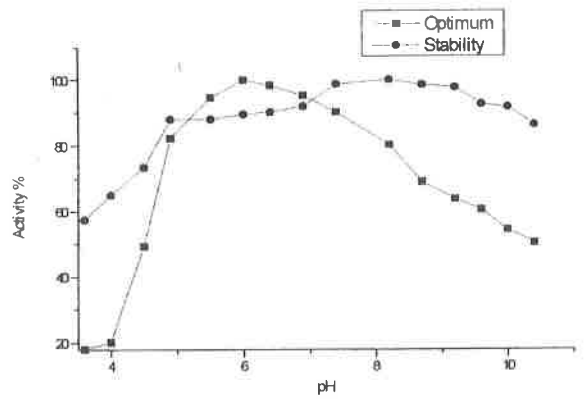

Figure 2 - Optimum pH and stability pH of *Bacillus* sp 77-2 crude xylanase

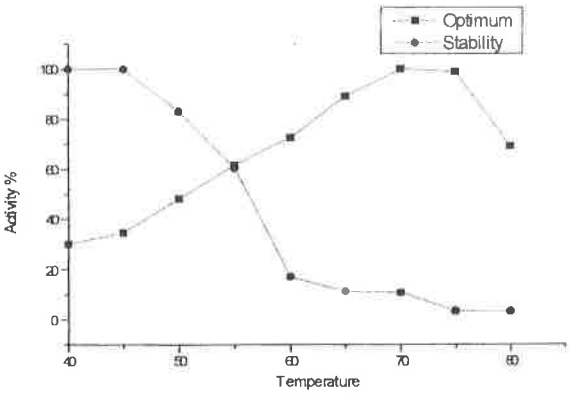

Figure 3 - Optimum temperature and stability temperature of *Bacillus* sp 77-2 crude xylanase

Hydrolysates identification. The products of xylan hydrolysis by *Bacillus* sp 77-2 are shown in Fig. 4. Xylotriose and xylobiose were obtained after 5 minutes of hydrolysis, and xylose was detectable after 1 hour of hydrolysis indicating activity of endo and exoxylanase in the crude supernatant (Fig. 4 A). The main product of pulp hydrolysis found was xylobiose, with xylose found in a smaller quantity (Fig. 4 B). In both cases glucose production was not detected, indicating that cellulase was not produced (Fig. 4 C). This is very important because the presence of cellulase degrade cellulose leading to a decrease in viscosity and yield. These enzymatic properties indicated that the *Bacillus* sp 77-2 crude xylanase is suitable for applications in pulp improvement.

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RESUMO

Caracterização de uma xilanase, livre de celulase, produzida por *Bacillus* para o biobranqueamento da pasta kraft

A aplicação de xilanase no estágio de branqueamento da pasta kraft tem mostrado ser um eficiente meio de decrescer o consumo de cloro no

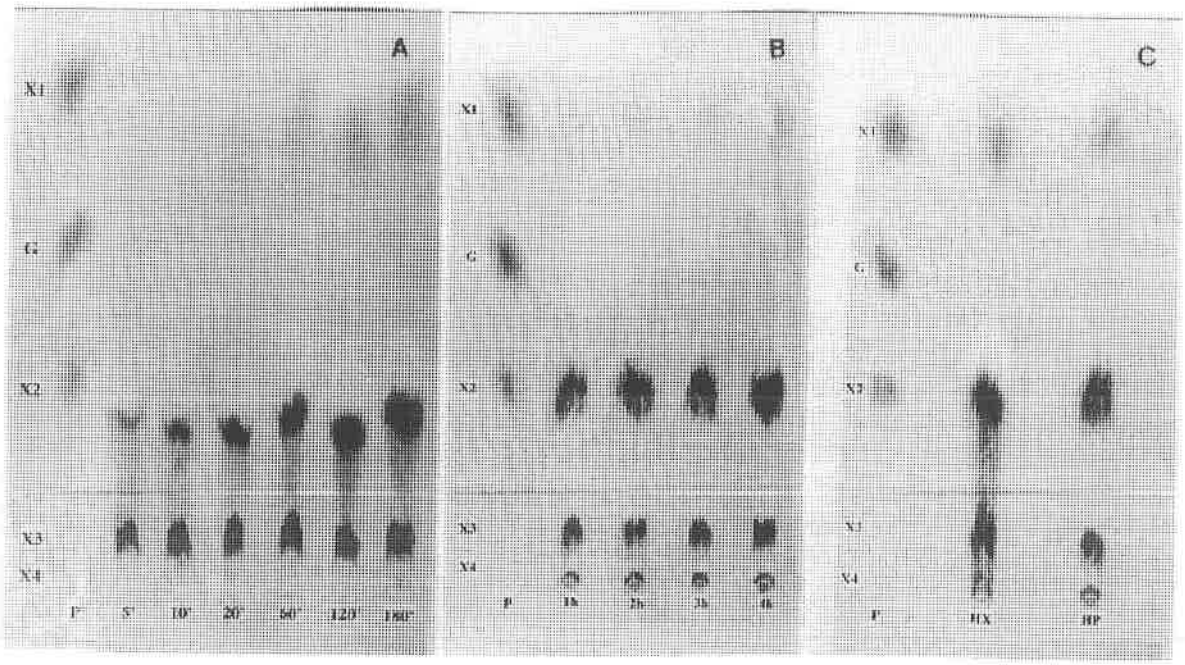

Figure 4 - Products of xylan and pulp hidrolyses by *Bacillus* sp 77-2 xylanase P = standard; X_1 = xylose; X_2 = xylobiose; X_3 = xylotriose; X_4 = xylotetraose; G = glucose; HX = hydrolyse of xylan; HP = hydrolyse of pulp

processo e de aumentar a alvura final da pasta branqueada. A xilanase para ser aplicada à pasta deve ser termostável e livre de celulase. Neste trabalho uma linhagem de *Bacillus* sp 77-2 alcalofílico e termófilo foi isolado e selecionado por apresentar alta produção de xilanase livre de celulase. A enzima apresentou pH ótimo de 6.0 (com cerca de 70% desta atividade em pH 9,0), temperatura ótima a 60°C, pH de estabilidade entre 5 e 10 e foi estável à temperatura de 50°C. Estas características são importantes para o branqueamento da pasta kraft, uma vez que são similares àquelas encontradas no ambiente de polpação industrial.

Palavras chaves: xilanase, pasta kraft, biobranqueamento

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AN ALTERNATIVE BIPHASIC CULTURE SYSTEM FOR RECOVERY OF MYCOBACTERIA AND FOR DIFFERENTIATION OF SPECIES OTHER THAN *M. TUBERCULOSIS* COMPLEX FROM BLOOD SPECIMENS

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ABSTRACT

Mycobacteremia is an increasingly frequent complication of late-stage infection with the Human Immunodeficiency Virus (HIV). Several different procedures have been used to detect and characterize mycobacteria in blood. However, most of these are expensive and time consuming. Our study addressed the application of an alternative biphasic blood culture system containing modified Middlebrook 7H9 broth and Lowenstein Jensen medium (mod 7H9/LJ) developed for direct recovery of mycobacteria from blood. Additionally, we evaluated the possibility of rapid discrimination of Mycobacterium other than tubercle bacilli (MOTT) organisms by differential growth after simultaneous inoculation into control media and media altered by the addition of ρ-nitrobenzoic acid. In the first part of this study mod 7H9/LJ was compared with conventional 7H9/LJ. Mycobacterial growth curves were generated for *M. tuberculosis* and *M. intracellulare* in both control and test medium. In the second part of this study mycobacteria were recovered from 64 of 537 cultured specimens (11.9%). Growth was detected in 64 (100.0%) of the 7H9/LJ, and 62 (96.9%) of the mod 7H9/LJ biphasic bottles.The mean time to mycobacterial detection in the two systems was the same. For the third part of the study, a total of 1091 blood specimens were cultured in mod 7H9/LJ and in mod 7H9/LJ containing 500 µg/ml of ρ-nitrobenzoic acid. A total of 72% of all *Mycobacterium avium* complex (MAC) isolated from blood were presumptively identified correctly as MOTT within 27 days. This study indicates that the alternative biphasic culture system has great potential for use under laboratory conditions that prevail in developing countries.

Key words: Mycobacteria, blood, detection, mycobacteremia diagnosis

INTRODUCTION

Though historically it has been possible to recover *M. tuberculosis* from the blood of patients with miliary disease or during the initial dissemination phase of tuberculous infection, until the advent of the HIV pandemic, mycobacteremia was rarely detected (1,4).

The frequency of both silent and symptomatic mycobacteremia in Acquired Immunodeficiency Syndrome (AIDS) patients and the range of species

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encountered makes the culture of blood and bone marrow for mycobacteria an essential diagnostic tool for the modern laboratory (2,3,4,12).

The clinical demand for the diagnosis of disseminated mycobacterial disease in AIDS has led to the development of a number of commercial and non-commercial systems aimed at the detection of mycobacteria in sterile body fluids including lysis centrifugation, radiometric broth culture, and nucleic acid amplification and detection (1,10,16,17,20,21). Though these tools have proven sensitive and clinically useful, they are sometimes cumbersome or time-consuming and moreover, are too expensive to be used routinely by laboratories in the developing world. We sought to develop a simplified alternative method that would avoid practical problems associated with other systems and that would be easily affordable. This report describes the laboratory and clinical evaluation of a biphasic system containing modified Middlebrook 7H9 broth and Lowenstein-Jensen medium.

The study was carried out in three phases: 1) the laboratory evaluation of an alternative inexpensive enrichment method for Middlebrook 7H9 broth, 2) a clinical study comparing the sensitivity of detection of two biphasic culture systems, and 3) the evaluation of a method for direct species differentiation of mycobacteria growing from blood cultures.

MATERIAL AND METHODS

Laboratory Evaluation of Modified Middlebrook Media. Middlebrook 7H9 broth medium was enriched according to a standard protocol by the addition of ADC - bovine albumin fraction V, glucose and catalase (Difco Laboratories, Detroit, Michigan, USA) 10% by volume. A modified Middlebrook 7H9 broth was prepared by replacing the Middlebrook ADC enrichment with bovine serum (heat inactivated for 30 minutes at 56°C) to a final concentration of 10% and pH 7,2. The ability of the two medium preparations to support mycobacterial growth was compared by inoculation with pedigreed strains and serial quantitative subculture. Standard inocula of *M. tuberculosis* H37Rv (ATCC 25177) and *M. intracellulare* (ATCC 13950) were prepared in the following manner. Each strain was harvested in log-phase growth from 15 day cultures on Lowenstein-Jensen slants and placed in a tube containing 5 ml of Middlebrook 7H9 broth and glass beads. A homogenous suspension was prepared by shaking in a Vortex mixer for 3 minutes and the resulting suspension was allowed to sit while larger clumps settled to the bottom of the tube. The supernatant was then placed in a second tube and left undisturbed for 15 minutes to allow further settling of clumped organisms. The supernatant from this smooth suspension was then transferred to another tube and adjusted to a density equal to a 0,1 nm by the addition of 7H9 broth using a nephelometer. *M. tuberculosis* and *M. intracellulare* standardized inocula (0,5 ml each) were added to culture tubes containing 4,5 ml of standard or modified Middlebrook 7H9 broth. The capped tubes were incubated at 37°C and growth curves were constructed by performing quantitative culture of the test media on Lowenstein-Jensen slants at days 0, 3, 6, 9, and 12 after inoculation.

Clinical Evaluation of Modified Middlebrook Media. Biphasic culture systems were prepared in glass bottles of 50 ml capacity using Lowenstein-Jensen (LJ) medium for the solid phase and either standard or modified Middlebrook 7H9 broth for the liquid phase. The LJ medium was inspissated as an 8 ml slant and a 20 ml volume of enriched 7H9 broth was added that left roughly half of the LJ slant submerged and half exposed. The biphasic system using standard Middlebrook 7H9 broth with ADC (7H9/LJ) was used as control in a comparison with an identical system replacing the modified 7H9 broth described above (mod7H9/LJ). Both broth preparations were supplemented with 0.025% sodium polyanetholsulfonate to prevent coagulation, and antibiotics (20 µg/ml trimethoprim, 50 µg/ml carbenicillin, 10 µg/ml amphotericin B) to prevent contamination.

A total of 537 blood specimens were obtained from patients with AIDS with suspected disseminated mycobacterial infection hospitalized at the AIDS Training and Reference Center (CRTA) of São Paulo, Brazil, from January 1991 to July 1993. Ten milliliters of blood collected by venipuncture without anticoagulant after iodophor disinfection of the skin was divided into two 5 ml aliquots and immediately inoculated into the control (7H9) and test (mod7H9/LJ) media. The cultures were incubated upright at 37°C and observed daily for growth for up to 8 weeks. Bottles were inverted daily during the first week for reinoculation of the exposed solid media. Positive cultures were those demonstrating visible growth in either the solid or liquid medium, confirmed to be mycobacterial by Ziehl-Neelsen (Z-N) staining. Broth from the cultures remaining negative after 8

weeks of incubation was Z-N stained and examined for acid-fast bacilli (AFB) before being discarded to rule out growth not discovered by visual inspection.

Evaluation of a System for Direct Mycobacterial Species Differentiation. A total of 1091 blood specimens were obtained from AIDS patients with suspicion of disseminated mycobacterial infection hospitalized at the CRTA during the period from September 1993 to April 1995. Ten milliliters of blood collected by venipuncture without anticoagulant after iodophor disinfection of the skin was divided into two 5 ml aliquots for inoculation into each of two biphasic mod 7H9/LJ medium bottles prepared as detailed above. One of the bottles also contained 500 µg/ml of ρ-nitrobenzoic acid (PNB). The cultures were incubated upright at 37°C and inspected for growth daily for the first two weeks, at which time they were inverted for reinoculation of the exposed solid medium, and then weekly for an additional 6 weeks without further inversion. Bacterial growth detected by visual inspection was confirmed to be mycobacterial or contaminant on the basis of microscopic examination of stained preparations using the Z-N method. For all positive cultures, mycobacterial growth was compared in the paired bottles and cultures growing in medium containing PNB were presumptively classified as MOTT.

Identification of Mycobacterial Isolates. *M. tuberculosis* isolates were identified on the basis of ρ-nitrobenzoic acid susceptibility, 2-thiophenecarboxylic acid hydrazide resistance, niacin production, nitrate reduction and colony morphology. (5,11) MOTT were identified at the species level using standard methods.

Statistical Methods. The detection sensitivity of the two biphasic systems was compared using the Kappa agreement coefficient (8).

RESULTS

This study was carried out in three phases. In the first phase a modified Middlebrook 7H9 broth enrichment method was tested, replacing with bovine serum the more expensive Middlebrook ADC enrichment. Mycobacterial growth curves were generated for *M. tuberculosis* and *M.intracellulare* in both control and test medium by quantitative subculture at 3-day intervals. As shown in Fig. 1, similar logarithmic growth (7) was seen in both media.

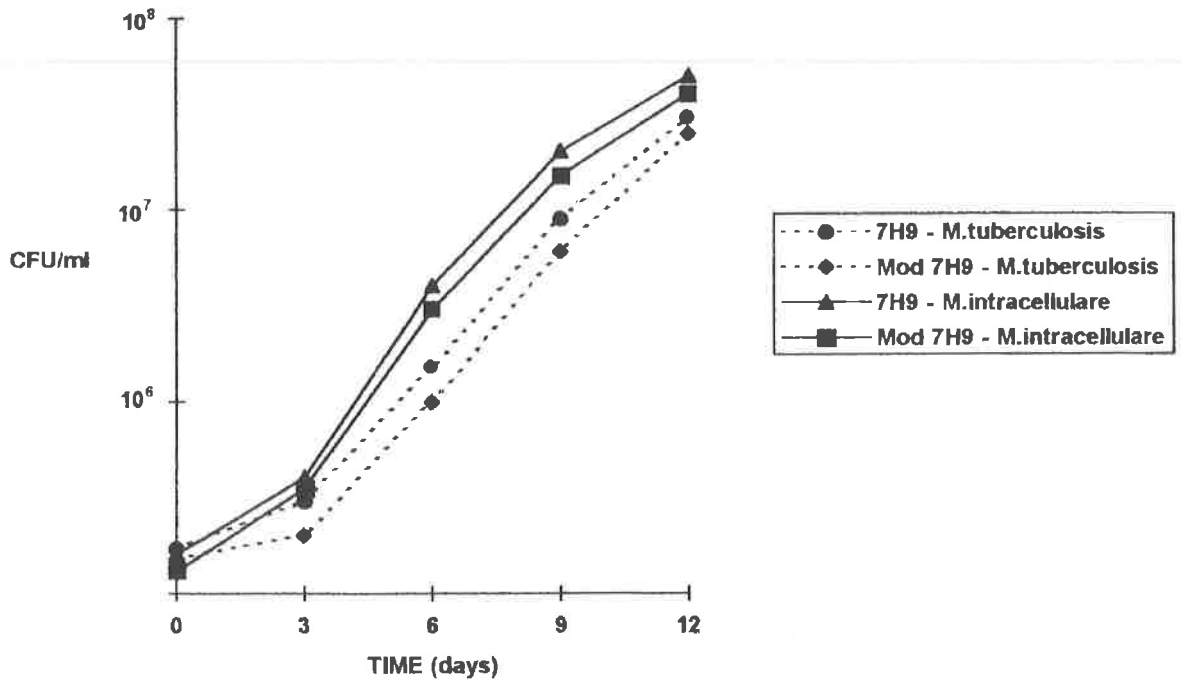

Figure 1 - Logarithmic growth of *M. tuberculosis* and *M. intracellulare* in Middlebrook 7H9 broth and Modified Middlebrook 7H9 medium.

In second phase of the study, 537 blood cultures were performed using a biphasic medium prepared with Lowenstein-Jensen and Middlebrook 7H9 broth. The cultures were performed in duplicate to compare mycobacterial recovery and contamination rates in bottles with standard ADC-enriched broth 7H9/LJ) to those of bottles with the modified broth described above (mod 7H9/LJ). Mycobacteria were recovered from 64 of the 537 cultures (11.9%), 64 of which (100.0%) were detected by the 7H9/LJ bottles and 62 (96.9%) by the mod 7H9/LJ bottles. Kappa coefficient calculation showed excellent agreement (91.0%) between systems.

Of the 64 isolates, 37 (57.8%) were MAC and 24 (37.5%) were *M. tuberculosis* complex organisms. Three isolates could not be identified at the species level because of contaminating overgrowth or lack of growth on subculture. Primary contamination occurred in 11 (2.0%) and 8 (1.5%) of the 7H9/LJ and mod 7H9/LJ biphasic systems, respectively (Table 1).

The number of days required for detection of positive cultures did not differ between the two systems (Table 2). Mycobacterial growth was detected by inspection of both the liquid and solid phases of the culture system.

In the final phase of the study a method of species differentiation directly from the inoculated culture medium was evaluated. Pairs of biphasic 7H9/LJ bottles, one containing PNB, a specific inhibitor of *M. tuberculosis*, were inoculated with 5 ml of blood from AIDS patients with suspicion of disseminated mycobacterial infection. Of 1091 cultures, 58 (5.3%) were positive for mycobacteria. Of the 43 isolates given a final idenfication of MAC, 31 (72.1%) could be presumptively identified as MOTT over an average of 27 days on the basis of growth in the PNB-containing bottle. The 12 MAC isolates that failed to grow in the PNB-containing systems were all subsequently shown to be PNB-resistant. Misidentification of *M. tuberculosis* did not occur and all of these 15 isolates were growth inhibited by the selective biphasic bottle (Table 3).

Five isolates could not be identfied at the species level because of either contaminating overgrowth or lack of growth on subculture. Primary contamination occurred in 12 (2.23%) biphasic systems.

Table 1 - Recovery of mycobacteria and contamination rates from 537 blood specimens in two biphasic culture systems

Biphasic system	Positive cultures n=64			Contaminated cultures n=12
	MAC	MT	NI	
7H9/LJ[a]	5	1	0	4
Mod. 7H9/LJ[b]	2	2	0	1
7H9/LJ and Mod. 7H9/LJ	32	23	3	7

[a] Middlebrook 7H9 broth and Lowenstein Jensen
[b] Modified Middlebrook 7H9 broth and Lowenstein Jensen
MAC - Mycobacterium avium complex
MT - Mycobacterium tuberculosis complex
NI - not identified species

DISCUSSION

The well-documented association of AIDS with Disseminated Mycobacterial Infection prompted the investigation of methods for the detection and identification of these organisms from blood specimens. Although biphasic blood cultures are commonly used for the isolation of bacteria and fungi, they are seldom used for the isolation of mycobacteria. The design of our study addressed the application of an alternative blood culture system for diagnosis and for monitoring the efficacy of therapy under the prevailing laboratory conditions in developing countries.

In this phase of our study, the simple replacement of ADC supplement with bovine serum to a final concentration of 10% in Middlebrook 7H9 medium did not affect the growth of *M. tuberculosis* or *M. intracellulare* (Fig. 1). This may be a useful substitution for containing costs (9), although, as with any other in-house media supplemented with serum or blood, factors may be present that possibly affect

Table 2 - Number of days required for detection of the *M. tuberculosis* and *M. avium* complex organisms.

Organism	N° of isolates[a]	7H9 / LJ		Mod 7H9 / LJ	
		Mean	Range	Mean	Range
M.tuberculosis	23	31.3	18-60	31.5	17-60
MAC[b]	32	27.1	14-60	27.2	15-60

[a] Excluding 10 isolates that could not be identified
[b] *Mycobacterium avium* complex

Table 3 - Screening for MOTT in 1091 blood specimens directly and simultaneously inoculated into the mod 7H9/LJ biphasic system (control) and into the 7H9/LJ biphasic system containing PNB (PNB).

Identification	PNB (+)		PNB (-)		Total
	Control (+)	Control (-)	Control (+)	Control (-)	
M. tuberculosis	0	0	15	1076	1091
MAC	29	2	12	1048	1091

Mod 7H9/LJ - Modified Middlebrook 7H9 / Lowenstein Jensen biphasic system
PNB - -nitrobenzoic acid
(+) mycobacterial growth
(-) absence of mycobacterial growth
MOTT - Mycobacterium other than tubercle bacilli
MAC - *Mycobacterium avium* complex

bacterial growth (14). Several methods have been used to recover mycobacteria from blood cultures. For example, Agy et al. (1), using the isolator system, recovered mycobacteria from 29 of 180 blood specimens submitted to culture (16.1%). Salfinger et al. (16) detected mycobacteria in 17.6% of the blood samples examined using sodium deoxycholate solution-lysis-centrifugation as the concentration technique, followed by inoculation of the sediments into radiometric BACTEC (7H13) (14). Strand et al. (18) reported that BACTEC 13A medium revealed the presence of mycobacteria in 63 of 1848 blood culture (3.4%). In the present study carried out for medium evaluation with clinical samples, mycobacteria were recovered from 64 of the 537 cultures (12.1%), 64 of which (100.0%) were detected with the 7H9/LJ bottles and 62 (96.9%) with the mod 7H9/LJ bottles. It should be noted also that our biphasic medium contained smaller amounts of liquid medium than usually recommended (3,6,11).

It is important to emphasize that factors outside the laboratory such as therapeutics and clinician indications for mycobacterial cultures may greatly influence the rate of recovery of mycobacteria from blood in these studies (1,4).

With respect to the time needed to detect mycobacterial growth, there was no significant difference between the biphasic systems studied. The wide range of growth detection times observed was probably due to the variable number of bacilli in the blood specimens. As demonstrated by Berlin et al. (3), the time of recovery is inversely proportional to the number of organisms inoculated.

The ability of mycobacteria to grow on media containing differentially inhibitory substances has repeatedly been shown to be of value for identification purposes (13,19).

The *M. tuberculosis* complex is inhibited by PNB at a concentration of 500 g/ml, whereas MOTT are resistant to this concentration. The latter, however, may present variation in sensitivity, with the existence of a small percentage of strains sensitive to certain drug concentrations, as proposed by Rastogi et al. (15) and Tsukamura et al. (19). In the present study, after a mean growth of 27 days in medium containing PNB, 31 strains (72.1%) were considered to be MOTT. This means that the laboratory can supply a presumptive MOTT result on the basis of the growth of these strains on medium containing PNB. All of these strains were later confirmed to be MAC on the basis of the results of biochemical tests.

With respect to cultures presenting growth only on control medium, i.e., strains sensitive to PNB, it was not possible to provide a presumptive result since 15 (100%) were identified as *M. tuberculosis* and 12 (27.9%) as MAC. One of the tests used for MOTT identification is resistance of the strain to 500 g/ml PNB. Resistance to this PNB concentration was observed in 12 MAC strains during their identification, suggesting that the bacillary load inoculated into the two flasks (control medium and medium mod. 7J9/LJ with PNB) was so low that it was inhibited by this drug concentration.

Two strains that presented growth in PNB-containing medium did not grow on control medium (with no PNB), suggesting that the samples were paucibacillary, permitting the occurrence of a difference between the inocula added to the two media.

For analysis of the agreement between methods for the presumptive identification of MOTT by growth on medium 7H9/LJ containing PNB and by MAC identification using biochemical methods, the Kappa coefficient was calculated with a 95% confidence interval, giving a value of 0.799 (0.696-0.901). This value may be considered a measure of the relative efficacy of the method of presumptive identification since it anticipates the definitive results obtained by the biochemical tests with reasonable concordance.

The differentiating ability of PNB-containing biphasic media appears to be sufficiently rapid and specific to be useful in a clinical laboratory, since identification of mycobacteria by conventional methods may take up to 1 month.

In conclusion, the alternative biphasic system is compact, self-contained, simple to use and

inexpensive. All of these advantages make it suitable for use under the prevailing laboratory conditions of developing countries.

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RESUMO

Sistema alternativo bifásico de cultura para o isolamento de micobactéria e triagem de espécies outras que não as do complexo *M.tuberculosis* a partir de espécimes de sangue

Micobacteremia constitui uma das manifestações oportunísticas mais frequentes dos estágios avançados da infecção pelo virus da imunodeficiência humana. Diversas metodologias tem sido propostas para o seu diagnóstico, no entanto, a maioria destas apresentam custo elevado ou complexidade de operação técnica. Baseados nestes fatos nos propusemos a avaliar um sistema alternativo de cultura bifásico contendo Middlebrook 7H9 modificado Lowenstein Jensen (mod 7H9/LJ), especificamente adaptados para (i) o isolamento de micobacterias, (ii) triagem de outras espécies que não as do complexo *Mycobacterium tuberculosis* (MOTT) através da adição de ácido ρ-nitrobenzoico aos meios (mod 7H9/LJ+PNB). Para a primeira etapa do estudo comparou-se os meios mod 7H9/LJ e 7H9/LJ convencional, realizando-se a curva de crescimento das espécies *M. tuberculosis* e *M. intracellulare* em ambos os meios. Na segunda etapa do estudo isolou-se um total de 64 cepas de micobacterias a partir de 537 espécimes de sangue (11,9%), das quais 64 (100,0%) em 7H9/LJ e 62 (96,9%) em mod 7H9/LJ. Verificou-se um tempo semelhante de detecção destas micobacterias em ambos os sistemas. Para a terceira etapa do estudo, cultivou-se um total de 1091 espécimes de sangue nos sistemas mod 7H9/LJ e 7H9 mod/LJ contendo 500 µg/ml de ácido ρ-nitrobenzoico, constatando-se que um total de 72% dos organismos isolados e pertencentes ao complexo *Mycobacterium avium* puderam ser presuntivamente identificados como MOTT em 27 dias. Estes resultados, associados a simplicidade e baixo custo destes sistemas bifásicos constituem elementos potenciais para sua aplicabilidade na rotina diagnóstica em países em desenvolvimento.

Palavras-chave: Micobactéria, sangue, detecção, diagnóstico, micobacteremia

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ADEQUACY OF DIFFERENT RESPIRATORY SPECIMENS AND CULTURE METHODS FOR BACTERIOLOGICAL DIAGNOSIS OF CYSTIC FIBROSIS

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ABSTRACT

To evaluate the sensitivity of different respiratory specimens and culture methods for the bacteriological monitoring of pulmonary infections in cystic fibrosis (CF) patients, we compared 41 specimens from oropharynx (OP), nasopharynx (NP) and sputum, collected simultaneously. We also analysed 144 sputum cultures processed by conventional and quantitative methods. The isolation rate of muccoid *P. aeruginosa* was 70% in sputum, 46% in OF and 4.9% in NP ($p < 0.05$). For non-muccoid (NM) *P. aeruginosa*, the rates were 32 % in sputum, 12% in OP and 7% in NP secretions ($p < 0.05$). For *S. aureus* and *H. influenzae*, the rates of recovery from the 3 specimens were similar ($p > 0.05$). The percentage of false negative results in OP or NP was high for all respiratory pathogens. *P. aeruginosa* NM, M and *H. influenzae* were not detected in some NP cultures (77%, 93% and 78% respectively) and were not isolated in about 60% of the OP cultures. Considering the sputum cultures, conventional and quantitative methods gave similar results, in more than 70% of the cases. We have also found false negative results in both methods. The conventional technique was less sensitive for *H. influenzae* (16.6%) and *P. aeruginosa* NM (15.9%) while the quantitative method was worse for *S. aureus* (13.1% of false negative). Concerning *P. aeruginosa* M, both cultures failed to detect 6% of the positive cases. Our results suggest that the microbiological analyses should be judicious in CF patients because the clinical specimen and culture methodology used may determine false negative results.

Keywords: *Pseudomonas aeruginosa*, respiratory infections, cystic fibrosis.

INTRODUCTION

Pulmonary disfunction accounts for most of deaths among Cystic Fibrosis patients (CF). The progressive decline of their pulmonary function results from chronic airway infections with intercurrent acute exacerbations (12) caused mainly by bacteria, although virus may also be involved (21).

The main bacterial pathogens associated with pulmonary infections are: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*. Other bacteria, such as *Enterobacteriaceae*, are less frequently found (7, 10, 11, 17). Recently, also *Bulkholderia cepacia* has been reported to be associated with pulmonary infections. Colonization of CF patients by these bacteria can

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result in an asymptomatic carriage, a slow and continuous decline in lung functions or a fatal necrotizing pneumonia (9, 10, 14, 18).

Any of these microorganisms can be found in patients of all ages but usually their higher prevalence is associated with a certain specific age. While *S. aureus* and *H. influenzae* are predominant in children younger than 2 years, in older children and adults, *P. aeruginosa* is the most important bacterial pathogen. Colonization of CF patient airways by *P. aeruginosa* begins with a classic non-mucoid strain which tends to become mucoid as the disease progresses. Although mucoid strains can also be found in other chronically colonized patients, they are typical of CF (9, 10, 12).

Due to the insidious nature of some lower respiratory tract infections in CF patients, monthly bacteriological monitoring of their respiratory secretions is deeply recommended (12, 18). Different factors may directly influence the results of the cultures, such as the nature (quality) of the clinical specimens. Clinical specimen most usually submitted to bacteriological culture is expectorated sputum. However, in young children, throat secretion or secretions obtained by endolaringeal suction and bronchoscopy are commonly used. The central question often raised is whether oropharyngeal cultures accurately reflect the presence or absence of bacterial pathogens in the lower respiratory tract of patients.

Different centers of attendance of CF patients differ in the methodology used for sputum culture (7, 12, 21). In most of them, the conventional technique is used. In these cases, clinical specimens are directly streaked onto the culture media, without any previous treatment, and the quantity of bacteria is estimated on a subjective basis. Alternatively, the quantitative sputum culture has been proposed not only as a method allowing the determination of the efficacy of the antimicrobial therapy, but also by allowing the detection of microorganisms with a slower growth rythm, such as *H. influenzae* (2, 8, 13, 16).

The proposal of the present study was to determine the sensitivity of oropharynx and nasopharynx secretion cultures, as well as to compare the sensitivity of the conventional and quantitative sputum culture methods in the recovery of bacteria more frequently found in respiratory infections in CF patients. We have also estimated the frequency of microbial flora of the respiratory tract both in CF and non-CF patients with pulmonary infections attended in two pediatric centers from Rio de Janeiro, Brazil.

MATERIALS AND METHODS

Clinical specimens. CF patients were from Instituto Fernandes Figueira (FIOCRUZ) and non-CF patients from Instituto de Puericultura Martagão Gesteira (UFRJ), Rio de Janeiro. In order to evaluate their microbial flora, 380 respiratory specimens were obtained from 90 CF patients and 250 from 121 non-CF patients. The diagnosis of CF has been established on a clinical basis, radiologial findings and high sweat eletrolytes levels (5).

The sensitivity of oropharynx (OP) and nasofarynx (NP) secretion cultures in detecting pulmonary pathogens was investigated by comparing the rates of recovery of bacteria from 41 specimens of OP, NP and sputum, obtained simultaneously from 13 CF patients.

The sensitivity of conventional and quantitative cultures was analyzed by collecting 144 sputum samples from 42 CF patients that were simultaneously processed by both culture methods.

OP and NP secretions were collected with sterile swabs, introduced into Stuart transport medium for periods no longer than 4 h. Sputa were obtained through spontaneous expectoration by using the protected technique with cotton tampons (3) in order to avoid spittle contamination, and were streaked within no more than 2h after collection. Before culturing, sputum samples were submitted to microscopic analysis, in order to evaluate the quality of the material, as proposed by Bartlett et al (1), and only those considered adequate were processed.

Methods of Culture. Conventional: The OP and NP secretions and sputum were sown by the classic streaking technique (22). Thereafter, the following criterion was used to estimated the bacterial growth:

- few: < 10 colony forming units (CFU) in the first inoculation area or > 10 CFU in the first inoculation area and < 10 CFU in the second inoculation area;
- moderate quantity: > 10 CFU in the third inoculation area
- many: > 10 CFU in the fourth inoculation area

Quantitative cultures were carried out as described by Monroe et al (19). Sputa were liquefied in a 2.5% (v/v) N-acetil-L cysteine solution, diluted in sterile isotonic saline solution and 0.1 ml of the different dilutions ranging from 10^{-2} to 10^{-9} were streaked across the surface of different culture media. Results were expressed as CFU per sputum ml.

All specimens were sow in Sheep-Blood-Agar, Eugon Agar (Difco) and Cystine-Lactose-

Electrolyte-Deficient Agar (Cled-Difco) and incubated at 35°C. The first two in 5-10% CO_2 atmosphere and the last one in aerobioses. Isolated microorganisms were identified by the conventional bacteriological methods (22).

Statistical Analysis. Mantel-Hansel Chi-square and Fisher's statistical tests were used. Tests were considered to be significant at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Microbiological profile of Respiratory Secretions from CF and non-CF Patients. The most frequent microorganisms in CF patients were *P. aeruginosa* and *S. aureus*, which were found in 77.0 % and 66.7% of our patients, respectively. Mucoid *P. aeruginosa* and *Enterobacteriaceae* were isolated from 30% of the patients, followed by *H. influenzae* (28.9%), *Candida* sp (27.8%) and *Streptococcus pneumoniae* (16.7%). The other species isolated showed a frequency of less than 10% (Fig. 1). These results are in agreement with the literature, although the frequency of recovery of these microorganisms in different centers of attendance of CF patients has been shown to vary (10, 16, 17, 19, 21). These variations can be attributed not only to the use of different laboratorial methodologies, but also to age and number of patients studied, to previous use of antibiotics and to the fact of being inpatients or outpatients.

Most of the microorganisms were isolated from CF and non-CF patients. However, the rates of isolation of *S. aureus* and *P. aeruginosa* were significantly higher in the CF group ($p < 0.05$). Mucoid *P. aeruginosa* were found only in CF patients, which emphasizes the close association of mucoid strains and CF. Possibly, the finding of a mucoid *P. aeruginosa* strain in patients whose CF diagnosis was not yet been determined points out the need for a deeper investigation.

Sensitivity of cultures from OP and NP secretions and from sputum. The survival of CF patients is closely related to both the precocity of the diagnosis and the etiology of the pulmonary infections. Since bacteria are the agents most often responsible for the progression of pulmonary dysfunction, different CF patient attendance centers have proposed the monthly bacteriological monitoring of CF respiratory secretions (6, 7, 10, 11, 12). Both the submitted clinical specimen and the culture methodology used to isolate pulmonary pathogens must be criteriously analysed in order to garantee that this monitoring will be effective.

Results of the cultures from 41 specimens (OP, NP secretions and sputum) collected simultaneously from 13 CF patients were compared. The rate of recovery of mucoid *P. aeruginosa* from sputa (70%) was significantly higher than the recovery from OP (46%) and NP (4.9%) secretions ($p < 0.05$). Similarly, the rates of recovery of non-mucoid *P. aeruginosa* from sputa (32%) was significantly higher than the recovery from OP (12%) and NP (7%) secretions ($p < 0.05$). For *S. aureus* and *H. influenzae*, the rates of recovery from the three specimens were similar ($p > 0.05$) (Fig. 2).

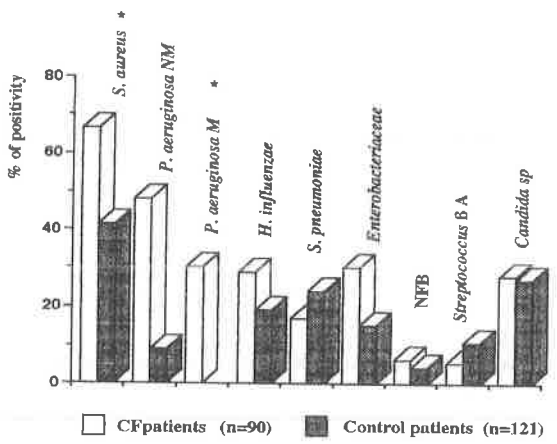

Figure 1. Microbiological profile of respiratory secretions from CF and non-CF patients. -*p<0.05.

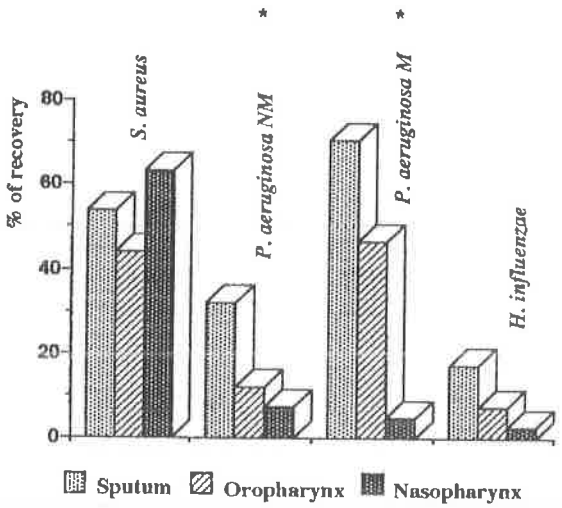

Figure 2. Rate of recovery of respiratory pathogens from different specimens (PO, and NP secretions and sputum) collected simultaneously from CF patients. *p<0.05.

Considering sputum as the gold standard specimen, in 59.5% of the cases *S. aureus* was isolated simultaneously in the 3 clinical specimens. Cultures of OP and NP secretions were simultaneously negative in 4.5% of sputum cultures yielding *S. aureus*, in 38.5% of cultures yielding non-mucoid *P.aeruginosa*, in 27.6% of cultures yielding mucoid *P. aeruginosa* and in 55.7% of sputum cultures yielding *H. influenzae*. In other cases, these microorganisms were identified in only one of the specimens from the upper respiratory tract (OP or NP) (Table 1). The false-negative results for all respiratory pathogens were higer than 55% in both OP and NP cultures, except for *S.aureus* (23% in both materials) and *P. aeruginosa* M (34.5% in OP secretions) (Table 1). Ramsey et al. (23) reported a lower percentage of false-negativity in OP cultures: 37%, 9% and 7% for *H. influenzae*, *S. aureus* and *P. aeruginosa*, respectively. To our knowledgement, our study is the first to compare results obtained simultaneously from NP and OP secretions and they suggest that the concomitant culturing of oropharingeal and nasopharingeal secretions from non-expectorant children should give higher rates of bacterial isolation.

Accordingly, if we had used only specimens from the upper respiratory tract for the diagnosis of pulmonary infections, as usually done in children with no expectoration, we would have failed to identify an important percentage of respiratory pathogens. However, even when they were used in association, both of them sometime failed to identify the pathogens.

Sensitivity of the conventional and quantitative sputum culture. CF patients airways are chronically colonized by a few microorganisms that are seldon erradicated, in spite of the adequacy of antimicrobial therapy. Accordingly, the therapeutic efficacy can not be estimated on the basis of the negativity of respiratory secretion cultures. The reduction in bacterial concentration in sputum has been used as a criterion to evaluate such efficacy. That is the main reason for the regular use of the quantitative sputum culture in CF patients. Moreover, this technique allows the characterization of germs with a slow growth rate, such as *H. influenzae* and *S. pneumoniae*. Notwithstanding, the conventional sputum method keeps on being used as the major culture method in different centers (4, 24, 25).

The analysis of 144 sputum samples processed simultaneously by the conventional and quantitative methodologies showed that the isolation rates of the main respiratory pathogens were higher than 80% for all the microorganisms. (Fig. 3). In most cases, there were high levels of agreement (> 70%) in results obtained with both methods. Agreement of the methods ranged from 88% of the cases, for mucoid *P. aeruginosa*, to 75% of the cases, for *H. influenzae*.

We found false-negative results on both culture methodologies. The conventional technique gave worse results for *H. influenzae* and for non-mucoid *P. aeruginosa* (16.6 % and 15.9% of false negativity, respectively). In contrast, the quantitative method was less sensitive for *S. aureus* (13.1% of false-negative results). Concerning mucoid *P. aeruginosa*, both techniques showed the same sensitivity, failing to identify 6% of the positive cases (Fig. 4).

The low sensitivity of the conventional method was in a certain way predictable for some germs, specially when associated with mucoid *P. aeruginosa*, in the same specimen which usually obscures the growth of the other bacteria. The non-homogeneous distribution of microorganisms in sputum cultured without any previous treatment probably also contributes to false-negative cases (15, 25).

Table 1. Isolation rate of respiratory pathogens from 41 respiratory specimens (OP and NP secretions and sputum) collected simultaneously from CF patients.

Microorganisms	Positive Sputum Cultures				False Negative Results	
	OP+ NP+ (%)	OP- NP- (%)	OP+ NP- (%)	OP- NP+ (%)	OP (%)	NP (%)
S. aureus	59.5	4.5	18	18.5	22.5	23
P. aeruginosa NM	0	38.5	38.5	23	61.5	77
P. aeruginosa M	0	27.6	65.5	6.9	34.5	93.1
H. influenzae	0	55.7	22.3	0	55.7	78

NM = Non mucoid strains
M = Mucoid strains
OP = Oropharynx
NP = Nasopharynx
+ = Positive results
- = Negative results

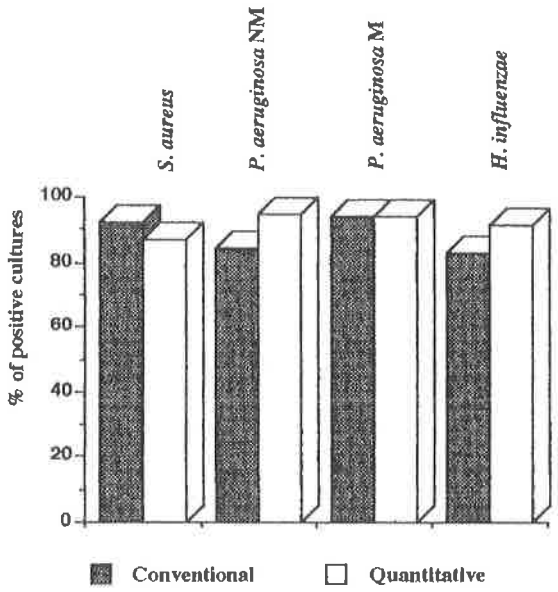

Figure 3. Sensitivity of 144 conventional and quantitative sputum cultures from CF patients.

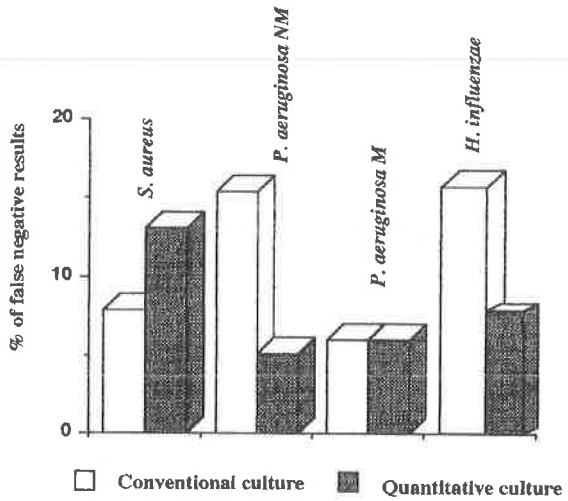

Figure 4. Frequency of false-negative results from conventional and quantitative sputum cultures from CF patients.

In our study the quantitative culture failed to detect a few bacteria that were identified in the conventional method. We speculate that in these cases, the bacterial concentration in sputum was inferior to 10^3 CFU/ml, the initial dilution plated, since in all cases, a small number of bacterial colonies were detected in the conventional culture. Alternatively, they can have resulted from the antibactericidal action of the mucolitic agent used to dilute sputum, reported before (20). Our findings demonstrate that these two culture techniques are complementary, as both failed to identify some of the pathogens.

The comparison of the quantity of germs obtained by both culture methodologies showed a good correlation, mainly in positive cases with bacterial counts higher than 10^5 CFU/ ml (Table 2).

The date presented here pointed out the necessity of a criterious analysis of the microbiological results obtained from CF patients, due to the possibility of false-negatives results, depending on the methodology and clinical specimens used. The interaction between the physichian and the microbiologist is important to determination of the most adequate bacteriological routine for such patients.

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RESUMO

Adequação de diferentes espécimes respiratórios e métodos de cultivo para o diagnóstico bacteriológico de Fibrose Cística

Para avaliar a sensibilidade de diferentes espécimes respiratórios e métodos de cultivo para o monitoramento bacteriológico das infecções pulmonares em pacientes com fibrose cística (FC), comparamos 41 espécimes de orofaringe (OF), nasofaringe (NF) e escarro, coletados simultaneamente. Analisamos também 144 culturas de escarro pelos métodos convencional e quantitativo. A *P. aeruginosa* mucóide (M) foi isolada em 70% das amostras de escarro, 46% de OF e 4,9% de NF ($p < 0,05$). Para *P. aeruginosa* não mucóide (NM) os índices foram de 32% no escarro, 12% no OF e 7% no NF ($p < 0,05$). Para *S. aureus* e *H. influenzae* os índices foram semelhantes nos 3 espécimes ($p > 0,05$). O percentual de resultados falso-negativos em OF e NF foi alto para todos os patógenos respiratórios. *P. aeruginosa* NM, M e *H. influenzae* não foram detectados em algumas culturas de NF (77%, 93% e 78%, respectivamente), não tendo sido encontrados

Table 2. Correlation between the quantities of respiratory pathogens recovered by conventional and quantitative sputum cultures from CF patients

Sputum Cultures		*S.aureus*		*P.aeruginosa* NM		*P.aeruginosa* M		*H.influenzae*	
Quantitative (CFU/ml)	Conventional	n	%*	n	%*	n	%*	n	%*
-	+	0	0	2	1.4	4	2.8	0	0
	+	7	4.9	3	2.1	0	0	1	0.7
	+++	3	2.1	1	0.7	1	0.7	5	3.5
10^3-10^4	-	1	0.7	0	0	0	0	1	0.7
	+	0	0	0	0	1	0.7	5	3.5
	+	0	0	0	0	0	0	3	2.1
	+++	0	0	0	0	1	0.7	0	0
$\geq 10^5$	-	4	2.8	12	8.3	5	3.5	10	6.9
	+	2	1.4	2	1.4	7	4.9	0	0
	+	12	8.3	20	13.9	15	10.4	7	4.9
	+++	45	31.2	41	28.5	50	34.7	44	30.5

* Percentage of 144 sputa processed by both techniques
NM = Non mucoid strains
M = Mucoid strains
+ = Few
++ = Moderate quantity
+++ = Many
CFU= Colony-Forming Units

em cerca de 60% das culturas de OF. As culturas de escarro convencional e quantitativa tiveram resultados similares, em mais de 70% dos casos, porém ambos mostraram resultados falso-negativos. A técnica convencional foi menos sensível para *H. influenzae* (16,6%) e *P. aeruginosa* NM (15,9%), enquanto que o método quantitativo foi pior para *S. aureus* (13,1% de falso-negativos). Ambas as culturas deixaram de identificar 6% dos casos positivos de *P. aeruginosa* M. Nossos resultados sugerem que a análise microbiológica em pacientes com FC deve ser criteriosa, uma vez que a escolha do espécime clínico e a metodologia de cultivo empregada são determinantes na frequência de resultados falso-negativos.

Palavras-chave: *Pseudomonas aeruginosa*, infecções respiratórias, fibrose cística.

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OCCURRENCE OF HYPHOMYCETES AND ACTINOMYCETES IN RED-YELLOW LATOSOL FROM A CERRADO REGION IN BRAZIL

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ABSTRACT

A study to quantify the populations of filamentous fungi and actinomycetes was undertaken in a cerrado area, in Corumbataí county, São Paulo, at different depths of medium-textured red-yellow latosol, correlating them to humidity, organic matter, pH of the soil and climatic factors. The technique used was the serial dilution of composite soil samples (n=4), using Martin's medium with streptomycin for filamentous fungi and a starch medium for actinomycetes. The number of filamentous fungi was more abundant in the first soil layer, 0-5 cm, compared to the other depths. The number of actinomycetes was higher between 5 cm and 50 cm depth. There were two-monthly variations in the number of filamentous fungi and actinomycetes. A sharp drop in fungi was found in May 1988 at all depths of the soil, when there was lower air temperature and an atypically high monthly rainfall for this period in the cerrado. Statistically significant and directly proportional correlations were obtained between the number of actinomycetes and the rainfall at depths of 0 to 5 cm and 50 to 100 cm. The results obtained in the different soil layers to 100 cm depth demonstrated the importance of the first 5 cm layer due to the heavy concentration of microorganisms, mainly the filamentous fungi, together with the highest organic matter content.

Key words: actinomycetes, cerrado, filamentous fungi, soil.

INTRODUCTION

The cerrado is an important ecosystem in São Paulo but is almost totally devastated. The lack of knowledge of the interaction occurring in soil under this type of vegetation and the carelessness with which it is handled contribute continuously to the loss of fertile land and tons of humus. Conserving the balance of the microbial populations in the soil of a cerrado area can mean the conservation of its fertility, since one is related to the other (10). Like other environments, the cerrados present their own characteristics, specifically in relation to their different types of soil, and their fertility is restricted to a shallow layer on the surface, in which most of the microbial activity and humus components (20) are found.

In previous studies it was found that the number of bacteria in cerrado soil was lower than in other environments while the number of actinomycetes was high, and similar to that of other types of soil (10). Soil fungi populations were studied in different types of cerrado (4, 11, 12, 18, 30). The application of vinasse in annual cumulative doses on filamentous fungi

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populations has affected the number of these microorganisms in the soil, but has not affected their diversity (28). Concerning the actinomycetes and bacteria populations, after cumulative doses of vinasse, there was a temporary increase in these populations in the soil under cerrado vegetation (26, 27). The addition of fertilizers and lime has caused a disproportional increase in the actinomycetes population in cerrado areas (9).

The aim of this study is to check the distribution of the filamentous fungi and actinomycetes at different depths of the cerrado soil and correlate it to the climatic conditions, organic matter content, pH, soil humidity present therein.

MATERIAL AND METHODS

The study area of 379,193 sq.m. has cerrado vegetation an is located in Corumbataí county, São Paulo, between longitudes 47°40' and 47°45' West and latitudes 22°10' and 22°15' South and belongs to FAPESP since 1962. The soil samples were collected with the help of a cup auger at depths of 0-5, 5-15, 15-30, 30-50 and 50-100 cm. In an area of cerrado *stricto sensu* measuring 10,000 sq.m.. The collection points were chosen at random. Four soil samples were made up of three sub-samples for each depth, in a total of twenty samples. A total of seven collections were made between September 1987 and September 1988.

The meteorological conditions of the region were supplied by the 7th Meteorological District of São Carlos, São Paulo, between latitude 22°01' South and 47°53' West. While the soil samples were being collected, the temperature and relative humidity of the air were measured at the surface of the soil. The temperature of the soil at 5 cm and 20 cm depth was also measured to determine some aspects of the microclimate of the portion under study. Every collection was done between 9:00 a.m. and 12:00.

The soil pH was measured in water in a 1:2.5 proportion using a potentiometer, after shaking for 30 minutes (14) and the organic matter content and soil humidity were obtained according to the methods mentioned in the literature (17).

The colony forming units of filamentous fungi (CFUFF) and actinomycetes (CFUA) were determined in Petri dishes using the serial dilution technique (6, 17). 10 g soil samples, previously passed through a 2.83 mm screen, were placed in 90 ml of previously sterilized (27) saline solution (NaCl, 0.85%). Five repetitions were used per sample in the 10^{-4} dilution, and aliquots of 1 ml were inoculated in Martin's medium with streptomycin (16) for the filamentous fungi, and in starch medium for the actinomycetes (7). The number of colony forming units was determined after incubation at 28°C for three days for the filamentous fungi and six days for the actinomycetes.

The results obtained were statistically analyzed using the variance test ANOVA with replicas and two factors. An attempt was made to compare the differences between the averages obtained at the varied soil depths, at different times of the year, using the Tukey's test. The Pearson correlation coefficient was calculated based on the averages of the parameters that were obtained (25).

RESULTS AND DISCUSSION

The climatic conditions are shown in the form of a climatogram (31). The results showed the existence in the study area of a hot wet season fallowed by a dry cold season (Fig. 1). Such results, together with others quoted in the literature (19, 27), showed that the climate is wet tropical or Cwa according to Köppen's classification. Furthermore, it was found that in November 1987 there was an interruption in the rainfall, and this phenomenon, known as winter seasonal may be common in a cerrado area (14). The values of relative humidity and air temperature (Table 1) showed greater seasonal variations in May 1988 and March 1988, respectively.

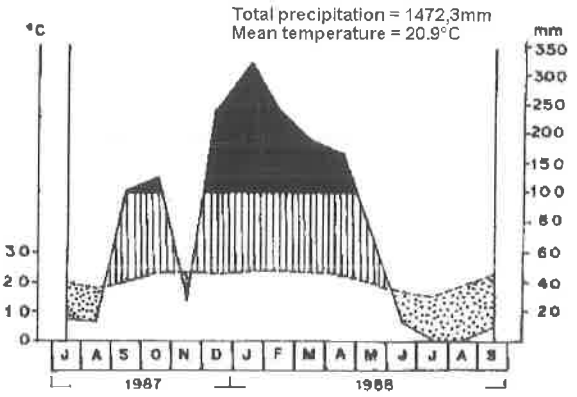

Figure 1. Climatogram of the region, with air temperature (°C) (-) and rainfall (mm): (■) high, (▥) normal, (□) low; obtained between September 1987 and September 1988.

The humidity, organic matter content and pH values of the soil also underwent seasonal variations which were greater in the months of May 1988 and July 1988. The pH of the soil was higher in March 1988. The values of these parameters were statistically different regarding the soil depths, with exception of the pH (Table 2).

The results showed a sharp seasonal variation in the populations of filamentous fungi and actinomycetes, with higher values in March 1988 for both populations. The ANOVA test with two factors and replica has shown highly significant statistical differences =1% (**) regarding the collections periods (F=19.495** for CFUFF and F=101.435** for CFUA). Such differences were also found in relation to the depths of soil (F=295.064** for CFUFF and F=80.497** for CFUA). The Tukey test showed that in relation to the populations of filamentous fungi the statistically significant differences occurred in depth 0-5 cm and in the other depths as well (Table 3). The same distribution was not found for the actinomycetes populations, which were numerically different in all depths studied and had a higher number below 5cm to 50 cm in depth (Table 3). These variations are probably related to the environmental conditions of the area under study. The close relation of the organic matter with the microorganisms is well known (13, 15) but it was not possible to statistically characterize any correlation of this parameter with the microorganisms studied here.

Considering that the study area has large trees, with an aspect of forest (5), the soil is constantly covered by a thick layer of tree litter. This fact can justify the occurrence of a large number of filamentous fungi due to the high content of organic matter in the soil, also described by other authors (5, 27) and with statistically significant and positive correlations obtained in other studies on the same reserve (29). Nutrient cycling in the study area is fast, a factor attributed to the nature of the natural organic matter and climate type, with a very short dry season (1, 22). The filamentous fungi did not present statistically significant correlations with the climatic factors, although such correlations have already been found in other studies in the area (29). Indirect evidence of the role of seasonality and tree litter on the filamentous fungi populations were found in May 1988 when the rainfall was high for the period (Fig. 1), associated with low temperatures (Table 1), resulting in a high accumulation of water in the soil (Table 2). Such factors have contributed to a decrease in the filamentous fungi populations (Table 3). A strong influence of the presence of vegetation on the microclimate of the area was found, since statistically significant inverse correlations between the relative humidity of the air and rainfall (r=-0.707), with the temperature of the air (r=-0.675) and, directly proportional to the humidity of the soil down to 5 cm (r=0.685), demonstrated the capacity of water storage by the tree litter.

Table 1. Relative humidity of the air (RU), temperature of the air (I) and soil to 5 (II) cm and 20 (III) cm deep under cerrado vegetation.

Months	RU (%)	Temperature °C		
		I	II	III
Sept.	76,5	30	25,3	14,8
Nov.	72	30,5	24,6	15,6
Jan	52	31,1	26,1	18,6
Mar	65	32,4	24,1	15,7
May	91,5	16,1	15,1	9,9
July	72	17,5	19,1	19,1
Sept.	75,5	28,5	25,5	24

Table 2. Content of soil humidity, organic matter content and pH, at different depths (cm): 0-5, 5-15, 15-30, 30-50, 50-100, under cerrado vegetation.

Months	Soil humidity (w/w)					Organic matter $g.dm^{-3}$					pH				
	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
Sep.	10,6	9,5	10,3	10,5	11,5	61	41	40	42	41	4,4	4,4	4,4	4,3	4,6
Nov.	8,99	9,5	10,5	10,4	11,2	44	39	39	41	39	4,5	4,5	4,4	4,4	4,5
Jan.	9,99	8,7	8,1	9,5	10,8	75	40	36	36	39	3,9	4,1	4	4,3	4,3
Mar.	8,8	10	10,3	11,2	11,3	69	43	40	41	41	4,3	4,4	4,6	4,3	4,6
May	16,7	12,4	12,7	14,7	12,	73	44	39	40	38	4,8	4,3	4,1	4,4	4,4
Jul.	10	7,4	7,2	7	6,6	82	43	46	44	44	4,1	4,4	4,5	4,4	4,4
Sep	8,7	8,3	9,4	9,6	8,6	52	40	40	39	39	4,5	4,4	4,3	4,5	4,6

Depts: 0-5 (I), 5-15 (II), 15-30 (III), 30-50 (IV), 50-100 (V)

Table 3. Colony forming units of isolated filamentous flungi and actinomycetes in average textured red-yellow latosol under cerrado vegetation at depts: 0-5, 5-10, 15-30, 30-50, 50-100 cm.

Months	CFUFF.g.solo^{-1} (p.w) x 10^3					CFUA.g.solo^{-1} (p.w) x 10^3				
	I	II	III	IV	V	I	II	III	IV	V
Sep.	8.5	2.8	1.8	0.8	0.5	40	48	53	49	15
Nov.	12.5	12.5	1.9	1.2	1.6	12	41	21	18	18
Jan.	12.7	5.9	5.8	1.4	0.7	26	25	34	32	19
Mar.	15	8.9	2.3	2.7	0.8	53	48	52	38	28
May	5.3	2.9	2.5	1.2	0.6	38	37	27	47	20
Jul.	15	4.9	2.9	1.5	0.7	15	25	25	23	16
Sep	10	7.5	1.1	1.8	1.5	20	41	17	12	8

Depts: 0-5 (I), 5-15 (II), 15-30 (III), 30-50 (IV), 50-100 (V)

The distribution the genera in the soil has shown the predominance of deuteromycetes at practically all depths. That the diversity of genera diminished considerably below 30 cm. The most frequent were *Penicillium* sp and *Trichoderma* sp, similarly to the genera found in another *cerrado* area (12). Fungi with non-sporulating mycelium were isolated at each depth of the soil, and their frequency increased with soil depth (Table 4). Fungi with non-sporulating mycelium occurred during the whole process of fungal succession as well as the genera noted above (2).

Unlike the filamentous fungi of actinomycetes were not abundant in the first 5 cm of soil depth (Table 3). Their distribution was over the whole soil profile but they mostly occurred in the 5 cm to 50 cm depth layers except at some periods, such as in March 1988. The variations in the number of actinomycetes in the first layer of soil showed the influence of the atmospheric conditions on such populations. Statistically significant correlations were obtained between the rainfall (r=0.858) and relative humidity of the air (r=-0.723) with the number of actinomycetes at 0-5 cm depth. The rainy season caused an increase in the number of actinomycetes At this period, the lowest values of relative humidity of the air, due to the high evapotranspiration at that time were also observed (19). In the soil layers at 0-5 cm and 5-15 cm depth, other statistically significant correlations were found between the rainfall and the pH values of the soil, (r=-0.685) and (r=-0.904) respectively, and between the air temperature (microclimate) and soil humidity (r=-0.764). These results also demonstrated the major influence of the climatic conditions on the first layers of soil, where the greatest activity occurs in this type of vegetation (20). A statistically significant correlation was also found between the rainfall (r=0.807) and number of actinomycetes at the depth between 50 and 100 cm.

The correlations demonstrated the importance of the rainfall on the actinomycetes populations. Their number dropped sharply in the periods with little rain, except at 5-15 cm depth, in November 1987 and September 1988. However, the excessive rainfall caused the decline of these microorganisms in the soil, as observed in January 1988. In other areas, high values of actinomycetes colony forming units were obtained at 11-15 cm depth in the soil, where the pH was closed to neutrality and the soil humidity was high (8). No relation between pH, soil humidity and actinomycetes was observed at 5-15 cm depth, but the rainfall influenced the soil pH, as was previously mentioned for depths of 0-5 cm and 5-15 cm.

The pH values (Table 2) were low, revealing a high soil acidity. The layers below 5 cm were less acid at most collection periods. Despite the lack of correlation between the number of actinomycetes and pH reported imprevious years the same study area, positive statistically significant correlation were found between the populations of this microorganism and the aluminum content, and negative correlation with the soil pH (29). In other cerrado areas, the number of actinomycetes was high in a medium adapted to pH 5.5, when compared to the result obtained with a medium adapted to pH 6.5 (7).

The distribution of the actinomycetes is conditioned to several factors. Most occurrences of these microorganisms were found in neutral soils tending to alkaline, with a reasonably high the organic matter content (8, 15). In other studies it was found that, despite the soil being acid, alkaline microsites can be formed during the process of organic matter decomposition. Under such conditions, although the fungi are more efficient in the decomposition of nitrogenous substrata added to the acid soils, with the release of ammonia, the *Streptomyces* sp later become more active in these microsites, where the pH was

Table 4. Occurrence in percentage (%) of hyphomycetes genera isolated in Petridish (10-4) in Martin medium with streptomycin of cerrado soil. The samples were taken at depths of: 0-5, 5-15, 15-30, 30-50, 50-100 cm.

Genera	Nov.					Jan.					Mar/				
	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
Absidia sp	3.1	3.3				3.2	3.2				14.8	5.1	7.7		
Ascomycetes not ident.	3.3						3.3	4.4					23.1	5	
Aspergillus sp	1.6	6.7	6.7			1.6	14.6	4.4	27.3		3.4		15.4		40
Aureobasidium sp			13.3	25				13.2				1.7			
Cephalosporium sp								4.4							
Chaetomium sp							3.2					3.5			20
Curvularia sp															
Fusarium sp	3.1					3.2									
Mucor sp	1.6					1.6	3.2					3.5			
Non-sporulanting myc.	17.2	13.3	40	25	75	17.4	13	30.3	27.3	100	3.4	9	30.7	40	40
Oedocephalum sp								8.6							
Paecilomyces sp								4.4	18.2		1.1				
Penicillium sp	56.2	40	33.3	50	25	55.5	38.7	30.3	27.3		34	52.6		15	
Sepedonium sp	1.6	10				1.6	6.4								
Trichoderma sp	14	23	6.7			14.3	14.4				43.3	24.6	23.1	40	
Verticillium sp	1.6					1.6									

Genera	May					Jul.					Sep.				
	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
Absidia sp	2.9	2.5				10.7	2.3				28.6	14.3	20		
Ascomycetes not ident.		9.6	5.9	20.4		1.8	4.7	13.3	22.2	30		8.2	20	26.3	40
Aspergillus sp				7.2			4.7			10			20	5.3	20
Aureobasidium sp								10							
Cephalosporium sp			3.5			7.1									
Chaetomium sp		7.3						3.4		10					
Curvularia sp														5.3	
Fusarium sp						1.8					9.5				
Mucor sp		7.3		7.2		1.8									
Non-sporulanting myc.	17.8	20	18	7.2	100	1.8	18.6	10	11.1		3.2	8.2		26.3	40
Oedocephalum sp	2.9		4												
Paecilomyces sp							2.3				3.2	6.1			
Penicillium sp	53.4	31.7	40	29		17.9	37.2	40	55.6	50	49.2	46.9	40	36.8	
Sepedonium sp															
Trichoderma sp	21	7.3	14	29		19.6	18.6	10	11.1		6.3	16.3			
Verticillium sp	2	14.3	14			37.5	11.6	13.3							

altered as a result of the release of ammonia (32). The occurrence of actinomycetes in acid soils, typical of cerrado regions, are not clearly explained in the literature, but their ecological role shows that they are able to decompose aromatic compounds (7), and interfere with the *Rhizobium* population through production of antibiotics in the soil (9, 21, 23, 24).

The distribution of the microorganisms in the soil involves a group of factors, and it is difficult to describe them separately. In desert areas, the existing fertile regions, linked to presence of few plant species, influenced the distribution of the microbial populations of filamentous fungi and actinomycetes. Their distribution in the soil profile can be related to the plant species that are present (3). The results obtained in this study permit us to conclude that in the cerrado, the layer down to 5 cm depth was the most important, because the filamentous fungi are concentrated there. This layer presents a high organic matter content, which in this environment has a

diversified origin. In this layer, there were also high numbers of actinomycetes but they were concentrated below the first layer, drawing attention to the occurrence of biological activity in deeper layers of soil.

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RESUMO

Ocorrência de fungos filamentosos e actinomicetos em latossolo vermelho-amarelo em uma região de cerrado no Brasil

Em área de cerrado, no município de Corumbataí, SP, foi realizado um estudo para quantificar as populações de fungos filamentosos e de actinomicetos, em diferentes profundidades de latossolo vermelho amarelo, textura média, correlacionando-se com a umidade, matéria orgânica, pH do solo e com os fatores climáticos. A técnica utilizada foi a diluição em série de amostras de solos compostas (n=4), utilizando-se o meio Martin com estreptomicina para fungos filamentosos e o meio de amido, para actinomicetos. Os fungos filamentosos foram mais abundantes na primeira camada do solo, 0-5 cm, em relação as demais profundidades. Houve variações bimestrais dos números de fungos filamentosos e de actinomicetos. Um decréscimo acentuado de fungos foi verificado no mês de maio/88 em todas as profundidades do solo, quando houve menor temperatura do ar anual e alto índice pluviométrico mensal, atípico para este período no cerrado. Correlações estatisticamente significativas e diretamente proporcionais foram obtidas entre o número de actinomicetos e o índice pluviométrico nas profundidades, 0-5 cm e 50-100 cm de profundidade. Os resultados obtidos nas diferentes camadas do solo até 100 cm de profundidade, evidenciaram a importância da primeira camada de 0-5 cm, devido a grande concentração de microrganismos, principalmente dos fungos filamentosos, juntamente com os maiores teores de matéria orgânica.

Palavras-chave: actinomicetos, cerrado, fungos filamentosos, latossolo.

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INTERACTION IN CULTURE MEDIUM OF ACTINOMYCETES AND NATIVE FUNGI WITH RHIZOBIA THAT FORM NODULES IN *NEONOTONIA WIGHTII* LACKLEY (PERENNIAL SOYBEAN)

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ABSTRACT

The relationship between soil fungi and actinomycetes, with rhizobia that produce nodulation in *Neonotonia wightii* (perennial soybean) was studied in culture medium in order to obtain material to be used in mixed inoculations of this legume. A field experiment was designed to provide soil samples and isolate microorganisms belonging to these groups that are capable of interfering in the development of two selected rhizobium strains. Results show that the *Bradyrhizobium* strains used in the experiment, which are efficient in nodulation of *Neonotonia wightii,* are highly sensitive to substances produced in the culture medium by actinomycetes and fungi.

Key words: *Bradyrhizobium,* growth inhibition, *Neonotonia wightii*, perennial soybean.

INTRODUCTION

Agricultural and production characteristics of the perennial soybean favor its use to form mixed pastures in São Paulo State. The symbiotic system of this legume is with rhizobia of the cowpea group (5), bacteria that today belong to the genus *Bradyrhizobium*. Rhizobia of the cowpea group possess soil survival traits and competitive ability that make them a frequently found bacterium (43), widely distributed in tropical soils. It forms nodules in a large number of legume species (27), usually resulting in low productivity because of a symbiotic system that is not always effective (28). According to some authors, this rhizobium is best adapted to stable environments such as pastures (20).

Certain traits of the symbiotic system formed with perennial soybean place this species in the class of legumes considered by Date (16) to be promiscuous and often inefficient, especially in the initial establishment phase in pastures.

Important factors that interfere with rhizobium survival in the soil and in establishing symbiosis are the competitive ability of the rhizobium strain for nodulation sites and the number of rhizobia in the soil (37), which depend on carbon sources available to the rhizobium (40), as well as local rainfall, temperature, pH (43) and biological agents in the soil (15, 22).

The number of rhizobia in the soil is only part of a larger picture, where many other microorganisms are interacting (10). The populations are kept in check, each species maintaining a similar numerical range over time, depending on soil management practices, and also conserving taxonomic, morphological, and especially, physiological heterogeneity (18). Considering the numerous types of known interactions that may occur, Sprent (36) concluded that the chances of modifying the infection process in the soil as a result

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of biotic factors are enormous and have been poorly studied.

We studied the relationship between native fungi and actinomycetes from soils planted with *Neonotonia wightii* (in the initial stage of establishment) and rhizobia that form nodules on this legume, in culture medium, in order to obtain biological material to be used in mixed inoculations.

MATERIAL AND METHODS

Field experiment. The field experiment was set up in the Ovine and Caprine Station, Zootechnical Institute, Itapetininga, São Paulo State (23°35'S; 48°02'W). A Koeppen Cfa type climate prevails here. The dark red ortho-latosol was collected to a depth of 20cm; soil chemistry was as follows: pH=4.6 (in $CaCl_2$); MO=5.1%; P=2.0µg/cm^3; V=31.4%; and, in meq/cm^3: K=0.32, Ca=0.55, Mg=0.78; H+AL=3.6. The site had been covered by *Axonopus compressus* Sw. for the past 20 years.

After plowing and leveling the soil, dolomite lime was applied at the rate of 3500kg/ha, 40 days before sowing, to increase base saturation (V) to 60% (45) following the chemical analysis of the soil. The fertilizer was placed in the planting rows, 10cm deep, using a mixture of simple superphosphate (700kg/ha=140kg P_2O_5/ha), potassium chloride (30kg/ha), zinc sulphate (20kg/ha), copper sulphate (10kg/ha), borax (5kg/ha), manganese sulphate (10kg/ha) and sodium molybdate (500g/ha).

According to the recommended planting method for this legume (25), the experiment was made up of four plots, each one with 100m^2, made up of 25 rows of seeds. Two plots were planted with Clarence cultivar and two with Tinaroo cultivar (day 10/23). During the experiment an irrigation system supplied the equivalent of 12mm of rain one day before each soil collection.

Growth of fungi and actinomycetes from the soil. Composite soil samples were collected in each plot to 15cm depth. Samples were also collected with a cylindrical auger after germination in the root region at 3-day intervals, until the first root nodules arose in half of the plants sampling (forty two days after planting). 10g of each one of the four composite samples was diluted by a factor of 1/10 in sterile physiological solution, and sown immediately on the surface of the culture medium, in order to obtain a greater variety of microorganisms (after Porter (31)). 0.1ml aliquots (diluted 10^{-4}) were scattered over the surface of the plates containing a starch culture medium for actinomycete growth, following Waksman (41), and at dilution 10^{-3} in plates with rose bengal medium (19), for fungi growth, with no streptomycin (as recommended by Corke and Chase (13), for a total of ten plates per sample of composite soil and each culture medium. The plates were incubated inverted at 28°C for four days for actinomycete growth and two days for fungi. Colonies were counted after this period.

Inhibition rings of rhizobium growth. To obtain inhibition rings of rhizobium growth, a modified technique by Panthier *et al.* (29) was used: 15ml of Yeast Manitol Agar (39) were poured over the plates with fungi and actinomycete growth (kept at 45-50°C). After solidification was poured over the culture medium 0.1ml of YM liquid medium, containing rhizobia in suspension after four days of growth (strain NO-65 = SMS-303, isolated from *N. wightii* and recommended for inoculating seeds of this legume when necessary, or isolate i-94b, a native rhizobium not catalogued, efficient in nitrogen fixation in perennial soybean). In each soil sample there was repetition of strains, five plates for each culture medium, for a total of 40 plates per collection day. After growing two days in inverted plates at 28°C, the inhibition rings were measured and the existence of resistant colonies within these rings was verified.

Estimating rhizobia MPN in soils. The same diluted soil samples were used on collection day to estimate the Most Probable Number (MPN) of native rhizobia in the soil samples by the Dilution and Infection of Plants Method (6) and the production of nodules formed by native rhizobia. Leonard pots were used containing a mixture of sand and vermiculite and a nutrient solution with no mineral nitrogen (11), previously sowed with soybean cultivar corresponding to that plot whose soil was diluted. In each pot 1ml of dilution 10^{-1} to 10^{-5} was inoculated, in duplicate, for a total of 44 pots per day of collection. Readings were taken 45 days after sowing.

RESULTS AND DISCUSSION

Figs. 1 and 2 show counts of fungi and actinomycetes from the samples. These estimates of numbers of soil microorganisms usually contain a large experimental error factor (1, 31). In our case, they should be used cautiously when analyzed quantitatively. The main objective of this study was to grow microorganisms for later testing of their

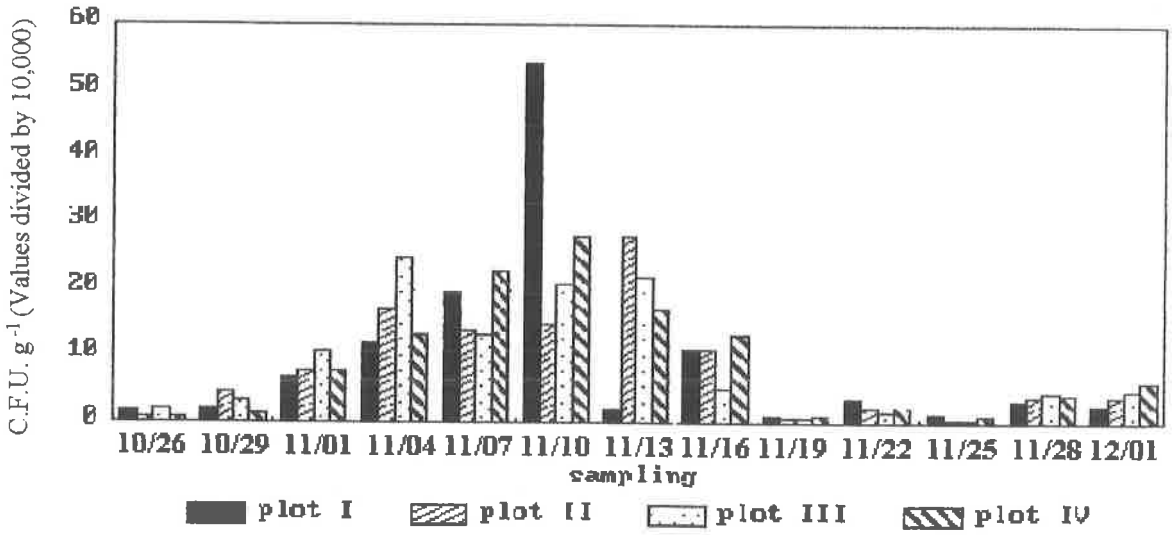

Figure 1: Fungi counts (mean of 10 plates) in dark red ortho-latosol plots with perennial soybean cultivars Clarence (I and III) and Tinaroo (II and IV).

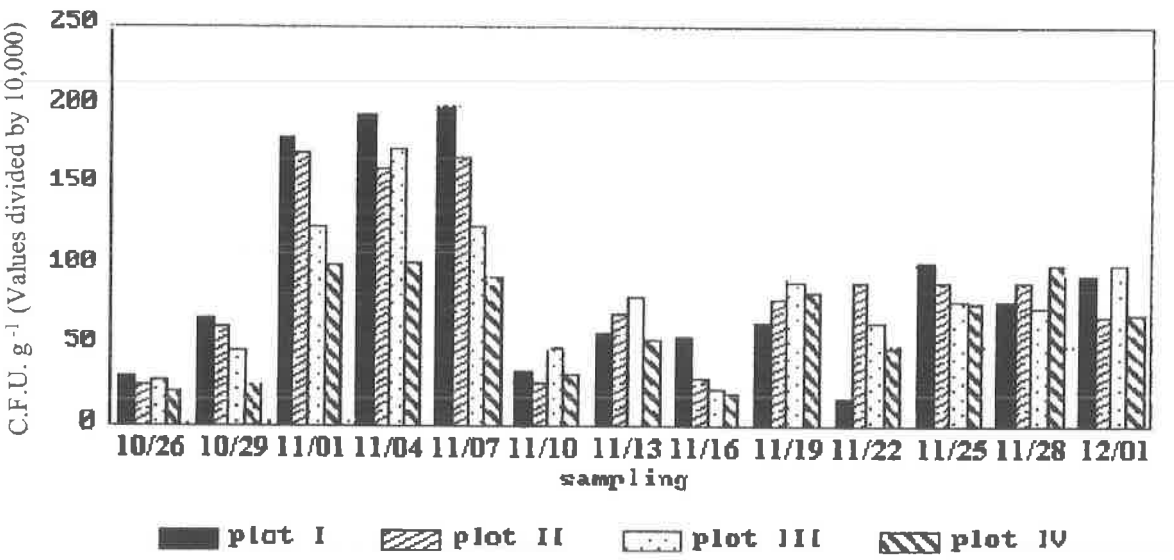

Figure 2: Actinomycete counts (mean of 10 plates) in dark red ortho-latosol plots with perennial soybean cultivars Clarence (I and III) and Tinaroo (II and IV).

interference in rhizobium growth. For this reason, readings of the number of colonies per plate were done for a shorter growth period than that recommended for characterization of colonies, because effusive growth, if present, would not permit observation of interference. Therefore, the mean number of colonies per count should be used comparatively and only in this study.

The relatively low counts shown in the figures may be attributed to plowing, removal of plant cover and the resulting exposure of microorganisms to temperature and moisture extremes (2) that prevailed in the field during soil preparation and planting. On the other hand, the short growing period before the reading may not have given slow-growing colonies of microorganisms time to appear.

The data in Figs. 1 and 2 show that after planting (26/Oct/90: First sampling) there was a nearly twofold increase in the number of colony forming units, probably because of the stimulus of microbial development due to greater availability of phosphorous in the soil (3, 17). As the sampling progressed, there were slight declines and more stable periods in the number of colony forming units for the two groups of microorganisms, in agreement with that observed by Siqueira (35). Peak fungal development occurred when the number of actinomycetes declined, between samplings 5 and 7. On these days, the plants began to emerge from the soil. Mean number of fungal colonies did not differ for the two cultivars, but the number of actinomycete colonies was consistently lower for the Tinaroo cultivar, until the ninth sampling.

The estimated number of native rhizobia (Table 1) was consistently low, with values less than 58 bacteria/g of soil. This is in agreement with data given by Lopes *et al.* (24) for perennial soybean and native rhizobia in different latosols of São Paulo State. In previous studies, numbers as high as 10^3 bacteria/g have been observed in other types of soils of the same State (33, 34).

The small estimated number of native rhizobia in this test may explain the poor nodulation observed in the field, which, according to Philpotts (30), may be due to the low number of these organisms in problem soils. The small estimated number of native rhizobia may be attributed to the methods used. Vidor (38) cites host specificity as a possible cause of underestimating rhizobia numbers in a sample by the Plant Dilution and Infection Method. This may affect the number of nodules produced by different strains of rhizobia, underestimating some while overestimating others. Kingdley and Ben Bohlool (21) reported data on the recovery of rhizobia innoculated in different types of soils, that varied from <1 to over 90%, depending in soil structure. The low number of rhizobia can be explained when soil type is taken into consideration, that is, dark red ortho latosols, clayey to very clayey in both the A and B horizon (4). The rhizobia may be not available for nodulation because of adsorption on the clay-ion complex (7, 8, 23, 26).

However, because a clayey soil was used, fewer macropores may have favored rhizobia dispersion (12), and compensated the low number of bacteria found in the field, so the low number of native rizobia may be not one limitant factor for the legume development.

Table 1: NMP of native Bradyrhizobium sp per g soil; plots I and II were sowed to cultivar Clarence; II and IV to cultivar Tinaroo.

soil samplings	plot I	plot II	plot III	plot IV
1	5,8	<5,0	<5,0	<5,0
2	<5,0	<5,0	<5,0	5,8
3	5,8	17,0	58,0	5,8
4	5,8	17,0	5,8	17,0
5	5,8	<5,0	<5,0	<5,0
6	5,8	<5,0	<5,0	<5,0
7	5,8	<5,0	5,8	5,8
8	<5,0	<5,0	<5,0	17,0
9	5,8	<5,0	<5,0	<5,0
10	<5,0	5,8	<5,0	<5,0
11	<5,0	<5,0	<5,0	<5,0
12	<5,0	<5,0	<5,0	<5,0
13	<5,0	<5,0	<5,0	<5,0

Microorganisms that produce substances which interfer with the growth of the rhizobia used as indicators in this study (Table 2) were obtained directly from the inoculation of soil dilutions in culture medium. Fifteen plates inoculated with the NO-65 rhizobium strain showed growth inhibition halos; 10 of these (3.85%) from actinomycetes and 5 (1.92%) from fungi. Twenty-four plates inoculated with the i-94b rhizobium strain also had growth inhibition halos; 18 (6.92%) from actinomycetes and 6 (2.3%) from fungi. In general, strain NO-65 produced colonies with mucous-like growth in plates where the medium had become acidified due to growth of other microorganisms (change in overall appearance of colony, dependent on medium pH, observed in some isolates of *Rhizobium* sp by Campuzano De Ramirez *et al.* (9), and forms resistent to inhibiting agent (or agents) activity inside the halos. Halos were formed in plates inoculated with strain i-94b that turned acidic or alkaline due to the growth of other microorganisms. In most of these, the rhizobium had mucous-like growth (when growing as a pure isolate in this medium, this rhizobium, like strain NO-65, presents a cowpea-group type growth) and had generally larger halos than those produced in the plates with strain NO-65, indicating greater susceptibility of the i-94b isolate to microorganisms that produce substances that are soluble in the culture medium.

The most outstanding feature of these halos was that some were over 20mm in diameter, an uncommon occurrence (41; E.C.Silva, pers. com.). Rangarajan *et al.* (36) cites interence diameters of between 3.0 and 17.5mm in a study of isolates of *Streptomyces* with

Table 2: Number of halos per rhizobium strain used as indicator: soil I and III = plots sowed to cultivar Clarence; II and IV to cultivar Tinaroo; a = rhizobium NO-65; b = rhizobium i-94b; A = plate with culture medium for actinomycetes and F = for fungi.

			soil samplings												
soil	micr	rizob	1	2	3	4	5	6	7	8	9	10	11	12	13	Σ
I	A	a	0	0	0	0	8	0	0	0	0	0	2	0	0	10
		b	2	0	0	10	1	0	3	0	1	>20	0	10	5	>52
	F	a	0	1	0	0	1	0	0	0	0	0	0	0	0	2
		b	0	0	0	0	0	0	0	0	0	0	0	0	0	0
II	A	a	12	0	0	0	0	0	0	0	>40	0	0	0	0	>52
		b	13	>21	0	0	1	0	0	0	3	0	0	0	1	>39
	F	a	0	0	2	0	0	0	0	0	0	0	0	0	0	2
		b	1	0	0	0	0	0	0	0	0	0	0	0	0	1
III	A	a	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		b	0	0	0	1	0	0	0	0	0	0	0	0	12	13
	F	a	1	0	0	0	0	0	0	0	0	0	0	0	0	1
		b	0	1	1	0	0	0	0	0	0	0	0	1	2	5
IV	A	a	0	10	0	0	0	0	0	2	2	0	0	4	>21	>39
		b	1	0	0	0	0	0	0	0	0	8	0	0	2	11
	F	a	3	0	0	0	0	0	0	0	0	0	0	0	0	3
		b	0	0	1	0	0	0	0	0	0	0	0	0	0	1
V	A	a	12	10	0	0	8	0	0	2	>42	4	2	4	>21	>101
		b	16	>21	0	11	2	0	3	0	4	>28	0	10	20	>115
	F	a	4	1	2	0	1	0	0	0	0	0	0	0	0	8
		b	1	1	2	0	0	0	0	0	0	0	0	1	2	7

Bradyrhizobium sp and *Bradyrhizobium japonicum*. In this study, the presence of halos with large diameters can be explained by the fact that rather low concentrations of bacteria were used (in the order of 10^5 to 10^6 bacteria/ml), as well as through the use of cultures in an exponential growth phase (14). However, the possibility that the microorganism that was tested is very antagonic to the rhizobium cannot be ignored.

We conclude that differences exist between rhizobium isolates regarding susceptibility to substances that are soluble in the culture medium produced by indigenous soil microorganisms. Also, in culture medium, native rhizobia are extremely sensitive to substances produced by actinomycetes and native fungi.

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RESUMO

Interação, em meio de cultura, entre actinomicetos e fungos nativos, com rizóbios eficientes na nodulação de *Neonotonia wightii* Lackley (soja-perene)

Foram feitos estudos, em meio de cultura, de relações entre fungos e actinomicetos do solo com rizóbios eficientes na nodulação de *Neonotonia wightii* (soja-perene), com o objetivo de obter material a ser utilizado em inoculações mistas desta leguminosa. Para este fim, foi instalado um ensaio de campo para coleta de amostras de solo e isolamento de microrganismos pertencentes a estes grupos, com capacidade de promover interferência no desenvolvimento de duas estirpes selecionadas de rizóbio. Os resultados indicam que as estirpes de *Bradyrhizobium* sp utilizadas e eficientes na nodulação de *Neonotonia wightii* apresentam grande sensibilidade, em meio de cultura, a substâncias produzidas nesses meios por actinomicetos e fungos.

Palavras-chave: *Bradyrhizobium*, inibição de crescimento, *Neonotonia wightii*, soja-perene.

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HEMOLYTIC ACTIVITY OF HUMAN AND MARMOSET *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* ISOLATES

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ABSTRACT

The hemolytic activity of 62 *Actinobacillus actinomycetemcomitans* strains isolated from oral cavity of marmosets (30 strains) or humans with and without periodontal disease (30 strains) as well two reference strains, was searched by culturing the bacteria in media containing human all types, sheep, horse, rabbit or cow blood. Alpha-hemolytic activity was detected in all strains and the expression of this activity seems to be stable in this microbial group. The hemolysis kinetics was determined for three *A. actinomycetemcomitans* strains and maximum hemolytic activity was detected during the stationary phase of bacterial growth .

Key words: Hemolytic activity, *Actinobacillus actinomycetemcomitans*, marmosets, periodontopathogen, human

INTRODUCTION

Hemolytic activity has been reported as a putative virulence marker for a wide variety of bacterial species (7), since it can potentialy increase the availability of iron for the "in vivo" growth and metabolism of resident or invading bacteria (4, 21). This activity is rarely noted in strains cultured from subgingival plaque of healthy periodontia, but is common in strains isolated from patients with active periodontal disease (8). *Actinobacillus actinomycetemcomitans* is a Gram-negative bacterium associated with a variety of infectious diseases such as brain abscess, urinary tract infections, periodontal disease and, particularly, cases of localized juvenile periodontitis (10, 15, 22). Although numerous virulence factors have been identified for this bacterium (6, 11, 13) there are very few papers regarding *A. actinomycetemcomitans* hemolytic activity, and with controversial results (1, 3, 5, 14).

Considering the potential relevance of hemolysins in pathogenicity (7) and its possible ecological importance, as well the lack of information about the spectrum of biological activity and ideal conditions for detection of this cytolisin production by *A. actinomycetemcomitans*, further studies concerning this periodontopathogen are necessary. Herein, the hemolytic activity (qualitative and quantitave aspects) of *A. actinomycetemcomitans* isolated from human and from marmoset oral cavity was searched using different human and animal blood. The hemolysis kinetics as well as the influence of some abiotic factors were also evaluated.

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MATERIALS AND METHODS

Bacterial strains. A total of 62 *A. actinomycetemcomitans* strains were studied: 30 were isolated from marmoset dental plaques (*Callithrix penicillata, C. jacchus* and *C. geoffroyi*), supported by the "Centro de Bioterismo, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais" (UFMG), 30 from patients with (23 strains) and without (7 strains) peridontal disease, attended in the "Clínica de Periodontia, Faculdade de Odontologia, UFMG" or in the "Clínicas de Periodontia Integrada, Universidade Federal do Piauí ", and two reference strains (Y4 and ATCC 29523).

Isolation, identification and biochemical characterization of the strains. The strains were isolated according to Slots (15) in the selective medium TSBV (Tryptic Soy Agar - Serum - Bacitracim - Vancomycin) after incubation for 72 hours at 37°C in anaerobiosis (CO_2 - 10% and N_2 - 90%), using Brewer-like anaerobic jars.The presumptive identification was based on the morphocolonial aspect, morphotintorial properties and catalase production. Biochemical complementary assays were also done according to Slots (16) and Slots et al (14). After characterization the strains were stored at -40°C in preservation medium containing inorganic salts and glycerine. For the experiments, the strains were inoculated in Tryptic Soy Agar (Difco or Biobrás), supplemented with 0.5% of yeast extract (TSAY) and 5% of blood, and incubated at 37°C in microaerophilia.

Qualitative detection of hemolytic activity (blood agar plate assay). The tests were done in TSAY supplemented with 5% of defibrinated blood (sheep, horse, or cow) or citrated blood (rabbit or human types O, A, B or AB, either Rh+ or Rh-). The strains were grown in Thioglycolate Broth supplemented with 0.5% yeast extract - TBY (37°C for 24 hours in microaerophilia) and the inoculum was standardized (approximately 10^5 cells per spot) using the Steers replicator (18), as described by Ávila-Campos et al. (2). The reading was done after 24, 48 and 72 hours of incubation, in microaerophilia or anaerobiosis, by observing the presence of an hemolytic zone around the spots.

Quantitative detection of hemolytic activity (agar overlay assay). The agar overlay assay was done as described by Janda and Abbott (9) with modifications. A volume of 20µl from overnight thioglycolate broth cultures, containing approximately 10^6 CFU/ ml, of 8 human strains, 8 marmoset strains, two reference strains and the strain of *Escherichia coli* HB101 (negative control), was spotted onto the surface of a TSAY medium. After the inoculum had dried, 5 ml of semi-solid phosphate buffered saline (PBS - 0.01M and 0.8% agar) containing 2% of blood from human (O, Rh+), sheep or horse, were layered over the TSAY medium. The plates were incubated at 37°C in microaerophilia or anaerobiosis and the hemolytic zone diameter was measured each hour during a 6-h period.

Hemolysis kinetics. *A. actinomycetemcomitans* strains Y4 (reference), P2-2 (patient with periodontal disease) and M16-2 (marmoset) were grown in TSBY (Tryptic Soy Broth supplemented with 0,5% of yeast extract) at 37°C for 24 h in microaerophilia. The cultures were then diluted in tubes containing 5ml of TSBY medium and 5 glass beads (2), to obtain 0,2 units of absorbance at 540 nm (approximately 10^6 CFU/ml) and the tubes were incubated at 37°C in microaerophilia. Assessment of growth was made by reading the absorbance at 540 nm (BECKMAN), as described by Miranda (12), at intervals of 0, 6, 12, 21, 24, 27, 36, 48, 72 and 96 h. Hemolytic activity was detected by agar overlay assay and the reading of the hemolysis zone diameter was performed within 5 h incubation. *Escherichia coli* HB101 was used as a negative control. All the assays were performed in duplicate.

Stability of hemolytic activity. To test the stability of hemolytic activity expression, all the strains were subcultured for 10 consecutive days in microaerophilia and the hemolytic activity was investigated by the blood agar plate assay with readings at 24 hour intervals as previosly described. We also searched for hemolytic activity in 10 representative clones of 8 human, 8 marmoset and two reference strains, which were randomly obtained by the pour plate method in TSAY medium. The blood agar plate assay was performed in microaerophilia, using human (O, Rh+), sheep, and horse blood.

RESULTS AND DISCUSSION

All 62 *A. actinomycetemcomitans* strains showed hemolytic activity which was characterized, in all blood types tested, as partial hemolysis or alpha-hemolysis.

When the blood agar plates (qualitative tests) were incubated in microaerophilia, hemolytic activity was detected within 24 h of incubation and the hemolysis

zones did not change until 72 h for all the strains studied. Hemolytic activity was also detected in *A. actinomycetemcomitans* strains grown under microaerophilia by Ávila-Campos et al. (3) and Ávila-Campos (1). Among the *A. actinomycetemcomitans* strains isolated from periodontal disease patients examined with human (all types), rabbit, and sheep blood, Ávila-Campos (1) found 2 non-hemolytic strains, 2 hemolytic strains only on human blood (one on AB, Rh+ and another on A, Rh+ and AB, Rh+), and 52 hemolytic strains on both rabbit and sheep blood agar. However, unlike the present study, that author showed no evidence of hemolytic activity in the reference strains Y4 and ATCC 29523 when agar plates containing sheep, rabbit or human (O, Rh+ and AB, Rh-) blood were used.

When the blood agar plates were incubated for 24 h in anaerobiosis it was also observed alpha-hemolytic activity in all *A. actinomycetencomitans* strains. However, after 48 and 72 h of incubation, the hemolysis zone of all strains became more diffuse, presenting a faint yellow-greenish colour, and difficult to detect on all blood types tested (human O Rh+, sheep and horse). This may explain why Slots (14) detected no hemolytic activity in strains grown on rabbit or sheep blood agar in anaerobiosis with reading at 96 h. Farias et al. (5) also did not detect hemolytic activity in 14 strains isolated from periodontal diseased patients using sheep blood agar and incubation at 37°C for 48 h in microaerophilia.

The results obtained for most of the strains studied were similar when the agar overlay assay was performed either in microaerophilia or in anaerobiosis with 3 different types of blood (Table 1). Hemolysis zones of 1-3mm of diameter, with a clear yellow-greenish aspect, were observed after 5h of incubation, while after 6h of incubation most strains produced hemolysis zone of 3-5 mm of diameter. As shown in Table 1, only three marmoset strains showed different results with the condition of the assay employed. For strains M16-45 and M20-1, a 3-5 mm

Table 1. Detection of hemolytic activity in *Actinobacillus actinomycetemcomitans* strains isolated from the oral cavity of humans with or without periodontal disease (PD) and marmosets by an agar overlay assay performed with different types of blood [a].

Strain	Hemolytic Activity in Medium Containing Blood From [b]		
	Human (O, Rh+)	Sheep	Horse
Reference			
Y4	+ +	+ +	+ +
ATCC 29523	+ +	+ +	+ +
Patients with PD			
P2-2	+ +	+ +	+ +
P2-4	+ +	+ +	+ +
P7-13	+	+	+
P7-23	+ +	+ +	+ +
P14-21	+ +	+ +	+ +
P14-39	+	+	+
Patient without PD			
P8-10	+ +	+ +	+ +
P8-12	+ +	+ +	+ +
Marmosets			
M16-2	+ +	+ +	+ +
M16-45	+	+ (++) [c]	+ +
M22-11	+ +	+ +	+ +
M22-23	++ (+)	+ +	++ (+)
M20-1	+ +	+ (++)	+ +
M20-6	+ +	+ +	+ +
M37-23	+ +	+ +	+ +
M38-8	+ +	+ +	+ +

a - Media were incubated in anaerobiosis or microaerophilia for 6 h;
b - Hemolytic zone diameter: - no zone detected; + zone diameter from 1 to 3mm;
++ zone diameter from 3 to 5mm
c - results in parenthesis show the differences presented by the strains when incubated in microaerophilia

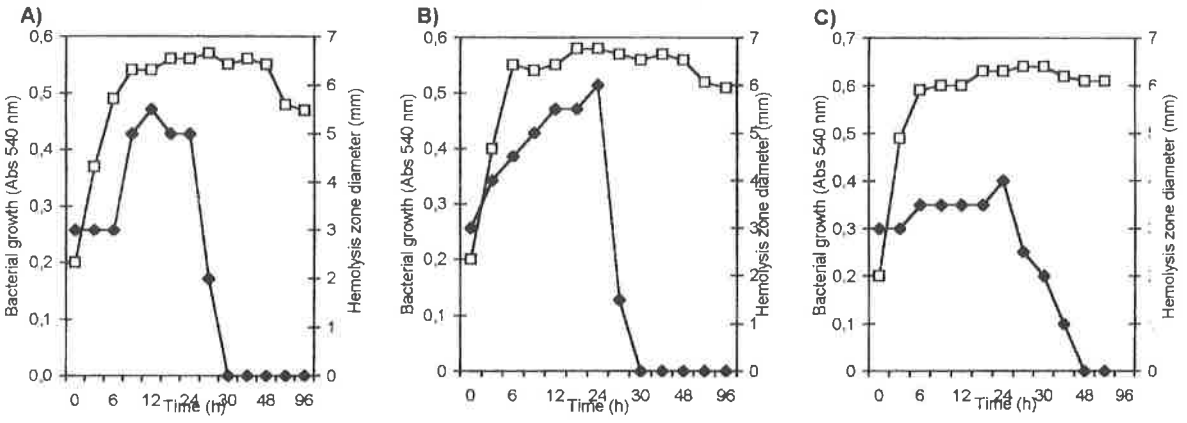

Figure 1. Growth Curve (□) and Hemolytic Activity (◆) of *Actinobacillus actinomycetemcomitans*. A) strain M16-2; B) strain P2-2; C) strain Y4

hemolytic zone was observed after 6h incubation in microaerophilia on sheep blood, while an hemolytic zone of 1-3mm was detected after incubation in anaerobiosis. For strain M22-23, it was observed on human (O Rh+) or horse blood a 1-3 mm hemolytic zone in microaerophilia and a 3-5mm hemolytic zone in anaerobiosis. Therefore, a single strain of *A. actinomycetemcomitans* can behave differently in distinct incubation atmospheres. *E. coli* HB101 (negative control) did not show hemolytic activity in microaerophilia or anaerobiosis. The influence of incubation atmosphere in hemolytic activity was also observed for several other bacterial species (9,17,20).

The results obtained in this study reveal that the blood agar plate and the agar overlay assays were appropriate to detect hemolytic activity in *A. actinomycetemcomitans* and no major differences were detected when the assays were performed with two atmosphere conditions and human or animal blood types. The reading time was critical when the blood agar plate assay was performed under anaerobiosis. The agar overlay assay seemed to be more sensitive than assays on blood agar plates, since the hemolytic activity could be detected within an incubation period of 5 h. Expression of this activity was stable in all strains and no populational heterogeneity was observed when subpopulations of human or animal isolates were examined.

The growth curves of M16-2, P2-2 and Y4 strains are shown in Fig. 1. The three strains did not show lag-phase probably because of the physiological state of bacteria in the inoculum. Strains Y4 (reference) and P2-2 (periodontal disease patient), both from human origin, reached log-phase peak within 6 h of incubation and strain M16-2 within 9 h of incubation. No significant alteration in absorbance was observed after log-phase for the three strains, which suggests that cellular death is not followed by lysis. These data are in agreement with those obtained by Miranda (12) who showed that the death-phase of *A. actinomycetemcomitans* aparently starts after incubation for 24 hours. With regard to hemolytic activity, it was maximum in 12 h cultures of the marmoset strain M16-2 and in 24 h cultures of Y4 and P2-2 strains (Fig. 1). Cultures of all three strains incubated for 27 h showed lower hemolytic acitivity and such activity was not detected after 36 h of incubation. These results suggest that expression of hemolytic activity by *A. actinomycetemcomitans* is probably dependent on cell viability.

Since the relevance of *A. actinomycetemcomitans* is already established in human pathology (10, 15, 22) and hemolytic activity expression is a common property of this microbial species, further studies are needed for the biochemical and molecular characterization of this activity, as well the definition of its role in pathogenicity.

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RESUMO

Atividade hemolítica de amostras de *Actinobacillus actinomycetemcomitans* isoladas de seres humanos e de saguis

A atividade hemolítica de 62 amostras de *Actinobacillus actinomycetemcomitans*, isolados da cavidade oral de saguis (30), de seres humanos com e sem doença periodontal (30) e duas amostras de referência foram testadas cultivando as bactérias em meio contendo sangue humano, de carneiro, de cavalo, de coelho e de boi. A atividade alfa-hemolítica foi detectada para todas as amostras e a expressão desta atividade parece ser estável neste grupo microbiano. A cinética de hemólise foi determinada para três amostras de *A. actinomycetemcomitans* e o máximo da atividade hemolítica foi detectada durante a fase estacionária.

Palavras-chave: atividade hemolítica, *Actinobacillus actinomycetemcomitans*, saguis, periodontopatógeno, humano.

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EPIDEMIOLOGICAL CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM *BOVINE MASTITIS* IN PORTO ALEGRE (RIO GRANDE DO SUL, BRAZIL)

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ABSTRACT

One hundred strains of *S. aureus* isolated from bovine mastitis in Porto Alegre (Brazil) were analysed for biotype, phage pattern and ability to produce protein A. Biotype could be defined in 76% of the isolates: 52% belonged to biotype C (bovine origin) and 24% to biotype A (human origin). Phage typing performed with the human basic set (Routine Test Dilution - RTD x 100) allowed the classification of 42% of the samples. Low titers of protein A were detectable in 89% of the strains.

Key words: *Staphylococcus aureus*; bovine mastitis; biotyping; phage typing; protein A.

INTRODUCTION

Staphylococcus aureus (S. aureus) is the most frequently bacterium isolated from bovine mastitis, being responsible for large economic losses all over the world.

Members of genus *Staphylococcus* present adaptation factors to their hosts, being able to be classified as the origin through biotyping (5,8). Blair and Williams (1) standardized phage typing, through which is possible to investigate the sources and propagation routes of staphylococcal infections. The join utilization of both techniques was suggested by Parker (9) for the performance of epidemiologic studies.

Since there is little information regarding the role of *S.aureus* as the etiological agent of bovine mastitis in Rio Grande do Sul (Brazil), the present paper aimed to characterize samples of this bacterium isolated from intramammary infections of bovines according to their biotype, phage pattern and ability to produce protein A.

MATERIALS AND METHODS

1. Bacteria and Growth Conditions. The 100 *S. aureus* isolates used in this study had been isolated from milk of cows from different short dairy properties around Porto Alegre. All cows suffered from subclinical mastitis. The isolates were identified as *S. aureus* following the recomendations (2).

2. Biotyping. The following assays were performed for biotyping of the *S. aureus* isolates.

Coagulation of bovine plasma. The investigated *S. aureus* isolates were grown for 18h at 37°C in brain hearth infusion broth (BHI, Merck, Darmstadt, FRG). 500 µl of these overnight suspensions was subsequently added to 500 µl citrate plasma from cattle. After incubation for 4 h at 37°C, this assay was evaluated for coagulation (8).

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Growth on crystal violet agar. The isolates were grown for 24h at 37°C on Columbia agar plates (Difco, Detroit, USA) containing crystal violet in a final concentration of $1:10^5$. Thereafter, yellow colonies were considered to be crystal violet-positive, while violet colonies were considered to be crystal violet-negative (5, 8).

Detection of fibrinolysin production. The isolates were grown on Columbia agar plates supplemented with 12% *(v/v)* human citrated plasma for 24h at 37°C. Fibrinolysin producing bacteria exhibited a cleared zone around the colonies (8).

Determination of hemolysin types. The isolates were incubated on 5% *(w/v)* sheep blood agar for 24h at 37°C and were analysed for their hemolysin production using the method described by Schalm et al. (10).

3. Phage Typing. Phage typing was performed according to Blair and Williams (1), in the Routine Test Diluition x 100 (RTD x 100). The following phages from the human basic set were utilized: 29, 52, 52A, 79, 80, 3A, 3C, 55, 71, 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85, 94, 96, 95, 81, 89, 90, D11 and HK2.

4. Production of Protein A. The isolates were incubed in BHI for 18h at 37°C. The suspension was centrifuged and the pellet dissolved in 1 ml Tris-HCl 0,05M NaCl 0,45M pH 7,5 (PBS). To this suspension was added 40 µl/ml of lysostaphin (Sigma). After incubation for 45 minutes at 37°C, this suspension was centrifuged and the supernatants used for protein A isolation (11). The protein A was detected by passive hemagglutination test using microtiter plates, as described by Carret et al. (2).

RESULTS

Seventy-six isolates were defined to the biotype: 52 isolates belonged to biotype C (bovine origin) and 24 isolates to biotype A (human origin). The isolates were classified into biotype C because they presented

Table 1. Biotypes from 100 strains of *S. aureus* isolated from bovine mastitis in Porto Alegre (1994)

Bovine coagulase	Crystal violet	Fibrinolysin	Haemolysin	No. of strains	Biotype
-	y	-	β	26	C
-	y	+	δ	16	A
+	y	-	α, β	10	C
-	vio	-	β	8	*
-	y	-	α	6	*
+	y	-	β	4	C
+	y	-	α, β, δ	4	C
+	y	-	α	3	C
-	y	-	absent	2	*
-	y	-	δ	2	*
-	y	+	α	2	A
-	vio	-	α, β, δ	2	*
-	vio	+	δ	2	A
+	y	+	α	1	*
-	y	+	β, δ	1	A
+	y	-	β, δ	1	C
-	y	-	α, β, δ	1	C
-	y	-	α, β	1	C
-	vio	-	α, β	1	*
+	y	-	α, δ	1	C
-	y	+	α, δ	1	A
-	vio	+	α, δ	1	A
-	vio	-	α	1	*
-	vio	+	α	1	A
-	vio	-	δ	1	*
+	vio	-	δ	1	C

- negative; + positive; y yellow; vio violet; * not classified

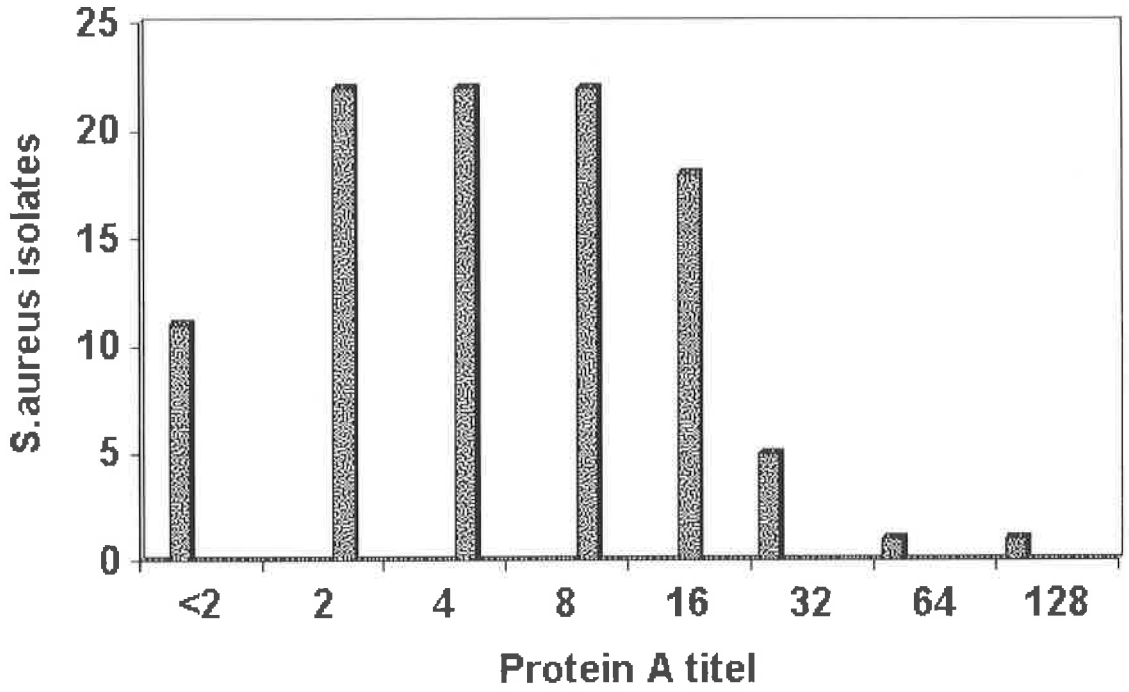

Figure 1. Protein A titel of 100 *S.aureus* isolates from bovine mastitis tested by passive hemagglutination test.

TABLE 2. Phage patterns of 100 strains of *S. aureus* isolated from bovine mastitis in Porto Alegre (1994)

Phage pattern	Phage group	No. of strains
NT	-	58
90	S	7
71/79	I, II	4
81	NC	4
79	I	3
71 (3A+)	II	3
6/42E/47/53/54//79/83A/85/D11/HK2	I, III, S	3
(81+)	NC	2
81/HK2	NC, S	1
6/42E/54/81/HK2	III, NC, S	1
6/53/79/83A/85/D11/HK2 (42E/81+)	III, S	1
85	III	1
53 (77/81/85+)	III	1
6/HK2 (42E/47/54+)	III, S	1
53/81/83A/85/D11/HK2 (90+)	III, NC, S	1
3A/6/42E/47/54/75/77/81/83A/84/94/D11/HK2	II, III, V, NC, S	1
29/81 (52+)	I, NC	1
6/85/HK2 (42E/47/54+)	III, S	1
53/71/79	I, II, III	1
6/79/83A/D11/HK2 (77+)	I, III, S	1
6/53/54/HK2 (83A/D11+)	III, S	1
(53+)	III	1
79+)	I	1
(6/HK2+)	III, S	1

NT not typable (--+) weak reaction (from 20 to 50 plaques); NC not classified; S special

production of bovine coagulase and/or yellow color on crystal violet agar associated to the production of beta haemolysin and abscence of fibrinolysin. All isolates producing fibrinolysin were classified into biotype A, independent of other characteristics. The different biotypes found are listed in Table 1.

Forty-two samples were typed in RTD x 100. The phage types found are listed under Table 2.

Protein A was found in 89 samples, with titers ranging from 1:2 up to 1:128 (Fig. 1).

DISCUSSION

A larger number of *S. aureus* of human origin (24%) was found in the present study, when compared to the results obtained (1,6%) also in Brazil by Lopes et al. (7). This difference might be related to the kind of dairy exploitation in the properties where the samples were collected. While Lopes et al. (7) obtained their samples in dairy farms, the samples utilized in this study came from small and less technically provided dairy properties, where the recommended sanitary milking rules are not usually followed.

In the present study, only 25% of the *S. aureus* samples coagulated bovine plasma. In opposition to the results obtained by Meyer (8) and Hajek and Marsalek (5), the present study has demonstrated that bovine coagulase is not an ever-present characteristic in *S. aureus* of bovine origin. Eventually exists a variation in the geographic distribution of this characteristic, as suggested by Wilson and Miles (13).

With respect to the color of the colonies on crystal violet agar, only 4 isolates of human origin (16,67%) presented the violet color in this agar, diverging from the results reported previously (5, 8). In the present study, the crystal violet test did not diferentiate isolates of bovine and human origin.

Twenty-four isolates could not be classified into biotypes. This classification difficulty was related to the discrepancies in the combination of results obtained from the different tests performed. Such discrepancies might be related to the genetic variation of the isolates or even to their adaptation to the host or to the region.

Comparing the results of the phage typing with those obtained in South Africa (12) and in Poland (6), the percentage of the typable strains remained quite below that found by those researchers with the human set of phages. It was, however, larger than the one found in Brazil (7) with the same set. A more detailed comparison would be possible by typing the samples with the bovine set of phages, not available in this study. The strains of *S.aureus* showed susceptibility to different groups of phage, and there was no predominant phage pattern. Most strains with uniform pattern of phage susceptibility were isolated from the same properties or regions, and they share common phenotypic characteristics.

The production of protein A could be observed in low titers in most of the isolated samples. This result is in accordance with Forsgren et al. (4), indicating that the level of protein A production is not high in *S. aureus* involved in chronic bovine mastitis.

From the present study it was possible to observe that biotyping of *S. aureus* isolated from bovine mastitis was not the best criterium for epidemiologic classification. Phage typing, which has advantages such as the high specificity and reproductibility, becomes many times difficult for the conditions of underdeveloped countries or for regions far from reference centers. Other criteria, like the profile of antimicrobial resistence, plasmidial and cromossomal DNA profile or presence of virulence factors, might be an alternative for the classification and epidemiologic tracking of *S. aureus* involved in bovine mastitis.

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