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GENETIC DIVERSITY OF HIV

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MINI REVIEW

ABSTRACT

The human immunodeficiency virus (HIV) is a retrovirus (Fam: *Retroviridae*), which is the causative agent of the devastating AIDS pandemics currently affecting millions of human beings. HIV rapidly evolves by several molecular mechanisms, such as mutation, hypermutation, insertions and deletions and recombination; all process actively contributing to its genetic diversity. This rapid evolutionary process, assisted by the rapid transmission through human populations, poses a significant challenge to the effective monitoring of HIV variation and AIDS control. Herein, the implications of genetic diversity related to the pathogenesis of HIV infection are addressed. The potential problems in its reliable detection by the antibody screening tests in current use, the role of a global genotyping surveillance, efforts to trace the progress of the worldwide epidemic and the rationale for future vaccines and drug trials will be discussed.

Key words: HIV-1, genetic diversity, molecular epidemiology

INTRODUCTION

The first indication that AIDS could be caused by a retrovirus came in 1983 when researches at the Pasteur Institute (1) recovered a reverse transcriptase containing virus from the lymph node of a man with persistent lymphoadenopathy syndrome. Since them, the human immunodeficiency virus has been considered the most significant emerging infectious pathogen of this century (25). In spite of the rapid ascertainment of the HIV epidemiology, and development of diagnostic tests and antiretroviral therapies, HIV continues its global spread. Probably the greatest challenge arises from the realization that the virus has a remarkable genetic heterogeneity. A major concern comes from the observation that HIV strains can elude detection by some of the most widely used serologic tests, escape immune surveillance, and rapidly develop resistance to antiviral compounds.

Individuals infected with HIV have been shown to harbor a large and complex mixture of different but related genomes. Eigen and colleagues (16) introduced the term "quasispecies" to refer to the diverse, rapidly evolving and competing RNA populations, postulated as earliest life forms on earth, and the formal principles they have elaborated for quasispecies evolution apply well to HIV populations today. The different mutants within such a quasispecies may exhibit marked differences with respect to biological properties such as cell tropism, cytopathic properties, replication rates, and surface antigen characteristics. The high and rapidly increasing level of heterogeneity seen in HIV strains can allow antigenic changes and immune system evasion, and development of effective resistance to antiviral therapeutics. Such diversity facilitates the establishment and progression of chronic HIV infections, with the appearance of strains of increased

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cytopathicity. The antigenic changes and immune system evasion is also of concern to the design of broadly cross-reactive vaccines, and in the assessment of the performance of diagnostic tests.

MECHANISMS OF SEQUENCE DIVERSITY

The mechanisms thought to contribute to the HIV diversity include mutation, hypermutation, insertions and deletions, and intergenic recombination. Mutations during reverse transcription have been demonstrated to occur *in vitro* (51) and *in vivo* (4, 29). The error rate has been estimated to be 5-10 misincorporations per HIV genome per replicative cycle (51, 46, 2). This high rate is consequence of the negligible proofreading exonuclease activity of the reverse transcriptase, that shows minimal sequence-correcting exonuclease activity. Hypermutation is defined as the process by which individual proviruses acquire several mutations in a single replication cycle. Hypermutation may underlie the preponderance of G to A substitutions commonly observed in HIV and the simian immunodeficiency virus (SIV). Moreover, G to C substitutions account for 90% of all substitution in the HIV genome ("dislocation mutagenesis") (59). Insertions and deletions, usually involving multiples of three nucleotides have been observed in virtually every study of HIV quasispecies. They predominate in the envelope (*env*) V5 origin. HIV-1 intergenic recombination has been documented in cell culture, where experimental manipulation can achieve dual infection by two defined parental strains differing at linked genetic loci (6, 57, 59). Recombination between different HIV-1 strains has also been demonstrated to occur *in vivo* by an unusual case of dual infection as a result of transfusion. We have recently described the unique case of dual infection in which an infant was concurrently transfused with two different HIV-1 seropositive units from two different donors, and we documented the first unequivocal evidence for dual infection, as well as recombination between the two infecting HIV-1 strains (11). Although the transfusion case was certainly unusual, dual infection may well be occurring often among people engaging in frequent high risk activities. Indeed, a case of alleged multiple infection as a result of homosexual activity (66), and reports of recombinants between different subtypes of HIV-1 (53), as well as HIV-2 (17), provide additional evidence suggesting that dual infection may be occurring relatively frequently. Several instances of recombination between members of the same quasispecies have also been reported (67, 8, 19, 24). Therefore, it is likely that recombination within and between strains may constitute a significant factor in the generation of HIV-1 diversity.

As noted above, the genetic diversification of HIV-1 is occurring over time within each infected individual. Of course, since the current pandemic constitutes the sum of infected individuals, HIV-1 has been diversifying over time and space at the level of the entire viral species as well. First, we will focus on the events, parameters, and ramifications of diversity at the individual level, and then move to the pandemic level.

INTRA-INDIVIDUAL DIVERSITY AND PATHOGENESIS OF AIDS

A few weeks after an individual is first infected with HIV, high titer viremia is detected, and high levels of virus replication can be documented in lymphoid tissue. With the appearance of antibody in the blood (i.e., seroconversion), it becomes difficult to isolate virus, and antigens decline to low or undetectable levels. Recent studies have shown that during the seemingly quiescent period following the primary infection with HIV, which often lasts a decade, the body is producing and destroying almost a billion virions a day. At the same time, about 100 million CD4-T cells are also being destroyed daily (22, 60). The body is also working hard, quickly replenishing most of the blood's supply of CD4-T cells. Consequently, the numbers of both the immune cells and virus remain stable, or nearly stable, for extended periods. Eventually the lymph nodes, where CD4-T cells are produced, wear out. Moreover, rapid replication of the virus means that many mutations arise, raising the chances that variants more cytopathic or resistant to treatment will emerge. Besides, the viral load after seroconversion predicts the likelihood of developing AIDS later (36).

Virological studies of primary infections indicate that HIV-1, present in acute seroconvertors, is relatively homogeneous in sequence and in phenotype (typically macrophage-tropic and non-syncytium-inducing [NSI]). Of note, studies of linked transfusion clusters indicate that the internal replicating strain is homogenous, even though the corresponding source (so-called "donor") harbors a mixture of genotypes and/or phenotypes (66, 64). It is not yet clear what accounts for this "bottleneck" during

transmission. Analyses of HIV-1 sequences in seroconvertors shows a stronger pressure to conserve the *env* gene than other viral core genes such *gag* (66, 64). Several hypotheses have been proposed to explain this discrepancy between the spectrum of virus in acute seroconvertors and transmitters. First, it is proposed that multiple HIV-1 variants penetrate in the new host, but only one is selected to become the dominant population because of its biological characteristics (selective amplification). This explanation is supported by the homogeneity found in seroconverting hemophiliacs who were presumably inoculated with multiple HIV-1 variants parenterally (64). Another model, termed selective transmission, proposes that one HIV-1 variant has an advantage in penetrating the mucosal barrier of the new host. This would explain not only the sequence homogeneity but also the finding that HIV-1 isolated during the primary infection is predominantly macrophage-tropic and NSI, i.e., macrophages or related antigen-processing cells in the submucosal space may allow NSI viruses, which are most efficient in replicating in these cells, to penetrate. A third possible explanation for bottlenecking is that multiple variants are transmitted with one strain predominating early following seroconversion. Even having low copy number at the time of contagion, the strain which will eventually dominates emerges at detectable levels later when immune pressures suppress abundant early replicating strains (9). This possibility would explain why a health care worker accidentally infected with a clone of HIV-1, showed an unusual homogeneity in the genotypes found up to 5 years after the primary infection (48). Furthermore, we have recently described a case where an individual was exposed to two different HIV-1 quasispecies by transfusion and got infected by the quasispecies of only one of the donors (13). This case exemplifies a previously unreported instance of transmission bottleneck selecting specific genomes from different inoculated HIV quasispecies.

In general, more virulent strains, i.e., those with cytopathic properties and high replication rates, tend to appear late in the asymptomatic period reviewed by Nowak (43). Thus HIV-1 isolates from asymptomatic carriers tend to grow slowly *in vitro* and yield low titers of p24 antigen and reverse transcriptase activity, whereas isolates from patients with AIDS or "AIDS related complex" grow rapidly, induce multinucleated cell syncytia more frequently, and show high levels of p24 antigen and reverse transcriptase activity (43). There is substantial evidence, particularly from studies of symptomatic patients, that the virus changes to "escape" immune antiviral responses in the host (35, 48). In general, serum from one individual is not able to neutralize the replicating strain in the person at that time as effectively as it neutralizes previous autologous strains or strains from other individuals (23, 18, 62). The generation of viruses that have "escaped" neutralization by serum antibodies has been demonstrated in *in vitro* studies, where HIV-1 strains cultured in the presence of neutralizing monoclonal antibodies mutate to become resistant to neutralization (35, 48, 50). A few nucleotide substitutions in the HIV-1 genome are enough to confer resistance to neutralization by antibodies (58, 61) or resistance to anti-retroviral drugs (49). *In vivo* studies show that after the decline of V3-specific antibodies, there is a simultaneous increase in genomic RNA levels and progression to AIDS, with the emergence of a new variant with major genomic changes, forming a new homogeneous population of sequences (61).

Thus, the increase in the number of virus variants may be a important factor leading to the breakdown of the immune system. There is also evidence that rapid disease progression might be related to lack of control of primary infection or higher viral burden stabilized after seroconversion (36, 31), or in rare cases where syncitium inducing (SI) viruses were shown in primary infection (40, 52). The study of a group of long term asymptomatic Australians who were infected with blood from a single blood donor has also emphasized the potential importance of the infecting viral strain on disease (30).

INTER-INDIVIDUAL DIVERSITY WITHIN SUBTYPES

Analyses of the rates of diversity within the *env* gene of HIV-1 between infected individuals within a point source cluster suggest that they may be diverging at rates of up to 1% per year (28, 5, 65). This intrapatient evolution contributed to the existence at the population level of many closely related but distinguishable "swarms" of HI-V variants (16). The average intrapatient *env* gene distance from randomly selected HIV-1 positive patients through the US back in the period between 1983-1985 was between 1 and 4%, while the interpatient distance ranged from 4 to 10 percent. The average intrapatient *env* distance for the US samples reached 6.5 percent in the period between 1990-1993 and the interpatient variation was

between 4 and 10 percent for the same period. The rates of intra-cluster genetic variation is always in between the intra and interpatient variation rates (14).

At a level beyond, and as a result of, this intra-individual genetic variation, HIV-1 is evolving over time and space in the human population. HIV-1 and HIV-2 are at the extremes of this subgroup diversity. Analyses of *gag* and *env* genes from HIV-1 genomes sampled at different times in the epidemic, and from different regions of the world, reveal HIV-1 as having evolved into distinct subtypes, or clades. A minimum of 9 distinct subtypes, or clades, of HIV-1, with an additional outlier clade designated HIV-1 group O (38, 42), are now circulating in the centers of the AIDS pandemic. It is conceivable that these distinct forms (in both *gag* and *env* sequences) may have diverged from a single common ancestor, as recently as 1960 (39). The HIV-2 family is similarly subdivided into at least 6 subtypes (17). The amino acid composition of each IIIV-1 clade differs from that of the others by at least 20% in the *env* region and 15% in the *gag* region (38). Their interrelationship has been portrayed by star-shaped phylogenetic trees, which are indicative of exponentially growing viral populations, further indicating the explosive nature of the AIDS pandemics. In contrast, group O variants are so diverse and distant from the others, averaging about 60% of nucleotide divergence, that they are better portrayed as outliers to the star-shaped topology, which includes the other HIV subtypes. These clades are widely distributed geographically and show changing distributions in different parts of the word, probably due to distinct "founder viruses" initiating localized epidemics in different regions (27, 17). Subtype A is found primarily in Central Africa and India, subtype D in Central Africa, subtype E in Thailand, subtype F in few isolates from intravenous drug users in Brazil (54) and all of the viruses characterized from children in Rumania (15). Other potential sequence subtypes, described as G, H, and I, include viruses from Africa, Russia, and Taiwan (38, 26, 3). All of the clades identified so far, can be found in Africa.

HIV-2 was discovered in 1985 in several countries in West Africa, and initially called HTLV-IV and LAV-2. More recently, HIV-2 has spread at low rates into Western Europe, where a number of infected blood donors have been detected and cases of transfusion transmission documented (44). HIV-2 is transmitted in the same manner as HIV-1, i.e., by sexual contact, IV drug use, and, at a lower rate than for HIV-1, from mother to child. HIV-2 causes progressive immunodeficiency, with susceptibility to a similar array of opportunistic infections as seen with HIV-1. Recent studies indicate that rates of disease progression and secondary viral transmission are lower in persons infected with HIV-2 as compared to HIV-1, possibly due to lower viral burden in HIV-2 infection (34).

In 1990, a divergent strain of HIV-1, now characterized as group O (38), was first reported from patients from west-central Africa (7, 21). This subtype is now endemic in the central African countries of Cameroon and Gabon and recently detected in Europe. However, these variants do not represent a single subtype and may be as different from each other as are the variants that make up subtypes A-I. The worldwide distribution of these divergent HIV-1 strains is not known yet, but they have been reported in Cameroon (7, 41), Gabon (41) and France (32). Despite being rare in France, the prevalence of HIV-1 group O among HIV-1 infected people was 3% in Gabon (41) and between 5 and 8% (41, 20, 63).

Migration of diverse forms of HIV-1 will inevitably play a major role in the molecular epidemiology of HIV-1 and HIV-2. Cocirculanting lineages of HIV-1 are being tracked in Thailand (45), Brazil (37, 33), Russia (3) and Central Africa, such as Gabon (41) and Cameroon (41). Besides group O, five additional HIV-1 subtypes (A, B, E, F, H) were identified in Cameroon (42).

SURVEILLANCE OF GENETIC VARIANTS

As summarized above, there is a phenomenal breadth of HIV diversity in Central Africa (42, 17) with increasing evidence of an accelerating "trafficking" of subtypes around the world. In mid-1994, French investigators first reported that a significant proportion of persons infected with HIV-1 subtype O tested negative on a number of HIV-1 and HIV-1/HIV-2 combination assays (32, 56). This lack of sensitivity observed with those antibodies assays was later confirmed by investigators at the Center for Disease Control (CDC) (55). Antibody assays employing synthetic peptides or recombinant antigens on the solid phase, and those using the so-called third-generation "antigen-sandwich" format, were particularly prone to false negative results. Although no HIV-1 subtype O infections have been reported in the U.S. to date (55), test manufacturers have moved quickly to enhance their assays' sensitivity to unusual variants like subtype O.

We have been monitoring the prevalence of HIV-1 subtypes in the US blood donor set using the heteroduplex mobility assay (HMA), a technique based on the mobility on DNA heteroduplex in polyacrylamide gels (10). We are currently analyzing samples from HIV-positive blood donors from 1984 and 1993/1994, and to date, there has been no detectable inroads of diverse HIV-1 strains in the US blood donors (12).

In sum, giving the rampant spread of HIV variants in the human population and the fast rate of evolution of the virus, a sustained high level of vigilance and careful assessment of the implications of the growing genetic diversity are needed if we are to have a chance of implementing effective control measures, including vaccination programs and antiviral drug therapies.

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RESUMO

Diversidade genética do HIV

O vírus da imunodeficiência humana (HIV) é um retrovírus (Fam: *Retroviridae*) que é o agente causador da devastadora pandemia da Síndrome da Imunodeficiência Adquirida (AIDS), atualmentte afetando milhões dos seres humanos. O HIV evolui rapidamente em decorrência do alto índice replicativo e de mecanismos como a mutação, hipermutação, inserções/deleções e recombinação: todos estes contribuindo ativamente para diversidade genética do vírus. Este processo evolucionário rápido, com a contribuição da transmissão muito rápida entre hospedeiros humanos, proporciona um fenomenal desafio no monitoramento eficaz da variabilidade do HIV e o ao controle da AIDS. Serão discutidas nesta revisão as implicações da diversidade genética do HIV na patogênese. São também abordados os problemas potenciais relacionados à detecção eficaz da infecção pelos testes de triagem atualmente em uso, o papel da epidemiologia molecular na vigilância da pandemia e as bases para estudos futuros de testes de vacinas e ensaios com drogas anti-retrovirais.

Palavras-chave: HIV-1, diversidade genética, epidemiologia molecular.

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PRODUCTION OF SLIME BY *STAPHYLOCOCCUS EPIDERMIDIS* AND ADHERENCE TO BIOMATERIALS

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ABSTRACT

The authors analysed the influence of the production of slime by *S. epidermidis* on the adherence to the biomaterials polivinylchloride (PVC), siliconised latex (LTX), teflon^R (TFN), poliurethane (PUT) and vialon^R (VLN). Six strains of *S. epidermidis* were compared, three slime producing and three not. With respect to PVC, LTX, PUT and VLN no significant difference between the two types of strains was noted. Teflon^R on the other hand, permitted an even greater amount of adherence of slime producing strains. All biomaterials and especially teflon^R, which generally presents low ratios of adherence for *S. epidermidis*, demonstrated higher levels of adherence of slime producing strains and have a greater potential for pathogenicity. The influence of production of slime by *S. epidermidis* on their adherence to plastic biomaterials, *in vivo*, should be better studied. The clinical and laboratorial evidences suggest that *S. epidermidis* isolated from patients using medical devices should be tested for their capacity to produce slime since this information can help in establishing the virulence of the organism and thus orient therapeutic measures.

Key words: nosocomial infection, slime, catheter, bacterial adherence, biomaterial.

INTRODUCTION

Hospital infections are a Public Health hazard that raises the cost of health care. In the USA, approximately 5% of all hospitalized patients acquire infections during stay in hospital (4). This fact has brought about various studies on the factors involved in these infections (21, 22).

According to Sheth *et al.* (30), infections related to the use of intravascular devices are the main complication in modern medical practice. Lopez-Lopez *et al.* (17, 18, 19) also demonstrated the importance of the material of which these biomaterials are made of, in altering the bactericidal activity of the defence cells of the human body.

Christensen *et al.* (8), while analyzing a rise in the incidence of sepsis associated to the use of intravascular catheters, found that 63% of the bacterial strains isolated produced slime and that only 37% of the bacteria isolated from contaminated cultures and from the skin also produced slime. The production of this mucoid substance could also be a microbial defense mechanism against antibacterial agents (13, 14, 24).

Goldmann (11) states that coagulase-negative *Staphylococci* are the commonest nosocomial pathogens isolated from patients using various prothesis and long-staying devices such as central venous indwelling catheters. With respect to *S. epidermidis*, foreign-body type infections seems to be associated to a polysaccharidic capsular adhesin which would mediate the adhesion to biomaterials. On the other hand, slime could promote persistent colonization to long-staying indwelling medical

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devices and protect *Staphylococci* from the scavenging action of host defence mechanisms.

There is a large probability that any individual will be submitted to a medical device during his lifetime, and the most dangerous complication of this procedure is an infection (29, 33). Various medical devices are made of different synthetic substances. At the present time those used are polyurethanes, with a series of physical properties which make them more adequate for the manufacture of catheters, although there already exist various other devices made of Teflon^{R}, polyvinylchloride and siliconised latex.

It is quite clear therefore, that some aspects related to the use of medical devices should be better clarified. Thus we decided to compare the adherence of slime producing strains with non producing strains of *Staphylococcus epidermidis* to various different biomaterials used in the manufacture of these medical devices.

MATERIALS AND METHODS

Bacteria. Six strains of coagulase-negative *Staphylococci* were used. They were isolated from venous catheters, skin and urine. Three strains were slime-producers and the other three did not produce slime. Strains were identified by standard methodology.

Bacterial inoculum. The bacteria used were radioactively labeled and inoculated into 5 ml of Mueller-Hinton broth added with 0.01 ml of ($^{2-3}$ H) adenine and incubated in an orbital agitator at 37°C for 18 hours. Subsequently the culture was centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was eliminated and the bacterial sediment was washed three times with cold phosphate buffered saline (PBS), in the same conditions of the centrifugation. The bacteria were then resuspended in PBS and adjusted spectrographically to an estimated concentration of 10^8 colony forming units per mililiter (CFU/ml).

Catheters. The catheters used were made of polyvinylchloride (PVC), Teflon^{R} (TFN), siliconised latex (LTX), polyurethane (PUT) and Vialon^{R}(VLN). The catheters were then cut into 1 cm pieces under sterile conditions. The internal and external areas of the pieces of catheter were calculated based on the measurements given by the manufacturer.

Identification of the production of slime (mucoid substance). The method used to identify the production of slime by the bacterial strains in this study was that of Christensen *et al.* (5). Two or three colonies of each strain were sown in glass tubes with 10 ml of trypticase soy broth (TSB), and incubated for 24 hours at rest at 37°C. Subsequently the content of the tubes was discarded and distilled water was added to a final volume of 10 ml. This same procedure was repeated three times with elimination of the eluates at the end. After drying at room temperature the material in the tubes was heat-fixed and dyed with a solution of safranine at 0.1% in distilled water. One minute later the tubes were emptied and the analysis was made. The production of slime can be considered positive by the visual identification of a film dyed with safranine covering the inside of the tubes. Each identification procedure was repeated three times, on different days and each time the result was obtained independently by a different observer. Strains of *S. epidermidis* HUS-C 16/87 (positive control) and HUS-C 9/87 (negative control) derived from clinical isolates and with which the test had already been done were used as standard.

Adherence to catheters. A modified quantitative method based on the technique described by Ashkenazi and Mirelman was used (2). The 1 cm long pieces of catheter were incubated in 10 ml of PBS at 37°C for one hour according to the technique of Pascual *et al.* (25). Two pieces of catheter were then put into a sterile screw-capped flask for each time of analysis. As inocculum a radiolabeled bacterial suspension in PBS with 10^8 CFU/ml, obtained as mentioned previously was used. Each flask was then inocculated with 10 ml of this suspension. The pieces of catheter were retrieved from the flasks at the predetermined time of analysis. Time zero was the moment of introduction of the pieces of catheter into the flasks. The flasks were then incubated at 37°C in repose. After the predetermined times the two pieces of catheter in each flask were retrieved, washed with cold PBS for five times, ascertaining that the PBS in the last washing was free of radioactivity, to assure that the washing had removed all bacteria not adherent to the segments of catheter. Each fragment of catheter was then put into a polipropilene tube into which 2.5 ml of scintillation fluid were added. The tube was shaken during 60 seconds and then submitted to a reading on the radiation counter. The standardized pattern corresponded to 10^7 bacteria. Finally the number of adhered bacteria for each square centimeter of material was calculated for easier comparison with different materials. Each procedure was repeated for a minimum of three times. To minimize the error only the mean values of the three readings were considered.

Table 1 - Adherence of slime and non slime producing *S. epidermidis* to biomaterials in different times of exposure. PVC (polyvinylchloride), LTX (siliconised latex), PUT (polyurethane), TFN (teflon^{R}), VLN (vialon^{R}). SD (standard deviation). Number of bacteria x $10^3/cm^2$.

	Time of Exposure									
	5 min		1 h		2 h		6 h		24 h	
Material	Slime	Non Slime	Slime	Non Slime	Slime	Non Slime	Slime	Non Slime	Slime	Non Slime
PVC	135 SD 14	149 SD 14	165 SD 11	178 SD 18	173 SD 11	182 SD 10	427 SD 9	572 SD 16	754 SD 15	970 SD 19
LTX	108 SD 11	99 SD 12	122 SD 12	132 SD 9	232 SD 13	270 SD 13	376 SD 15	412 SD 22	880 SD 13	906 SD 17
PUT	110 SD 13	105 SD 13	151 SD 14	130 SD 15	156 SD 8	138 SD 9	180 SD 13	187 SD 16	408 SD 19	448 SD 11
TFN	123 SD 14	111 SD 13	462 SD 19	165 SD 14	503 SD 14	178 SD 9	985 SD 18	268 SD 15	947 SD 15	515 SD 13
VLN	102 SD 10	94 SD 11	146 SD 13	135 SD 9	201 SD 13	167 SD 12	300 SD 20	230 SD 20	751 SD 24	538 SD 17

The results were submitted to the Student "t" test.

RESULTS

Table 1 shows the kinetics of adherence of slime producing and non slime producing strains related respectively to PVC, LTX, PUT, TFL and VLN, all exposed for 5 minutes, 1h, 2h, 6h and 24h.

The results of the adherence were expressed by the mean values of both the slime producing and non producing strains of *S. epidermidis* expressed in number of bacteria x$10^3/cm^2$ of exposed material by each respective time of exposure.

The statistical analysis demonstrated that slime producing strains of *S. epidermidis* adhered more readily to TFL than non slime producing strains in all times of exposure ($p<0,000001$). About the other biomaterials there was no significant difference.

Table 1 and Fig. 1 demonstrate that slime producing strains adhere more intensively to TFL even with small times of exposure (1 hour), reaching a maximum of adherence in 6 hours which persisted through the 24 hours of study. In the times of exposure of 1, 2, and 6 hours the number of slime producing bacteria adhering to TFL was approximately three times greater than the number of non slime producing bacteria, maintaining twice as much adherence at 24 hours of exposure ($p<0,000001$).

DISCUSSION

The rapid progress of medical science together with the technological advances have furthered the appearance of numerous medical devices with their respective uses and complications.

In the last decades the manufacture of intravenous solutions and catheters as well as their design and material have been benefitted greatly by technological advances in those fields. Nonetheless, the rise in the incidence of infections in patients receiving therapeutic infusions remains quite important due to, on one hand, the factors related to the host (a rise in immunossupressive treatment, more agressive surgical interventions, longer survival of critically ill patients) and on the other hand the influence of longstaying indwelling catheters. The interaction of catheters and microorganisms leads to their adherence, persistence, to infection and dissemination (sepsis), and seems to be caused by various factors (12).

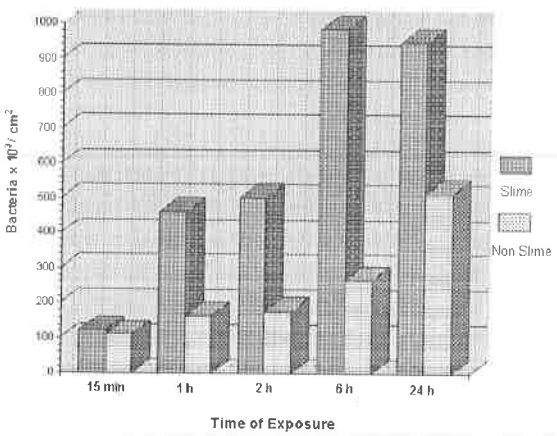

Figure 1 - Adherence of slime and non slime producing *S. epidermidis* to Teflon^{R} in different times of exposure.

Adherence to biomaterials is the first step necessary for the development of infection. There is also evidence that surface proteins on coagulase negative *Staphylococci* are essential for the inicial phase of the process of adherence to biomaterials (10).

Staphylococci continue to predominate as the most commonly encountered pathogen in medical device related infections (3, 7, 15, 34). In the last decades, coagulase negative *Staphylococci* have demonstrated their pathogenicity in patients using medical devices (valvular and orthopedic prosthesis, catheters and others) as well as in immunologically compromised patients (premature children, cancerous patients and transplant recipients) (6, 7, 9).

Coagulase negative *Staphylococci* strains isolated from indwelling venous catheters have a greater capacity to adhere to human epithelial cells than those isolated from other clinical sites. The physical and chemical properties, the hydrophobicity and the negative surface charges, as well as the type of material and the surface covering of the catheters, contribute greatly to the adherence of *Staphylococci* to biomaterials (16).

Innumerous authors have highlighted the importance of the production of slime by bacteria as an important factor in promoting their adherence to biomaterials (5, 23, 26, 32). This last factor could enhance not only the colonizing capacity of bacteria but also their virulence to the host.

Holmes and Evans (13) concluded that exopolysaccharides seem to confer unique biological properties to microorganisms such as resistance to host defences and to antimicrobials furthering new factors which enhance our understanding of the mechanisms of why infections associated to medical devices are so refractorial to treatment.

In order to establish the influence of the production of slime on the capacity of bacteria to adhere to plastic biomaterials, we compared strains of slime producing and non producing *Staphylococcus epidermidis*.

We noted that slime producing strains demonstrated a much higher capacity of adherence to TFL than non producing strains, particularly during the initial time of exposure from 1 to 6 hours, as shown in Table 1. The same phenomenon can be observed in Fig. 1 and demonstrates that all biomaterials facilitate the adherence of slime producing bacteria thus increasing the risk of infection to patients exposed to those bacteria in these circunstances. This fact has great importance when we are reminded that TFL catheters are largely used in medical practice as well as that this biomaterial is also frequently used in the manufacture of other medical devices.

Lopez-Lopez *et al.* (20) demonstrated that TFL is the biomaterial with the least degree of adherence to *Staphylococcus epidermidis*, when compared to other biomaterials, thus our present findings have even more importance since it is exactly with this same biomaterial that slime producing strains of *Staphylococcus epidermidis* (theoretically more pathogenic) showed a greater degree of adherence.

Ishak *et al.* (4), analysing the importance of slime in the pathogenesis of nosocomial bacteremias caused by coagulase negative *Staphylococci*, found a greater incidence of slime producing microorganisms in those bacteremias which caused disease. On the other hand they could not demonstrate a significant relationship between the adherence to TFL catheters and the production of slime. This last correlation was found to exist in our study.

Another important fact, relating to slime, besides its participation in the process of bacterial adherence, would be the protection it appears to confer to bacteria included in its matrix, against the action of antibiotics. Thus we would have a pool of microorganisms ready to promote recurrence of infections. Arizono *et al.* (1) concluded that the resistance of *S.epidermidis* to antibiotics rises after they adhere to the biomaterial and form a protective slime layer.

Evans and Holmes (8), analysing cases of recurrent peritonitis in patients submitted to chronic ambulatorial peritoneal dialysis (CAPD), demonstrated that *S.epidermidis* strains immersed in a biofilm adhering to silicone were not destroyed by the action of vancomycin, even in much higher concentrations than the minimal bactericidal concentration for those same microorganisms.

Similar results to those described above were found by various other authors (24, 27, 28, 31) who concluded that these findings contributed to the explanation for the resistance to catheter associated infections in the presence of antibiotics that are effective in "in vitro" tests, thus stimulating investigations to develop biomaterials resistant to colonization.

Our studies with TFL suggest that slime producing strains of *S.epidermidis* could have other properties which, associated with the production of slime, would enhance their adherence and cause them to be more virulent. With the knowledge of other studies, our data, demonstrating high levels of adherence in short times of exposure (1 to 6 hours), cannot be solely

attributed to the production of slime, which needs a longer period of time to occur (26). On the other hand production of slime is surely not a sudden occurrence but a progressive phenomenon, thus it could be produced in small quantities which could evade detection by the methods used, but still promote a greater level of adherence to TFL.

The mechanisms by which the production of slime by coagulase negative *Staphylococci* cause adherence to plastic biomaterials "in vivo" is not totally defined. It is thus necessary to continue the investigations so that this mechanism becomes definitively known. Nonetheless in view of these clinical and laboratorial evidences, every strain of coagulase negative *Staphylococci* isolated from patients using medical devices should be tested with respect to their capacity to produce slime, for such information could help in defining the virulence of the microorganism and the therapeutic procedures to be taken.

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RESUMO

Produção de *slime* por *S. epidermidis* e aderência a biomateriais

Os autores avaliaram a influência da produção de *slime* por *S. epidermidis* na aderência aos biomateriais policloreto de vinila (PVC), latex siliconizado (LTX), Teflon^{R} (TFL), poliuretano (PUT) e Vialon^{R} (VLN). Para isso, compararam a aderência de cepas produtoras e não produtoras de *slime* aos biomateriais citados. Em relação a PVC, LTX, PUT e VLN não houve diferença significativa de aderência, porém em relação a Teflon^{R} houve um aumento significativo da aderência das cepas produtoras de *slime*, em todos os tempos de exposição. Portanto, todos os biomateriais e, em especial, o biomaterial Teflon^{R}, que de um modo geral apresenta baixos índices de aderência para *S. epidermidis*, apresentam maiores níveis de aderência para cepas produtoras de *slime*, que têm, em geral, maior potencial de patogenicidade. O papel da produção de *slime* por *S. epidermidis*, na aderência aos biomateriais plásticos *in vivo*, deveria ser melhor estudado, porém as evidências clínicas e laboratoriais indicam que os *S. epidermidis* isolados de pacientes portadores de dispositivos médicos deveriam ser testados quanto à produção ou não de *slime*, pois tal dado poderia orientar quanto à virulência e quanto à decisão terapêutica a ser tomada.

Palavras-chave: infecção hospitalar, slime, cateter, aderência bacteriana, biomaterial.

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DETECTION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN STETHOSCOPES

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SHORT COMMUNICATION

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) was searched in thirty stethoscopes. The instruments were submitted to two different enrichment procedures. MRSA was detected in 70% of the stethoscopes. The best results were obtained in enrichment broth containing 4% NaCl, incubated at 30°C during 48 hours. The influence of the bacteriological procedure on the detection of MRSA in stethoscopes is stressed.

Key words: Stethoscope, MRSA, nosocomial infection, methicillin-resistant *Staphylococcus aureus*

Stethoscopes are among the most used medical instruments. They are part of daily activities of many physicians and other medical staff. Studies carried out in the past showed that 98 to 100% of stethoscopes presented bacterial contaminants (6). In despite of this, stethoscopes are not considered an important source of cross-infections among patients. In addition, it was verified that stethoscopes were not connected with a specific outbreak of methicillin-and-tobramycin-resistant *Staphylococcus aureus* (5).

The presence of bacteria in stethoscopes has been searched by different methods. There is no standardized bacteriological procedure to investigate the presence of bacteria in such instruments. This study was developed to compare the yield of two distinct enrichment procedures (brain heart infusion, incubated at 35°C during 24 hours, and brain heart infusion containing 4% of NaCl and 6 μg/ml of oxacillin incubated at 30°C for 48 hours) for detection of MRSA in stethoscopes.

A total of 30 stethoscopes was studied, they were used by physicians (n=11), medical students (n=11), nurses (n=3) and respiratory therapists (n=3) and not reported (n=2), in an adult clinical ward at Santa Casa de Misericordia (a 1,100-bed university hospital) in Porto Alegre. All samples were collected in the same day during a non-epidemic situation.

The surfaces of the diaphragms of stethoscopes were divided in two halves and each one was submitted to a distinct enrichment procedure. One half was rubbed with a swab previously wet in brain heart infusion (BHI). This swab was then incubated in BHI during 24 hours at 35°C. After this period, the specimen was subcultured on three different agar plates: trypticase soy agar with 5% sheep blood (BA), manitol salt agar (MSA) and Mueller-Hinton agar containing 6 μg/ml of oxacillin and 4% NaCl (MHSO). Plates were incubated for 24 hours at 35°C and examined for bacterial growth.

The other half of the diaphragm of each stethoscope was rubbed with a swab moistened with BHI in which 6 μg/ml of oxacillin and 4% NaCl had been added (BHI-OXS). Swabs were incubated in BHI-OXS during 48 hours at a lower temperature

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(30°C). The specimen was then inoculated on BA, MSA and MHSO. After 24 hours of incubation at 35°C, the plates were examined. Susceptibilities of staphylococci to oxacillin were determined by agar diffusion procedure according to recommendations of the National Committee for Clinical Laboratory Standards (8).

MRSA was detected in 21 (70%) stethoscopes. Among the stethoscopes in which MRSA was not present, methicillin-resistant coagulase-negative staphylococci (MRCNS) were detected in four instruments. In 14 (46,7%) sthetoscopes MRSA and MRCNS were present in the same instrument. In the five remaining stethoscopes, coagulase-negative staphylococci susceptible to methicillin (3 stethoscopes), *S. aureus* susceptible to methicillin (1 stethoscope), and *Acinetobacter iwoffii* (1 stethoscope) were observed. Additionaly, *Enterococcus* sp was found in an stethoscope in which both MRSA and MRCNS were present.

In all cases in which MRSA were detected by agar diffusion test no zone of inhibition was present. Growth was confluent around oxacillin disk.

The best result in the detection of MRSA was obtained when swabs were incubated at 30°C during 48 hours in BHI-OXS. We detected the presence of MRSA in 20 stethoscopes (16 from BA, 13 from MSA, and 12 from MHSO subculture) when such procedures were used. The procedure in which swabs were incubated in BHI at 35°C during 24 hours revealed MRSA in only 5 stethoscopes.

Hanged on necks, deeped into pockets, and, of course, in contact with the skin of patients, stethoscopes are almost omnipresent in the medical world. Some investigators showed that these instruments can be colonized by different microorganisms, including the pathogenic ones (2,6). On the other hand, other investigators were not able to incriminate stethoscopes as sources of cross-infection in a specific outbreak of MRSA (5). Based on this, there is no strict recommendation for cleaning stethoscopes (1). Some authors consider prudent to clean the bell and the diaphragm of such intruments regularly with 70% alcohol (9). Cleaning procedures using a commercial product containing alcohol (2), and chrohexidine in isopropyl alcohol (6) were considered effective in reducing the number of contaminants in stethoscopes.

Procedures to detect bacteria in stethoscopes vary significantly. Gerken, Cavanagh and Winner(6) used direct blot on blood agar plates and swabs moistened in peptone solution rubbed over the surface of bell and diaphragm. In another study bell and diaphragm of stethoscopes were bloted directly on the surface of a selective cullture medium (trypticase soy agar with tobramycin, 20 mg/ml) (5). Using such procedure to investigate a possible role of stethoscopes in an outbreak of methicillin and tobramycin resistant *S. aureus*, the study detected the epidemic strain in only 1/32 stethoscopes investigated. Based on these data, the role of stethoscopes in the transmission of *S. aureus* was considered to be minimal (5). Other investigators used a similar bacteriological procedure and detected 5/29 stethoscopes colonized with *S. aureus* (2).

Incubation at lower temperature for a prolonged period, and use of a higher concentration of NaCl in media are well known to increase MRSA and MRCNS detection *in vitro* (3,4,7). Our study incorporated such variables in the procedures and found a much greater proportion of stethoscopes contaminated with MRSA in a non epidemic situation.

Bacteriological surveys of inanimate objects, like stethoscopes, may be worthless if not supported epidemiologically. A question that arises is the following: how sensitive the procedure used to detect bacteria - MRSA in particular - in stethoscopes and other inanimate objects must be? From our results it is possible one suppose that the procedure used for MRSA screen has a strong influence on the results and may give a different idea concerning the investigation of an outbreak.

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RESUMO

Detecção de *Shaphylococcus aureus* resistentes à meticilina (MRSA) em estetoscópios

Trinta estetoscópios foram analisados quanto à presença de *Staphylococcus aureus* resistentes à meticilina (MRSA) através de duas diferentes técnicas de enriquecimento. MRSA foi detectado em 70% dos estetoscópios. O maior número de isolamentos de MRSA foi obtido em caldo de enriquecimento contendo 4% NaCl, com incubação a 30°C por 48 horas. A influência do procedimento

usado para detecção de MRSA em estetoscópios é destacada.

Palavras-chave: Estetoscópio, MRSA, infecção hospitalar, *Staphylococcus aureus* meticilina resistente

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STAPHYLOCOCCAL CARRIAGE AND ANTIBODIES TO TOXIC SHOCK SYNDROME TOXIN 1 IN BRAZILIAN WOMEN

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ABSTRACT

A study of 215 women from different socioeconomic backgrounds in Botucatu, Brazil, was conducted to reveal possible clues why toxic shock syndrome (TSS) is seldom diagnosed in Brazil. Of the 215 women, 79 were colonized with *Staphylococcus aureus* either in the nasal passages and/or in the vaginal area, which is comparable to the colonization of individuals in the developed countries. Thirteen of the women were colonized with *S. aureus* that produced toxic shock syndrome toxin-1 (TSST-1), the toxin responsible for the majority of cases of TSS. Eleven strains produced enterotoxin B, the only enterotoxin implicated in TSS, primarily in non-menstrual TSS. Enterotoxin A was produced by 15 strains and is commonly associated with the production of TSST-1, but has not been implicated in TSS. Seven strains produced enterotoxin D and one strain produced enterotoxin C, but these have not been implicated in TSS. Only 9 women used tampons which may be a major reason for the lack of menstrual TSS in Brazil. Only two of the 49 women whose sera were examined for the presence of antibodies to TSST-1 had no or very low antibody titers, the major protection against the development of TSS, both menstrual and non-menstrual TSS. This is a lower percentage than has been observed in the developed countries. Although another possibility for the lack of TSS in Brazil is the failure to recognize the disease, however, the results of this limited study indicate the importance of low usage of tampons and the high percentage of individuals with antibodies to TSST-1. The socioeconomic backgrounds of the participants were of little significance.

Key words: toxic shock syndrome, TSST-1, antibodies, staphylococci, tampons.

INTRODUCTION

Toxic shock syndrome (TSS) was first identified by Todd *et al.* (16). It was associated with children and resulted from staphylococcal infections. It did not receive recognition until late 1979 when young women, primarily teenagers, developed an illness that was associated with the use of tampons during menstruation (10,15). European countries began experiencing similar illnesses that were associated with the use of tampons during menstruation. Initially, over 90% of cases were menstrually related; however, later it was recognized that TSS could be associated with any type of staphylococcal infection with at least 50% being non-menstrual (10). No such illnesses have been reported from the so-called developing countries such as Brazil. The failure to observe menstrual TSS may be due to the infrequent use of tampons in these countries, however, this could not be the reason for the lack of TSS cases from other types of staphylococcal infections. One possibility is that a high percentage of the population have antibodies to toxic shock

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syndrome toxin (TSST-1), which have been shown to be protective against TSS (8). Another possibility is that the physicians in these countries are not familiar with the symptoms and are not identifying such illnesses as TSS.

In this study, 215 women residing in a wide area surrounding Botucatu, Brazil, were examined for the carriage of staphylococci and the presence of antibodies to TSST-1 in their sera. Each woman was given a thorough examination and pertinent information was recorded.

MATERIALS AND METHODS

Participating women. Women who reported to the University Hospital at the São Paulo State-UNESP University campus of Botucatu for minor health reasons were solicited for inclusion in the study. In all, 215 women of different ages and socioeconomic backgrounds were included in the study (Table 1). Cultures were taken from the nares, the labia, the vagina, and the cervix. Blood samples were taken for examination for antibodies to TSST-1. Any recent illnesses, use of antibiotics, menstrual period, length and severity of menstruation, marriage status, number of children, frequency of intercourse, use of contraceptives, and use of tampons were recorded.

Isolation of staphylococci. The following areas were swabbed aseptically: vaginal introitus (labial), both lateral walls of the vagina (vaginal), posterior fornix and cervix (cervix), and from the anterior nares (nasal). *Staphylococcus aureus* was isolated by immersing the swabs into tubes of brain heart infusion (BHI) broth containing 6.5% NaCl. After an overnight incubation at 37°C, the cultures were plated on Baird-Parker medium, mannitol salt agar plus egg yolk emulsion (3%), and Columbia blood agar base supplemented with 5% defribinated sheep blood. Typical *S. aureus* colonies were examined for coagulase and thermonuclease (TNase) production and anaerobic mannitol fermentation. Colonies positive for these characteristics were recorded as *S. aureus*.

Examination of staphylococci for production of toxins. Production of the enterotoxins and TSST were by the sac culture method (14). In this method, a dialysis sac containing 50 ml BHI is placed in the bottom of a 250 ml erlenmeyer flask. After autoclaving, 20 ml of 0.02 M $NaHPO_4$, pH 7.4, in 0.9% NaCl is placed in the flask and inoculated with a staphylococcal culture. Incubation is carried out with shaking for 24 hours at 37°C. The cultures are centrifuged and the supernatant fluids analyzed for enterotoxin.

Examination of culture filtrates for toxins. The optimum-sensitivity-plate (OSP) method was used (15). In this method, 3 ml of agar (1.2%) is placed in 50 mm plastic petri plates with tight lids: wells are cut according to the original specifications. Specific antisera is placed in the center well, enterotoxin (4 mg/ml) is placed in the two smaller wells, and culture supernatant fluids are placed in the four larger outer wells. Different plates are required for each enterotoxin (SEA, SEB, SEC, SED) and TSST-1. The plates are placed in a humidified container and incubated overnight at 37°C. Positive reactions are determined by formation of precipitin lines by the culture supernatant fluids that join with the control lines.

Examination of sera for antibodies. The sera, undiluted and diluted 1:10 and 1:100 were added to the

Table 1. Classification of participants in toxic shock syndrome study[a]

Race:		White			Black			Total
Socialeconomic class[b]		Low	Medium	High	Low	Medium	High	
Age groups:	16-19	-	7	1	1	1	-	10
	20-29	18	48	5	4	8	1	84
	30-39	16	44	9	7	6	2	84
	40-49	7	15	4	4	2	1	33
	50-59	1	3	-	-	-	-	4
Single women[c]		1	9	3	1	2	-	16
Tampon use[d]		4	3	2	-	-	-	9

a - 215 women from the Botucatu area of Brazil who came to the hospital clinic at the Universidade Estadual Paulista, Campus de Botucatu, for minor aliments.
b - Low ≈ $32/week; medium: ≈ 3-5 x $32/week; high >5 x $32/week.
c - Age, white: low, 49; medium, 16, 19, 20, 22, 23, 39, 32, 35, 42; high, 19, 20, 38; black: low, 32; medium, 22, 31.
d - Age, white: low, 28, 30, 35, 38; medium, 21, 26, 32; high, 19, 38.

wells of a microtiter plate that had been coated with TSST-1 (300 ng/ml) followed by anti-human IgG labeled with alkaline phosphatase (Sigma Diagnostics Universal Human IgG Kit, SH405, Sigma Chemical Co., St. Louis, MO, USA). The enzyme substrate, p-nitrophenyl phosphate, was added; development was for 30 min. The concentration of the yellow color produced was determined by reading in a microtiter plate reader at 405 nm (1).

RESULTS AND DISCUSSION

Participating women. The classification of the 215 women participating in this study is given in Table 1. The majority of the women were in the 20 to 39 age groups, the ages at which most cases of menstrual TSS occur in association with the use of tampons. It is generally thought that a smaller percentage of women in developing countries use tampons, which was indicated as a possibility for Brazil as only 9 (4.2%) of the 215 women participating in this study used tampons. The indicated smaller use of tampons could be a contributing factor to the lack of menstrual TSS in Brazil.

Isolation of staphylococci. A total of 129 *S. aureus* strains were isolated from 79 (36.7%) women, 51 (23.7%) from the nares and 45 (20.5%) from the vaginal area, with 17 (7.9%) isolated from both areas. In some women *S. aureus* was isolated from all the vaginal area sites. Distribution between the age groups was 30.0%, 38.1%, 32.1%, 45.5%, and 25.0%, youngest to oldest, respectively. If the vaginal area is not included, only 52 (24.2%) women were colonized in the nasal passages. Only 3 (18.8%) of the 16 single women were colonized with staphylococci, whereas 5 (55.6%) of the 9 tampon users were colonized, with none producing TSST-1.

TSST-1 production by the *S. aureus* strains. The fact that TSST-1-producing *S. aureus* were isolated from the women shows that TSST-1 producing staphylococci are present in the environment in Brazil. Normally people who carry TSST-1-producing staphylococci are not likely candidates for TSS because they normally have antibodies to TSST-1 (11,13). The origin of the staphylococci causing TSS is not usually known, except in cases where they have been transferred from one individual, such as a surgeon, to the individual undergoing the operation (4,12), or from one family member to another (2). In these cases the individual colonized with the TSST-1-staphylococci has protective antibodies to the toxin.

Enterotoxin production by the *S. aureus* strains. Of the 108 staphylococcal strains (from 70 women) examined for enterotoxin production, 28 strains (from 24 women) produced one or more enterotoxins. The majority of the strains produced either SEA (15 strains) or SEB (11 strains), the most common types produced by strains isolated from humans. Seven strains produced SED and one strain produced SEC.

The second most important toxin involved in TSS is SEB (9) although SEA is the major enterotoxin produced in conjunction with TSST-1; however, seldom has a strain producing only SEA been involved in TSS. Only one nasal strain produced SEB, however, 8 women were colonized with SEB-producing strains in the vaginal area. This toxin has been implicated most frequently in nonmenstrual TSS (8), but very few nonmenstrual cases have been observed in the developing countries, such as Brazil. This cannot be due to the lack of *S. aureus* strains that produce either TSST-1 or SEB.

Antibodies to TSST-1 in the women's sera. Only one (2%) of the 49 women whose sera was tested had no antibodies to TSST-1, as there was essentially no difference in the OD between the undiluted sera (0.027) and the 1:100 dilution (0.018) (Fig. 1). One (2%) other woman had a very low titer, with an OD at the 1:10 dilution of 0.093 and an OD of 0.023 at the 1:100 dilution. The OD's for the sera from the remaining women were 0.100 or higher at the 1:100 dilution. Antibody titers were considered to be protective at an optical density (OD) of 0.100 at a 1:100 serum dilution. Selection of this as the protective level was arbitrary, originally based on the titers of the acute sera from TSS patients (3,8). A positive reaction at a 1:100 dilution of the sera was chosen as the protective level. The 1:100 dilution has been adopted as the standard by other investigators (6,7).

The first TSST-1 antibody testing was done in Bergdoll's laboratory by the radioimmunoassay method (3). There were no controls available because no one had ever tested for these antibodies. It was assumed that if tests were negative, no antibodies were present, and if tests were positive, antibodies were present. This was confirmed when essentially all of the TSS patients tested negative for antibodies to TSST-1 (8) and the majority of controls tested positive for the antibodies (8). It was assumed that in this study that negative results indicated the lack of antibodies and positive results indicated the presence of antibodies to TSST-1.

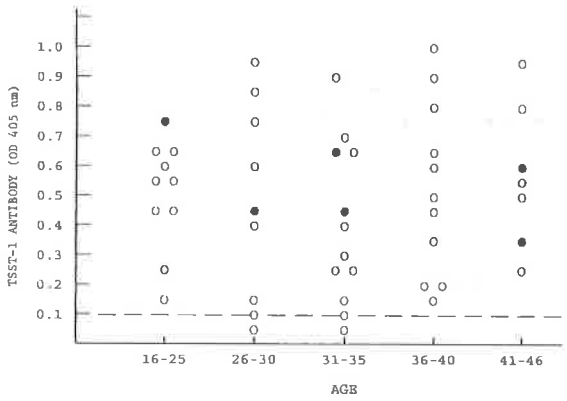

Figure 1. Antibody to TSST-1 in 49 generally healthy Brazilian women determined by an ELISA method. Optical density readings at 405 nm. The readings are recorded accourding to the age of participants. O, white women, ●, black women. Protective level: an OD of 0.100 or above.

The two women with the low OD readings represent a smaller percentage (4%) of women without protective antibodies than has been reported, with the possible exception of a group of women from New York City with a comparable percentage (4.0%) for 101 women (5). At the time that survey was done, no cases of menstrual TSS had been reported from New York City. It was concluded that the major reason for this was the high percentage of women who had protective antibody titers. This may be one reason why TSS is seldom observed in developing countries, such as Brazil.

It has been noted that a high percentage of individuals, especially those over the age of 30 years, have relatively high antibody titers to TSST-1 (5). The percentage of individuals with antibody titers increases with age, at least in the United States with the lowest percentage with antibodies being the youngest (5). The two women with the low OD readings in this study were in the 26 to 35 age group, with no women in the younger age groups having negative OD readings. The socioeconomic classification did not appear to have any significance as the OD readings were fairly uniform throughout the different groups. Neither of the women who had low OD readings were colonized with staphylococci, nor were 30 (61.2%) of the 49 women analyzed for the presence of TSST-1 antibodies. Although men were not included in this study, in other studies little or no difference in the antibody titers to TSST-1 in men and women was observed (5). This is important, as men are as vulnerable for nonmemstrual TSS as are women and many men in the developed countries have been diagnosed with TSS.

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RESUMO

Percentual de portadoras de *Staphylococcus aureus* produtor da toxina-1 da síndrome do choque tóxico e níveis de antitoxina em mulheres no Brasil

215 mulheres de diferentes classes sócio econômicas foram avaliadas quanto aos indicios através dos quais a síndrome do choque tóxico (TSS) é raramente diagnosticada no Brasil. Das 215 mulheres estudadas, 79 estavam colonizadas pelo *Staphylococcus aureus* no conduto nasal e/ou no trato vaginal, comparável com o nível de colocanização observado nos países desenvolvidos. 13 mulheres demonstraram colonização pelo *S. aureus* produtor da toxina-1 (TSST-1) da sindrome do choque tóxico. Somente duas entre 49 mulheres cujos soros foram examinados quanto a presença de anticorpos para a TSST-1, não tinham ou apresentavam títulos muito baixos de anticorpos. Este percentual é considerado menor que o observado em países desenvolvidos e pode ser um fator que contribua para a baixa incidência da TSS no Brazil. Somente nove mulheres usavam tampões absorventes o que pode constituir-se na principal causa da falta da TSS menstrual no Brasil. A classificação sócio-econômica das mulheres participantes foi considerada de pouca relevância nos resultados.

Palavras-chave: Síndrome do choque tóxico, TSST-1, anticorpos, estafilococos, tampões.

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PARTIAL PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR LIPASE FROM A NEWLY ISOLATED STRAIN OF *GEOTRICHUM* sp

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ABSTRACT

Among six hundred yeast strains screened for their ability to produce lipase, *Geotrichum* sp was selected as the highest enzyme producer. The extracellular lipase was purified by ammonium sulfate fractionation and DEAE- Sephadex A-50 chromatography. The purified preparation showed higher activity within the pH range 7.5-9.0 and an optimum temperature of 45°C. The presence of isoenzymes with molecular weights of 52 Kdal and 57 Kdal was identified.

Key words: *Geotrichum* sp, lipase production, lipase purification

INTRODUCTION

Lipases belong to the serine hydrolase group of enzymes (1) (E.C. 3.1.1.3). Triglycerides are their natural substrates and their mode of action is related to their interfacial properties in biphasic systems. Lipases, especially those from microorganisms have recently received increased attention due to their application in pharmaceutical and food industries (Table 1). It has been shown that the physicochemical properties and the substrate specificity of lipases obtained from different organisms were quite different from each other. Lipases produced by yeasts have been described in the literature. The lipase from *Geotrichum candidum* is currently the subject of intense investigation. The industrial interest is based on the fact that this enzyme shows a strong preference for fatty acids that contain a cis -9 double bond. Concerning lipases production, Iwai *et al.* (5) reported the lipid requirement for the production of lipase by *Geotrichum candidum* Link. They found that this microorganism produced lipase only in the presence of high concentration of fatty acid or oil in the culture medium. Jacobsen *et al.* (6) obtained a partially purified lipase preparation with an apparent molecular weight of 55 kDa from *Geotrichum candidum* by gel permeation chromatography. Sugihara *et al.* (14) reported the characterization of two molecular forms of *Geotrichum candidum* lipase. The two lipases showed essentially the same specific activities, substrate especificities, pH stabilities and optimal temperatures, but different optima pH and thermal stabilities.

According to Shimada *et al.* (11 long-chain fatty acid and their esters induced the lipase production by *Geotrichum candidum*, while middle and short-chain fatty acids and their esters did not show a similar effect. Lipase production increased with the chain length of the fatty acid, and long-chain unsaturated esters were the most effective.

Sonnet *et al.* (13) reported the selectivity of lipases from *Geotrichum candidum*, and studied the relative reactivities of several long-chain fatty acids in esterifications with 1-butanol catalyzed by lipases of this microorganism. It discriminated against fatty acids having a chain length greater than C-18 such as erucic acid.

In the present work a strain of *Geotrichum* was selected as the highest lipase producer. The extracellular enzyme was studied concerning its optimum pH and temperature and its pH and temperature stability.

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MATERIALS AND METHODS

Isolation of microorganisms: The samples of soil, plants or waste water were suspended in sterilized water and the suspension was spread directly on agar plates (2% peptone, 2% glucose, 1% yeast extract and 2% agar). The colonies were isolated and stored for lipase production tests.

Growth medium for lipase production: The liquid medium contained 1.5% deffated soybean meal, 1% wheat powder, 0.3% yeast extract and 0.2% NH_4NO_3, pH 5.0. It was autoclaved at 121°C for 20 minutes. The 50 ml flasks containing 15 ml of the culture medium were incubated in a reciprocal shaker at 30°C, for 48 hours. Cell separation was carried out by centrifugation at 5°C, 10,000 rpm during 10 min. Lipase activity was analyzed in the culture supernatant (lipase crude extract).

Lipase assay: Lipase assay was performed with olive oil emulsion which was prepared as follows: 25 ml of olive oil and 75 ml of 7% arabic gum solution were emulsified in a homogenizer for 5 min at 500 rpm. The reaction mixture containing 5 ml of olive oil emulsion, 2 ml of 0.1 M phosfate buffer (pH 7.0) and 1 ml of the culture supernatant (lipase crude extract) was incubated at 37°C for 30 min with orbital shaking. Immediately after incubation, the emulsion was disrupted by the addition of 15 ml of acetone-ethanol mixture (1:1 v/v), and the liberated free fatty acids were titrated with 0.05 N NaOH. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol of fatty acids per minute (3,10).

Determination of protein: Protein concentration was determinated according to the method of Lowry *et al.* (8).

Table 1. Potential uses of lipases in food and pharmaceutical industries. (2)

Industrial Field	Effect	Product
Food		
Dairy	Hydrolysis	Flavoring agents
Bakery	Improvement of flavor	Bakery products
Brewing	Improvement of flavor, aceleration of fermentation	Alcohol beverages, etc
Pharmaceutical	Digestion of oil and fats in food	Digestant
Medical	Blood triglyceride assay	Diagnostics
Cosmetic	Removal of lipids	Cosmetics in general

Purification of lipase: *Ammonium sulfate fractionation*: Solid ammonium sulfate was added to the culture supernatant up to 80% saturation and the precipitate was separated by centrifugation at 5°C, 10,000 rpm for 20 min. The precipitate was dissolved in a minimal amount of 0.05 M acetate buffer pH 5.0 and dialyzed against the same buffer at 4°C. *DEAE-Sephadex A-50-column chromatography*: The desalted enzyme solution was applied to a DEAE-Sephadex A-50 (2.5 x 53 cm) column which was equilibrated with 0.05 M acetate buffer, pH 5.0. After being throughly washed with the same buffer, the column was eluted using a gradient concentration of NaCl (0.2 - 0.8 M) and a flow rate of 20 ml per hour. The effluent was fractionated into 10 ml portions using Buchler alfa-200 Fraction Collector. The fractions showing lipolytic activity (n° 115-137) were combined and dialyzed against 0.05 M acetate buffer pH 5.0 at 4°C.

Effect of pH on lipase activity: The lipase assays were carried out in the pH range 3 to 9 using: 0.1 M acetate buffer (pH 3.0 to 5.6), 0.1 M phosfate buffer (pH 6.0 to 8.0), 0.1 M borate buffer (pH 8.0 to 9.0) and 0.1 M tris-HCl buffer (pH 9.0 to10.0).

Effect of temperature on lipase activity: The lipase assays were carried out at 25, 30, 37, 40, 45, 50 and 60°C.

pH stability of lipase: The enzyme solutions were diluted in 2ml of the relevant buffer (0.1 M acetate buffer pH 3.0 to 5.6, 0.1 M phosfate buffer pH 6.0 to 8.0, 0.1 M borate buffer pH 8.0 to 9.0 and 0.1 M tris-HCl buffer pH 9.0 to10.0) and incubated at 25°C for 24 hours. The remaining activities were measured by the standard assay method.

Thermal stability of lipase: Enzyme solutions were incubated at 25, 30, 40, 70 and 90°C, for 1 hour and the remaining activities were measured by the standard assay method.

Determination of molecular weight: The molecular weight was determined by sulfate polyacrilamide gel electrophoresis (SDS - PAGE) using standard proteins with molecular weight between 66,000 to 14,200 Da (7).

RESULTS AND DISCUSSION

Strain selection and identification

Among the 600 yeast strains which were isolated, strain n° 212 was identified as the highest lipase producer (Table 2). On the basis of taxonomical studies performed according to Itsuka and Koto (4), the yeast belongs to the genus *Geotrichum*.

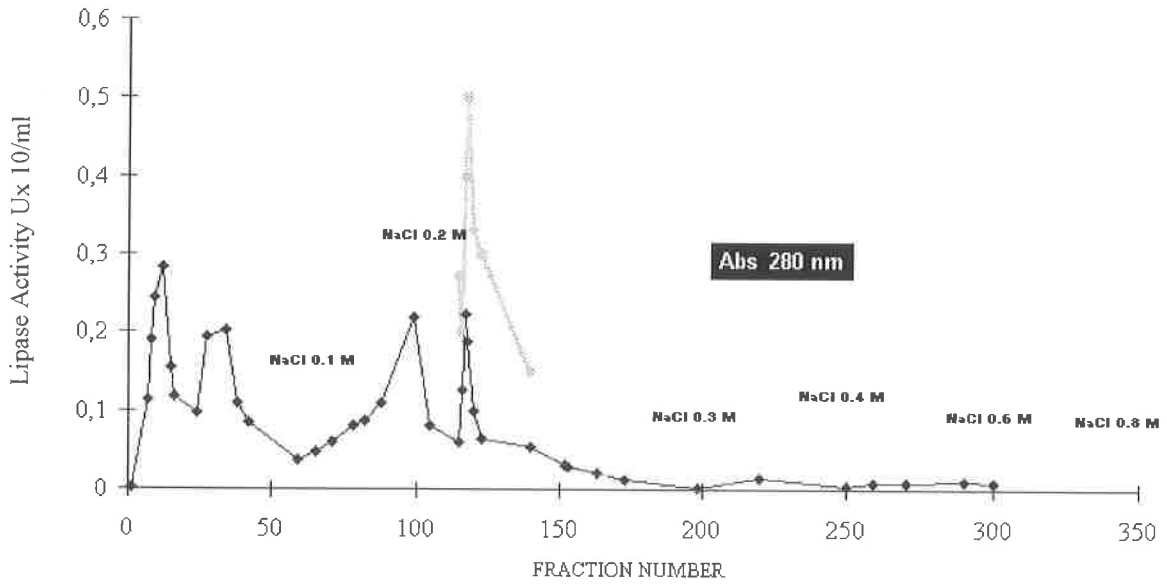

Figure 1: DEAE - Sephadex A-50 cromatography of *Geotrichum* sp lipase.

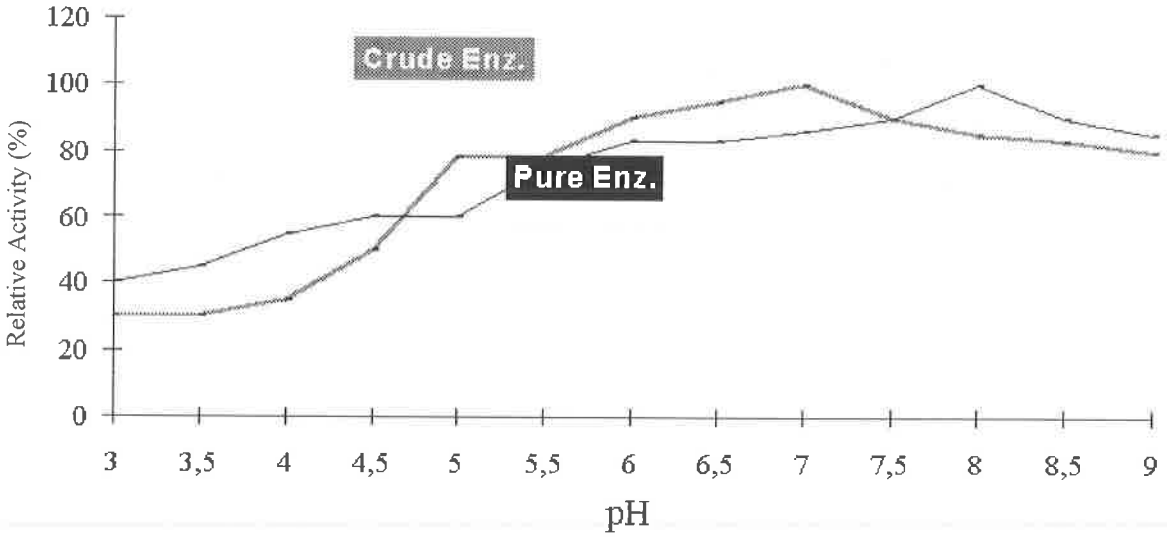

Figure 2: Lipase activity as a function of pH.

Enzyme purification

The purification steps are shown in Table 3. The enzyme was purified 6 fold after ammonium sulfate fractionation, and 16 fold after DEAE-Sephadex A-50 column chromatography. Fig. 1 shows the column elution profile. Four protein peaks were observed although lipase activity was only observed in a peak which was eluted with 0.2 M NaCl (fractions 115-137). This result was not in accordance to Jacobsen *et al.* (6) that reported the purification of

Table 2. Main lipase producer yeasts.

Strain #	Lipase activity (U/ml)
212	6,22
334	1.66
559	3,56
566	3,32
588	3,35
Candida rugosa (NRRL 1446)*	3.40

* Standard strain tested in the same conditions

Table 3. Purification of lipase from *Geotrichum sp.*

Purification Step	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification factor (%)	Yield %
Crude extract	800.0	1360	5920	0,23	1	100
Amonium Sulfate	22.0	880	660	1,33	6	64,7
DEAE-Sephadex A-50	37.5	495	133	3.72	16	36,4

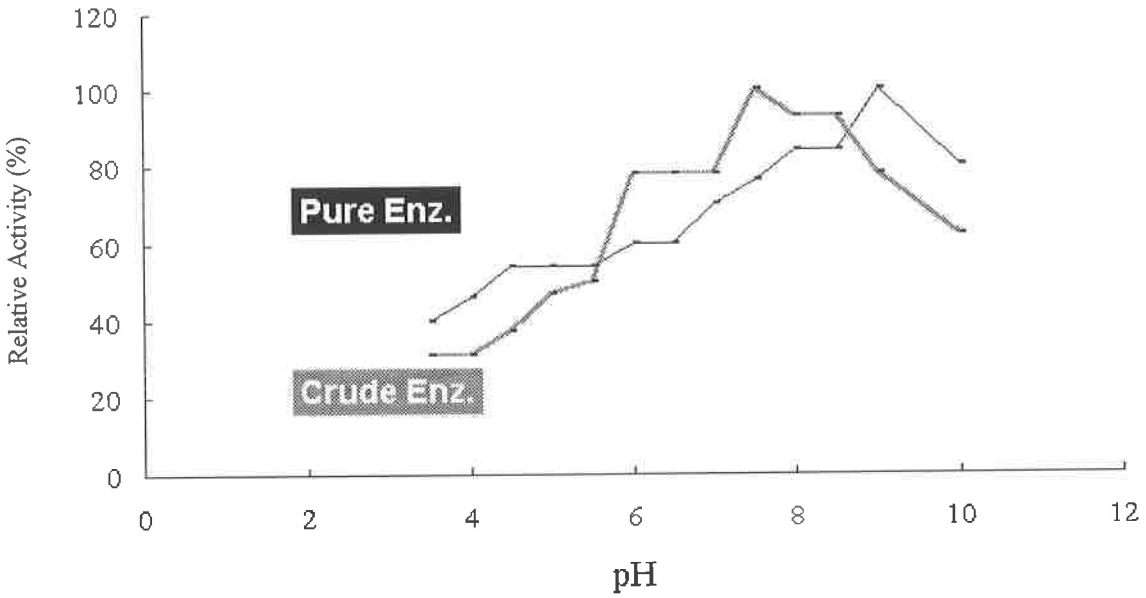

Figure 3: Lipase stability as a function of pH.

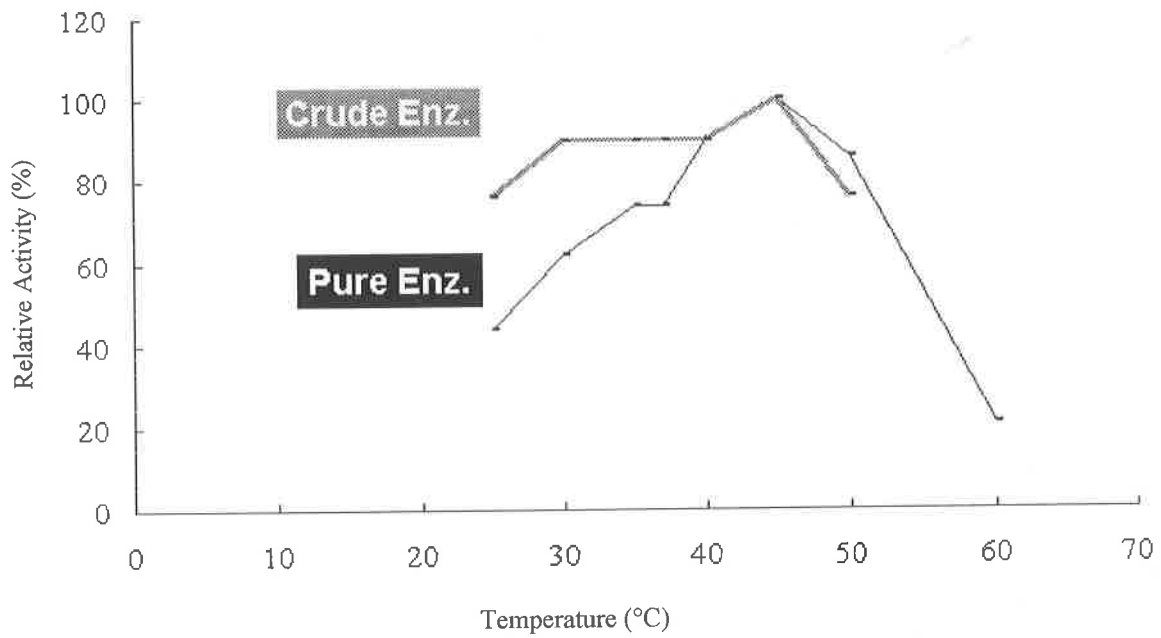

Figure 4: Lipase activity as a function of temperature

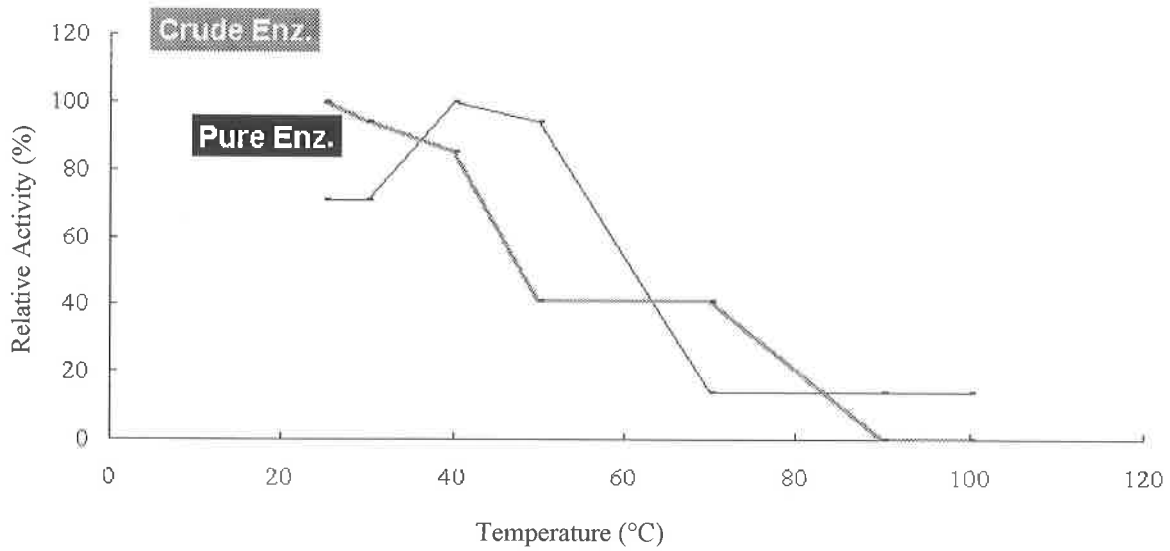

Figure 5: Lipase heat inactivation.

Geotrichum candidum lipase and found two peaks with lipolytic activity. Sidebottom *et al.* (12) also reported the presence of two isoenzymes in lipase preparations *Geotrichum candidum* ATCC 34614 and CMICC 335426.

Some properties of crude and purified enzyme

Effect of pH: Optimum pH values for crude and purified lipase from *Geotrichum* sp were different. Purified lipase was most active between pH 7.5-9.0 and crude enzyme between 5.0-7.0. Fig. 2 shows the values.The optimum pH range for the purified enzyme (8.1-8.5) was similar to the *Geotrichum candidum* lipase studied by Marks *et al.* (9).

Effect of pH on lipase stability: The crude enzyme was stable in pH range 5.5 to 8.5 at 25°C, after 24 hours of incubation. The purified enzyme was stable in pH range 7.5 to 9.0. The lipase remained more than 80% active within the pH range 7.0-8.0 for the crude preparation and 8.0-9.0 for the purified enzyme as shown in Fig. 3.

Effect of temperature on the enzyme activity: Temperature values within 25 and 60°C were studied. The optimum temperature was 45°C for crude and for purified enzyme as shown in Fig. 4. Sugihara *et al.* (14) reported two molecular forms of lipase from *Geotrichum candidum* and these enzymes showed optimum temperature between 30-40°C.

Effects of temperature on lipase stability: Fig. 5 shows that purified lipase presented good thermoestability. It retained 95% of its activity when it was heated at 50°C for 1 hour in pH 7.0. The crude enzyme retained 41% of its activity when heated at 50°C for 1 hour in pH 7.0. Tsujisaka *et al.* (15) reported the effects of temperature on stability of lipases produced by *Aspergillus niger, Rhizopus delemar, Geotrichum candidum* and *Penicillium cyclopium*. These enzymes were stable at 40, 45, 50 and 65°C respectively, when heated for 15 min at pH 5.6.

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RESUMO

Purificação parcial e caracterização de lipase extracelular de uma nova linhagem de *Geotrichum* sp

Foram testadas quanto à capacidade de produzir lipase extracelular, 600 linhagens de leveduras isoladas de várias fontes. Uma linhagem selecionada como alta produtora foi identificada como *Geotrichum* sp. A lipase produzida foi purificada por fracionamento com sulfato de amônio e cromatografia em DEAE-Sephadex A-50. A enzima apresenta maior atividade na faixa de pH de 7.5-9.0 e temperatura ótima de 45°C.

Palavras-chave: *Geotrichum* sp, produção de lipase, purificação de lipase

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EFFECT OF THE DISSOLVED OXYGEN LEVEL ON THE METABOLIC ACTIVITY OF *BACILLUS STEAROTHERMOPHILUS* WHEN GROWING ON GLYCEROL IN CHEMOSTAT CULTURE

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ABSTRACT

In glycerol chemostat cultures of *Bacillus stearothermophilus* var *non-diastaticus* the glycerol consumption rate decreased when the dissolved oxygen tension (d.o.t.) was lowered from 50% to 5% air saturation. Analysis of the enzymes glycerol kinase and glycerol dehydrogenase revealed that both enzymes were induced during growth on glycerol, and their relative cellular levels were highly dependent of the dissolved oxygen concentration of growing cell culture. The highest activities of glycerol kinase, except to NH_3 limited cultures, were found at a d.o.t. of 50% air saturation and in contrast, the highest activities of glycerol dehydrogenase were found when the d.o.t. was lowered to 5% air saturation. The latter enzyme which originally was thought not to be present in *Bacillus stearothermophilus* during aerobic growth in glycerol, appears to have a role as important as glycerol kinase. In addition, an investigation of the selected enzymes of the TCA cycle was carried out. With exception of aconitase and fumarase, the activities of all the enzymes that were measured decreased markedly with a lowering of the oxygen supply.

Key words: Glycerol kinase, Glycerol dehydrogenase, *Bacillus stearothermophilus*, oxygen limitation, TCA enzymes.

INTRODUCTION

Two pathways are available in bacteria for the dissimilation of glycerol (18); one pathway involves the use of a $NADP^+$-linked glycerol dehydrogenase which converts glycerol to glyceraldehyde (20), and the second entails the oxidation of glycerol to dihydroxyacetone with the concomitant reduction of NAD^+ to NADH (10).

In some organisms, like *Klebsiella aerogenes (K. pneumoniae)*, the primary enzyme of glycerol metabolism is either the high-affinity glycerol kinase or the low-affinity glycerol dehydrogenase, depending upon whether the culture is, respectively, glycerol-limited or glycerol sufficient (14). According to Burke and Tempest (4), this appeared not to be the case with *Bacillus stearothermophilus* which seemingly was unable to synthesize the dehydrogenase. However, in the present work, measurements of both enzymes showed that they were induced during growth on glycerol and that their relative cellular levels were highly dependent on the d.o.t. of growing culture. The results obtained are contained herein.

MATERIALS AND METHODS

Organism and culture conditions. The organism used in this study was a near-prototrophic strain of *Bacillus stearothermophilus* that apart from the carbon

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and energy source, required only biotin as an organic suplement. It was obtained from the Laboratory of Microbiology, University of Amsterdam, and named *Bacillus stearothermophilus* Amsterdam. In all its characteristics it appeared to be identical with the strain *B. stearothermophilus* var. *non-diastaticus* described by Epstein and Grossowicz (5).

Organisms were routinely grown in chemostats (LH Fermentation 500 series, 1 litre growth vessel with a 700 ml working volume) in defined simple salts media (6) at 55°C and pH 7.0. Glycerol was provided as the carbon and energy source and, in general, all non-limiting nutrients were present in at least a 3-fold excess. The basal medium was prepared in 20 l batches and sterilized by autoclaving at 121°C for 30 min. The required amount of glycerol was made up as a 150 mM, and autoclaved togheter with the medium at 121°C for 30 min. The temperature and pH values of the cultures were controlled automatically and foaming was suppresed by the addition of a silicone-based antifoaming agent, on demand, as sensed by a foam probe. Dissolved oxygen was monitored by means of a galvanic oxygen electrode (Uniprobe Instruments) and its concentration adjusted and maintained at the desired degree of saturation by varying the stirrer speed.

Procedure. Organisms were grown at a specific rate of 0.2 h^{-1} (i. e. 0.15 μ_{max}) under fixed steady-state conditions (55°C, pH 7.0), in five different glycerol-sufficient media (growth limiting with respect to ammonia, sulphate, phosphate, magnesium and potassium) as well as in a glycerol-limiting medium. The d.o.t. was first set and controlled, at about 50% saturation, then subsequently lowered to 5% air saturation. After equilibration at each d.o.t. for 1-2 days, samples of culture (10-20 ml) were withdrawn from the fermenter and analysed for glycerol. From the results of these assays, the rates of glycerol consumption were determined, and carbon balances constructed.

Analyses. Oxygen consumed and carbon dioxide produced by the cultures were determined by passing the effluent gas through an oxygen analyser (Taylor Servomex type OA 272) and a carbon dioxide analyser (Servomex IR gas analyser PA 404 - Servomex Ltd., Crowborough, Sussex, England). The rates of respiration were then calculated as specified by Pennock and Tempest (17), bacterial dry weights being assessed by the procedure of Herbert *et al.* (9).

Enzyme activities. In the preparation of cell-free extracts, suspensions of organisms from steady state chemostat cultures were centrifuged (5,000 g for 10 min), washed with 50 mM potassium phosphate buffer (pH 7,0) and sonified six times for 30 s at 75 W in a B-12 sonifier (MSE, Model 60 w, Branson) with intermittent 30 s at 75 W in an ice/water mixture. The extracts were then centrifuged at 20,000 g for 10 min and the supernatant fraction used for the determination of enzyme activities. Activities were determined according to the following procedures: glycerol kinase (1), glycerol dehydrogenase (3), aconitase, isocitrate dehydrogenase and malate dehydrogenase (20), fumarase (10). Protein was determined using the method of Lowry, as modified by Peterson (18). All enzyme activities are expressed in units of μmol min^{-1} mg $protein^{-1}$.

RESULTS AND DISCUSSION

Most strains of *Bacillus stearothermophilus* will grows on a wide range of carbon substrates including sugars, polyols and organic acids (4). The strain used in this work possesses this property, but displayed a remarkable sensitivity to glycerol, even when it was present in only a slight excess of the growth requirement. During the studies of the effect of the dissolved oxygen tension on the physiology of this organism when growing on glycerol in chemostat culture, it was found that such cultures were extremely difficult to establish. The sensitivity to glycerol of glycerol-sufficient cells was not uniform but varied with the nature of the growth limitation. Potassium-limited cells were more sensitive whereas the sulphate-limited cultures were less sensitive. From the results contained in the Table 1, it is clear that when *B. stearothermophilus* was growing on glycerol in chemostat culture at a d.o.t. of 5% air saturation it consumed less glycerol than at a d.o.t. of 50% air saturation. The glycerol sufficient cultures utilized glycerol at a higher rate than the rate of a corresponding glycerol limited culture. The extra glycerol consumed was oxidized mainly to CO_2. Therefore, changes in the metabolic rate were observed as a consequence of variations in the dissolved oxygen tension. In order to determine whether the observed changes in glycerol flux rate, following the lowering of the d.o.t., implicated changes in the cellular content of the enzymes of glycerol metabolism or in their activities, or both, measurements were made of the activities of enzymes of this pathway in cell free extracts from the variously-limited chemostat cultures of *B. stearothermophilus* grown with high and low aeration.

Table 1. Influence of the dissolved oxygen level on the rates of glycerol and O_2 consumption and on CO_2 production expressed by glycerol-limited and glycerol-sufficient cultures of *Bacillus stearothermophilus* growing at a fixed dilution rate (0.2 h^{-1}) and at 55°C and pH 7.0.

Limitation	d.o.t. (% Sat.)	Glycerol uptake	O_2 uptake	CO_2 output	Carbon recovery (%)
		[mmol h^{-1} (DCW cells)$^{-1}$]			
Glycerol	50	5.10	14.20	8.20	107
	5	4.69	16.10	7.40	108
Potassium	50	11.50	26.50	23.10	91
	5	7.90	27.70	14.03	96
Ammonia	50	11.80	22.30	17.30	72
	5	6.40	23.90	10.41	97
Sulphate	50	10.70	20.50	15.70	75
	5	6.20	27.40	15.70	75
Phosphate	50	7.60	23.30	16.30	108
	5	7.03	26.10	11.60	94
Magnesium	50	9.80	25.50	20.60	98
	5	7.60	31.20	18.05	115

Table 2. Influence of the dissolved oxygen level on the activities of glycerol kinase and glycerol dehydrogenase in cells from glycerol-limited and glycerol sufficient cultures of *Bacillus stearothermophilus* growing at a fixed dilution rate (0.2 h^{-1}) at 55°C and pH 7.0.

Limitation	Activity [µmol min^{-1} (mg protein)$^{-1}$]		
	d.o.t. (% Sat.)	Glycerol Kinase	Glycerol Dehydrogenase
Glycerol	50	16.40	2.60
	5	7.80	19.96
Potassium	50	11.20	0.78
	5	3.51	11.13
Ammonia	50	2.80	1.35
	5	3.90	13.80
Sulphate	50	10.58	0.19
	5	0.83	11.03
Phosphate	50	5.1	0.55
	5	2.2	11.25
Magnesium	50	15.40	0.48
	5	1.78	6.70

Influence of the dissolved oxygen level on the activities of the enzymes of glycerol metabolism. Glycerol kinase and glycerol dehydrogenase assays were carried out on extracts of *Bacillus stearothermophilus* cells grown at a dissolved oxygen level of 50% and 5% air saturation (Table 2). The glycerol kinase assay measured the reduction of NAD at pH 9.5 and required the addition of the coupling enzyme glycerol-3-phosphate dehydrogenase. The results shows that they were induced during growth on glycerol and that their relative cellular levels were highly dependent on the d.o.t. of growing culture: a high glycerol kinase activity was manifested in cells grown under fully aerobic conditions whereas a high glycerol dehydrogenase activity was present in cells grown under oxygen-limited conditions. Burke and Tempest (4) however, detected activity only of glycerol kinase, in cell extracts of *B. stearothermophilus* growing aerobically in continuous culture in a minimal salts medium containing glycerol as sole carbon source. Activities as high as 82.5 µmol. min^{-1} (mg Protein)$^{-1}$ were present and the specific activity of the purified glycerol kinase was 220 µmol. min^{-1} (mg Protein)$^{-1}$, indicating that the enzyme constituted almost 40% (w/w) of the total cell protein. Glycerol dehydrogenase in *Klebsiella pneumoniae* (*aerogenes*) is a catabolic enzyme whose activity reportedly was lost irreversibly under aerobic conditions. However, a total loss of enzyme activity in the cells is impeded by each of several compounds that inhibit the aerobic generation of metabolic energy. Moreover, this enzyme was reportedly present and functional in aerobic glycerol sufficient cultures of *K. pneumoniae* (15). Because the affinity of glycerol dehydrogenase for glycerol is low, an additional branching point of fermentation is required when this organism is growing anaerobically in a glycerol-limited environment. After glycerol has entered the cell by facilitated diffusion, it can be either oxidized to dihydroxyacetone, and subsequently phosphorylated to yield dihydroxyacetone phosphate or it can be dehydrated to 3-hydroxypropionaldehyde. This compound acts as a powerful electron acceptor which effectively re-oxidizes $NADH_2$, being reduced to 1,3-propanediol, a process that does not generate ATP. It thus seems that upon admission of molecular oxygen into an anaerobic culture, glycerol dehydrogenase is specifically inactivated by an energy-requiring process (11). Although some strains of *B. stearothermophilus* are known to be facultatively anaerobic (7), *B. stearothermophilus* var. *non-diastaticus* is reportedly an obligate aerobe (5,13). However, when growing on glycerol in oxygen-limited environments the synthesis of glycerol kinase is repressed and the synthesis of glycerol dehydrogenase induced. This suggests that the latter enzyme appears to play a similar role to that described for *K. aerogenes*. Thus, glycerol dehydrogenase has a

role as important as glycerol kinase during growth of this organism in glycerol.

Influence of the dissolved oxygen level on the activities of selected enzymes of the tricarboxilic acid cycle. A decreased in TCA cycle activity is, of course, inevitable when the oxygen supply becomes limiting because cells then are no longer able to rapidly oxidize the NAD(P)H arising from the isocitrate, 2-oxoglutarate and malate dehydrogenase reactions, and these reactions thus are hindered kinetically. It does not necessarily follow that there should be a corresponding change in the cellular content of the TCA cycle enzymes, even though this is known to occur when cultures of facultatively anaerobic organisms like *Escherichia coli* are grown anaerobically (22). The influence of the dissolved oxygen level on the activities of selected enzymes of the tricarboxilic acid, aconitase (ACON), isocitrate dehydrogenase (ICD), fumarase and malate dehydrogenase (MDH) were assayed in extracts of cells from variously limited cultures of *Bacillus stearothermophilus* that had been grown first with an excess of oxygen and then with oxygen 'shadowing' (Table 3). As expected, though with notable exceptions, the cellular content of TCA cycle enzymes decreased substantially following growth in oxygen-depleted environments. The exception was aconitase and fumarase. The measured activity of the latter enzyme was higher to glycerol-limited cultures at a d.o.t. of 5% air saturation. These results are similar to cells of *Escherichia coli* and *Klebsiella aerogenes* grown anaerobically, in which, the TCA cycle enzyme activities are much decreased (8,15). In addition, decreses in the synthesis of TCA cycle enzymes were found in several organisms when grown anaerobically or at low oxygen tension (2,16).

Table 3. Effect of the dissolved oxygen level on the activities of selected enzymes of the TCA cycle present in *Bacillus stearothermophilus* grown under standard conditions: D=0.2 h^{-1}; 55°C; pH 7.0.

Limitation	d.o.t. (% Sat.)	*Activities: μmol min^{-1} (mg protein)$^{-1}$			
		ACON	ICD	FUM	MDH
Glycerol	50	0.31	3.78	0.85	12.5
	5	0.33	3.10	1.24	12.8
Ammonia	50	0.28	5.50	1.32	10.5
	5	0.30	3.30	1.31	8.9
Potassium	50	0.25	8.20	2.71	12.8
	5	0.40	4.70	2.46	11.3
Magnesium	50	0.19	2.80	1.90	10.4
	5	0.18	1.60	0.90	6.5
Phosphate	50	0.22	4.10	1.29	7.7
	5	0.38	1.77	0.83	6.8
Sulphate	50	0.28	3.20	1.38	10.0
	5	0.27	2.71	1.22	8.7

* Abbreviations are as follows: ACON= Aconitase, ICD= Isocitrate dehydrogenase, FUM= Fumarase, MDH= Malate dehydrogenase.

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RESUMO

Efeito do oxigênio na atividade metabólica de *Bacillus stearothermophilus* crescido em glicerol em cultura quimiostática

Quando a concentração de oxigênio (d.o.t.) de uma cultura quimiostática de *Bacillus stearothermophilus* var *non-diastaticus* crescendo em glicerol como única fonte de carbono, foi reduzida de 50 para 5% de saturação com ar, foi observado que a taxa de consumo deste substrato diminuiu. Análise das enzimas glicerol quinase e glicerol desidrogenase revelaram que ambas enzimas foram induzidas durante crescimento em glicerol, e que seus níveis intracelulares foram altamente dependentes do grau de aeração da cultura. As atividades mais altas de glicerol quinase, exceto para culturas limitadas de NH_3, foram encontradas a d.o.t. de 50% de saturação com ar e em contraste, as atividades mais altas de glicerol desidrogenase foram encontradas quando a tensão de oxigênio foi reduzida para 5% de saturação com ar. Esta última enzima, que acreditava-se originalmente não estar presente em *B. stearothermophilus* durante seu crescimento em glicerol em aerobiose, parece desempenhar um papel tão importante quanto glicerol kinase durante o crescimento deste organismo em glicerol. Uma investigação das enzimas do ciclo de Krebs também foi feita. Com exceção da aconitase e fumarase, todas as enzimas que foram medidas, diminuiram sua atividade em função da redução da disponibilidade de oxigênio.

Palavras-chave: Glicerol quinase, glicerol desidrogenase, *Bacillus stearothermophilus*, limitação de oxigênio, enzimas do Ciclo de Krebs.

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RELATIONSHIP BETWEEN CARBON SOURCE, PRODUCTION AND PATTERN ACTION OF α-AMILASE FROM *RHIZOPUS* SP

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ABSTRACT

This paper discusses the inducer effect of corn soluble starch and the individual components (amylose and amylopectin) from corn and potatoes starch for α-amylase production by a strain of *Rhizopus* sp. The following decreasing order in the enzyme production was obtained: corn amylose > potatoes amylose > corn amylopectin > potatoes amylopectin > starch > maltose, coinciding with the ability of the enzyme to release reducing units, except the soluble starch that was more softly hydrolysed. However, when the enzyme action was measured by the iodine binding method, an inverse order of enzyme activity was obtained, that is: amylopectins > starch > amylosis. The results suggest that: a) branched structures in substrate affect the enzyme production; b) corn amylose and corn amylopectin are better inducers than their respectives homologous from potatoes; c) α-amylase from *Rhizopus* sp has different action patterns on substrates with straight or branched chains: from the former, it removes only reducing units with lower molecular weight (G1-G3); from the latter it also removes oligosaccharides with higher molecular weight (G5 - G6).

Key words: α-amylase, *Rhizopus* sp, induction, starch, amylopectin, amylose.

INTRODUCTION

α-amylase (α-1,4--D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyses starch by cleaving the internal α(1--->4) glucosidic bonds, producing a range of maltooligosaccharides (4,7,14,16). This enzyme is largely distributed in nature and has been found in cereal seeds, plant roots, animal organs and microorganisms.

Because it is one of the most widely used enzyme in industry, there has been most interest in the mechanism of the enzyme synthesis in plant and microorganisms. In fungi and bacteria, induction and repression effects of the carbon source are very important in the regulation of α-amylase biosynthesis (10,17). Starch and amylaceous raw material have been described as the most adequate carbon sources for high enzyme productivity by some strains of fungi and bacteria (9,10,17). The size of starch granules, amylose and amylopectin contents and molecular weight of these starch components are known to vary according to the starch source (3,15). Thus, the starch-amylases interaction may differ in accordance with the starch source. Besides, the isolated contribution of individual components of the starch for amylases synthesis is not known. In this paper the relationship between carbon source structure, α-amylase production by *Rhizopus* sp and its action pattern is discussed.

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MATERIALS AND METHODS

Microorganism isolation and enzyme production. Amounts of 1 g of soil collected in wheat fields were suspended in 100 ml of sterile water, slightly agitated for 2 h and inoculated in a plate medium-potatoes, dextrose, agar (PDA - Difco). The plates were incubated at 30°C, for 48 h; spores of each colony were transferred to slants with the same medium and researched for α-amylase production. The best enzyme producer was identified as *Rhizopus* sp by the Laboratório de Microbiologia - Setor de Micologia of the Universidade de São Paulo and selected for the work sequence. For α-amylase production, in submerged fermentation, 0.1 ml of a spores suspension, containing 4 $x10^7$ spores/ml, previously adapted to the liquid medium, was inoculated in flasks with 40 ml of a medium composed by 2.0% carbon source, 1.5% peptones, 0.5% yeast extract, 0.05% $MgSO_4.7H_20$, 0.5% K_2HPO_4, 0.2% $NaNO_3$ and final pH 5.0. The flasks were agitated at 200 rpm for 144 h at 30°C and the growth medium was analysed as enzyme source. Amylose and amylopectin from potatoes and corn (Sigma), soluble corn starch (Reagent) and maltose (Difco) were utilized to study the effect of carbon source. Samples of 0.1 ml of the growth medium of each experimental condition were removed at every 24 h and analysed for α-amylase activity.

α-Amylase assay. Enzyme activity was determined by measuring the decrease in iodine-binding capacity of a soluble starch solution, as described by Brumm and Teagle (1). The reaction system was composed by an aliquot of 0.1ml of enzyme solution and 1.0 ml of 0.5% soluble starch in 50mM acetate buffer and 25 mM $CaCl_2$, pH 5.5. After 10 min of reaction at 60°C, a sample with 0.05 ml was removed, added to 4.8 ml of 0.1N HCl and 0.15 ml of iodine reagent (0.05% Iodine plus 0.10% KI). The mixture was slightly agitated for 5 min and the absorbance measured at 620 nm. One unit of enzyme (U) was defined as the amount of enzyme required to hydrolyse 10 mg of soluble starch in 10 min at 60°C. The Somogyi and Nelson method (11) was utilized for determination of the released reducing sugar, expressed as glucose equivalent. Proteins were determined by the Lowry method modified by Hartree (6).

Enzyme action on various substrates. Amounts of 1.0 ml with 0.5% of soluble starch, maltose, amylose and amylopectin from corn and from potatoes added with 25mM $CaCl_2$ were incubated with 1.64 U of the purified enzyme (32.74 U/mg). After 10 minutes, the enzyme action was estimated by measuring the decrease in iodine-binding capacity and by Somogyi and Nelson method (11). The glucose oxidase method was utilized to measure the released glucose, when maltose was utilized as substrate (GLUCOX - Doles Reagentes e Equipamentos para Laboratórios Ltda), as described by Cruz *et al.* (2). For paper chromatography analysis, samples of 5 μl of each experimental condition were applied in Whatman paper and the chromatograms were developed after 20 h in a solvent system composed of n-butanol-pyridine-water (6:4:3) and the spots revealed with a silver nitrate solution.

RESULTS AND DISCUSSION

Enzyme production. *Rhizopus* sp produces α-amylase extracellularly when inoculated in media formulated with starch and different starch by-products, as shown in Fig. 1. The best results were observed when amylose from corn or potatoes was utilized as carbon sources. In both, the maximum activity was observed at 120 h of fermentation and in presence of corn amylose the enzyme production was about 10% higher. In their respective peaks, the α-amylase activity in the medium formulated with amylose from corn was 39% higher than that observed in the medium with amylopectin and 48% higher than that produced in soluble starch of the same source. This means that the branched structure present in starch and in amylopectin decreases the α-amylase synthesis, probably because the α(1--->6) glucosidic linkages restrict the enzyme action on those substrates, yielding dextrins and oligosaccharides and so limiting the carbon source availability for the microorganism metabolic processes. Park and Rollings (12), working with α-amylase from *Bacillus licheniformis*, have observed that the enzymatic hydrolysis is affected by the presence of branch points in starch substrate structure. However, only from this standpoint, is difficult to explain why the enzyme production was smaller in presence of corn soluble starch than in the amylopetin media.

In Fig.1, it is also possible to see that amylopectin from corn is a better inducer than its homologous from potatoes. It advanced the start of the enzyme synthesis to 24 h of fermentation against 48 h observed for the experiment with amylopectin from potatoes; in its peak of maximum activity (observed at 120 h) the enzyme

Table 1: Dextrinizing action of the α-amylase (1.64 U) from *Rhizopus* sp on corn starch, and starch by products from corn and potatoes.

Product	Reducing sugar (ug/ml)*	Absorbance before enzyme treatment	Absorbance after enzyme treatment	Dextrinized product (%)**
Corn starch	1,531	0,363	0,082	77.4
Corn amylose	1,422	0,488	0,144	72,7
Potatoes amylose	1,390	0,692	0,197	70,6
Corn amylopectin	1,270	0,050	0,006	88,0
Potatoes amylopectin	1,248	0,177	0,033	81,3
Maltose* **	187,5	-	-	-

* Somogyi and Nelson method; ** Iodine binding method; *** Glucose oxidase method

production was 20% higher than that verified with amylopectin from the tubercle (peak at 96 h). It is possible that the higher activity of the enzyme in amylopectin from corn, as shown in Table 1, is the responsible for this observation. These results suggest that, besides the afinnity of the enzyme with the glucosidic linkage, the molecular structure of the carbon source affects decisively the enzyme induction.

The lowest enzyme activity was observed in the growth medium formulated with maltose. In this experimental condition, the highest enzyme activity was observed at 72 h of fermentation and corresponds to only 37% of the enzyme produced in medium formulated with amylose from corn in its peak (120 h). It is possible that the difficulty of the enzyme to hydrolyse the disaccharide explains this behaviour. Maltose sometimes appears as inducer of the α-amylase synthesis (13), but sometimes as its inhibitor (8). With regard to amylose and amylopectin, references have not been found in the specialized literature.

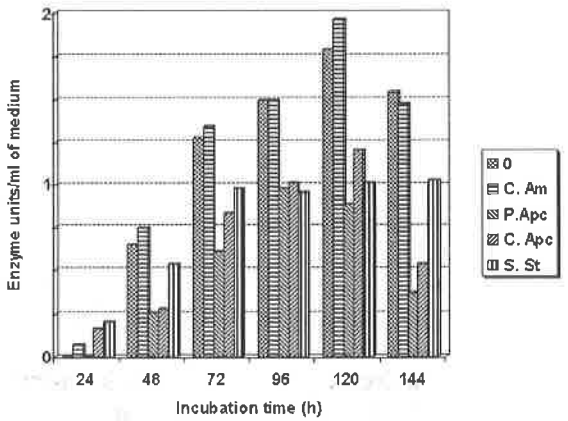

Figure 1. Carbon source effect in alpha-amylase production by *Rhizopus* sp.
P.Am - potatoes amylose; C. Am - corn amylose; P. Apc - potatoes amylopectin; C.Apc -corn amylopectin; S.St - soluble starch; Mt - maltose.

Enzyme action on starch, amylose and amylopectin. As shown in Table 1, amylose, starch and amylopectins, in decreasing order, demonstrated ability to bind to iodine. Despite the affinity of iodine with starch to be specific for the amylose, a dyed amylopectin-iodine complexe (purple color) was obtained. This is possible when the ion concentration is higher than 5 mg of iodine for g of polysaccharide (3), as used in this study. The amylose ability to dye with iodine is due to the inclusion of the polyiodide ions in the central core of amylose helix and it becomes greater concurrently to the extension of the glucose units chain (3). Thus, the greatest optical density verified in the iodine-amylose complexe from potatoes in confrontation with the amylose from corn can imply that the polysaccharide from the tubercle is composed by longer chains. It is possible that the same happens with the branches of the amylopectin from potatoes. The results concerning the lenght of the chains of amylopectin obtained by methylation analysis in accordance with Meyer and co-warkers, mentioned by French (3), suggest that the branches of amylopectin from potatoes starch are longer than those of amylopectin from corn.

According to Table 1, when starch and its individual components were treated with purified α-amylase from *Rhizopus* sp., the dextrinizing power of the enzyme was observed in the following sequence: corn amylopectin > potatoes amylopectin > corn starch > corn amylose > potatoes amylose. These results are analogous to those described by Stefanova and Emanuilova (15) and Ivanova *et al.* (8), who studied the action pattern of α-amylase from *B. brevis* and *B. licheniformis*, respectively.

When the enzyme action on the same products was evaluated by reducing sugar release, a different magnitude order from the aforementioned data was obtained, as folows: starch > corn amylose > potatoes amylose > corn amylopectin > potatoes amylopectin.

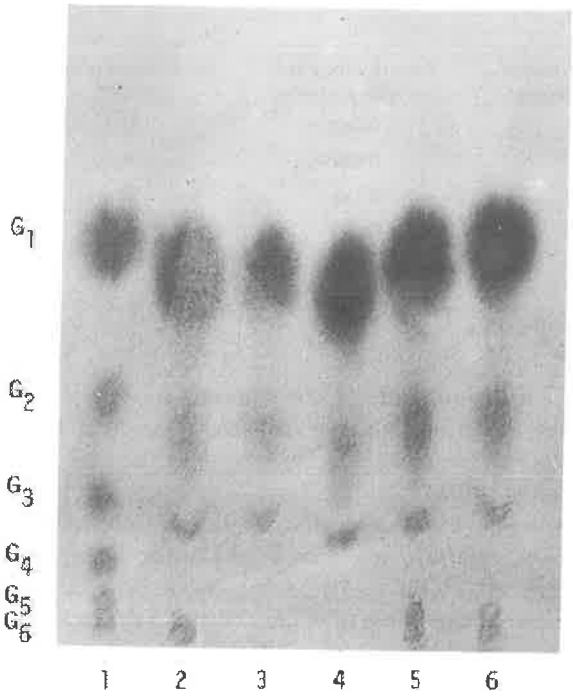

Figure 2. Chromatografic analysis of *Rhizopus* sp α-amylase action on soluble starch and their individual components. G1, G2, G3, G4, G5 and G6 are glucose, maltose, maltotriose, maltotetraose, maltopentahose e maltohexaose, respectively. 1 - Patterns; 2 - Soluble starch; 3 - corn amylose; 4 - potato amylose, 5 - corn amylopectin; 6 - potato amylopectin.

A greater liberation of reducing units from high-amylose substrates than those exhibiting increased quantities of branched amylopectin has already been reported by Inglet (7) and Rupp and Schwarts (14). According to these authors, while the enzyme attacks randomly the starch and amylose chains and releases large amounts of oligosaccharides with low molecular weight, its action pattern on amylopectin is more rigid, producing oligosaccharides with higher molecular weight. The chromatogram shown in Fig. 2 is in accordance with these findings. α-amylase from *Rhizopus* sp. released only glucose, maltose and maltotriose when acting on amylosis, similarly to the enzyme produced by *Bacillus apiarius* (5). Besides this, it produced oligosaccharides with higher molecular weight when incubated in starch and amylopectins. This observation elucidates the apparent distinct order of action when the enzyme activity is measured by the dextrinizing power or by reducing substance liberation.

CONCLUSIONS

A strain of *Rhizopus* sp isolated from soil yields α-amylase with a mechanism of endo-action when grown in medium formulated with starch and its biopolymeric components. The greatest enzyme productivity was found in presence of amylosis, polysaccharides composed only by α(1--->4) glicosidic bonds, meaning that the presence of branched points in the substrates affects negatively the enzyme synthesis. However, unlike what is expected in theory, starch, presenting the smallest quantity of branched linkages, showed less induction ability than amylopectins. Significant differences of enzyme productivity in media formulated with amylose from diverse sources were also found. These results suggest that, besides the enzyme affinity for the glucosidic linkage, other differences in the molecular structure of the polysaccharides used as carbon sources also affect the enzyme synthesis. For elucidation of the relationship between the structure of starch and its individual components and α-amylase synthesis further researches are recommended.

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RESUMO

Interrelação entre fontes de carbono, produção e modo de ação de α-amilase produzida por *Rhizopus* sp.

Este trabalho discute o efeito indutor do amido solúvel de milho e dos componentes individuais do amido de milho e de batata para a produção de α-amilase por uma cepa de *Rhizopus* sp. Foi obtida a seguinte ordem decrescente na produção da enzima: amilose de milho > amilose de batata > amilopectina de milho > amilopectina de batata > amido solúvel > maltose, coincidindo com a capacidade de liberação de unidades redutores pela enzima, com exceção do

amido solúvel que foi mais facilmente degradado. Entretanto, quando a atividade enzimática foi medida pelo método da descoloração do complexo com o iodo, foi obtida uma ordem inversa, ou seja, amilopectinas > amido > amiloses. Os resultados sugerem que: a) estruturas ramificadas presentes no substrato afetam a síntese da enzima; b) amilose e amilopectina de milho são melhor indutoras que as respectivas homólogas da batata; c) a α-amilase *de Rhizopus* sp apresenta diferentes padrões de ação ao atuar sobre substratos de cadeias retas e ramificadas. Daqueles remove apenas unidades redutoras de baixo peso molecular (G1 a G3); destes, também, remove oligossacárides de maior peso molecular (G5 e G6).

Palavras-chave: α-amilase, *Rhizopus* sp, indução, amido, amilopectina, amilose

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BIOFILM FORMATION MONITORING IN AN INDUSTRIAL OPEN WATER COOLING SYSTEM

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ABSTRACT

Coupons of stainless steel type AISI-304 were installed in the seawater reservoir of an industrial plant and then removed for analysis at two, four and eight-days in order to detect the different groups of microorganisms present in the biofilms that formed upon the metal surface. During this period, a quantitative variation of the groups of microorganisms was observed. The aerobic bacteria, which made up 95.3 percent of the total microorganisms at the start of the experiment, represented only 0.1 percent of these on the eighth day of exposure. In contrast, anaerobic bacteria, initially constituting only 4.7 percent of the microorganisms, corresponded to 96.3 percent of the total number of microorganisms after the same eight-day exposure. Sulfate-reducing bacteria (SRB) were present and also underwent variations, as did the total sulfide content. Analysis by scanning electron microscopy revealed the presence of microorganisms adhered to the metal surface, including microalgae.

Key words: biocorrosion, biofilms, cooling water

INTRODUCTION

Microorganisms occur in communities where the individual members show a great variety of biochemical and physiological functions (10). Biofilms are complex structures consisting of cells of mainly microbial origin and extracellular materials resulting from the metabolic activity of these microorganisms.

Microorganisms and their diverse metabolic activities have been studied and correlated with processes of corrosion, showing that these activities are responsible for accelerating deterioration (2).

A large number of industries built on coastal locations use seawater as a coolant fluid for part of their equipment. The water is taken directly from the ocean and is used in open or closed systems. Besides containing organic material favoring the proliferation of microorganisms, this water also contains relatively high concentrations of salts which can bring about electrochemical corrosion. Furthermore, this process may be facilitated by the presence of biofilm-forming microorganisms.

Stainless steel type AISI - 304, despite its sensitivity to the corrosive action of salt water, is still employed in the cooling systems of petrochemical plants.

In a previous paper (4) it was reported that the attachment of microorganisms to metalic coupons began during the first week of exposure to the industrial cooling water, but the maximum value was not achieved. The main goal of this research was to quantify the various groups of adherent microorganisms throughout the exposure period.

MATERIALS AND METHODS

Field Experiments. Field experiments were conducted at the PETROFLEX petrochemical plant, located on Guanabara Bay, in the city of Rio de

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Janeiro, Brazil. This industrial plant uses salt water to cool a number of heat exchangers in an open system.

The test samples were made of stainless steel type AISI - 304 and were installed in the PETROFLEX salt water intake basin. This water is frequently not submitted to previous treatment, and it is subject to contamination from neighboring plants. Analysis of the water revealed the following composition: Mg 740 mg/l; Al < 1.0 mg/l; Ca 323 mg/l; Fe 0.35 mg/l; Mn 0.1 mg/l; K 253 mg/l; Na 6.4 g/l; and Cl^- 12.85 g/l (4).

The metal coupons were removed for analysis at two, four, and eight days.

Quantitative determinations. Suspensions were made according to the technique described by Lutterbach and de França, 1996 (9).

Quantification of aerobic bacteria: Nutrient broth (Merck) was used as culture medium, and the aerobic bacteria were quantified according to the technique of the most probable number (MPN) (7). The incubation period was 2 days at 32°C ± 1°C.

Quantification of anaerobic bacteria: the MPN technique was used for quantification, and thioglycollate fluid (Difco) was the culture medium. The tests were conducted under anaerobic conditions and incubation was at 32°C ± 1°C for 28 days.

Quantification of sulfate-reducing bacteria: the same technique was used as described above, substituting Postgate B medium (11) as the culture medium.

Quantification of fungi: these were quantified by enumeration of colony forming units (cfu) on Petri dishes on Sabouraud medium (Merck). Incubation was for seven days at 25°C ± 1°C.

Quantification of total sulfide: total sulfide content was determined using a colorimetric method according to APHA, 1972.

Scanning Electron Microscopy (SEM)

Examinations were conducted on metal samples previously fixed for preservation of the biological material and treated according to the technique described by Coutinho, 1993.

RESULTS AND DISCUSSION

After two days of exposure to seawater from the industrial system, the presence of biofilms on the metal coupons was observed. Table 1 shows that at this time the lowest number of anaerobic microorganisms was present. This is probably due to the anaerobic environment not being totally developed upon the biofilm base, formed mainly by aerobic microorganisms.

After four days of exposure, a significant increase of SRB was observed among the anaerobic microorganisms. On the other hand, aerobic bacteria and fungi remained practically constant during this entire period of exposure (Table 1).

It is worthwhile noting that on the second day of exposure, 95.3 percent of the adhered cells were aerobic bacteria which diminished with time. The opposite was observed in relation to the anaerobic bacteria. The proportion of SRB fell from 38.9 percent to 3.6 percent in a four-day interval, although the cell number increased with the time of exposure (Table 1). The fungi remained constant, and their percentage was insignificant compared with the other microorganisms (Fig. 1).

An increase in sulfide accompanied larger amounts of SRB. This is to be expected, as sulfide results from the metabolic activity of these bacteria.

SEM revealed the adhesion of microorganisms to the metal surface during this period. A significant number of bacteria was detected, as well as extracellular material (Fig. 2) and algae of the genus *Diatomaceae* (Fig. 3).

The composition of the biofilms can show variations according environmentals parameters (8). The biofilm formation and its correlation with microbiolly influenced corrosion (MIC) are frequently

Table 1: Number of microorganisms and total sulfide content detected in the biofilms on the surface of stainless steel type AISI-304.

Days	Bacteria cells/cm^2			Fungi cfu/cm^2	Sulfide mcg/cm^2
	Sulfate reducing	Anaerobic	Aerobic		
2	3.0* ± 1.0**	$3.1 \pm 0.9 \times 10^3$	$6.3 \pm 0.9 \times 10^4$	$3.6 \pm 0.5 \times 10^2$	-
4	$5.5 \pm 0.7 \times 10^4$	$6.9 \pm 0.8 \times 10^4$	$7.5 \pm 0.4 \times 10^3$	$3.0 \pm 0.6 \times 10^2$	91.54
8	$2.0 \pm 0.5 \times 10^6$	$5.3 \pm 0.5 \times 10^7$	$5.8 \pm 1.0 \times 10^3$	$3.2 \pm 0.5 \times 10^2$	112.55

*Mean value of 4 experiments; ** Standard deviation of mean

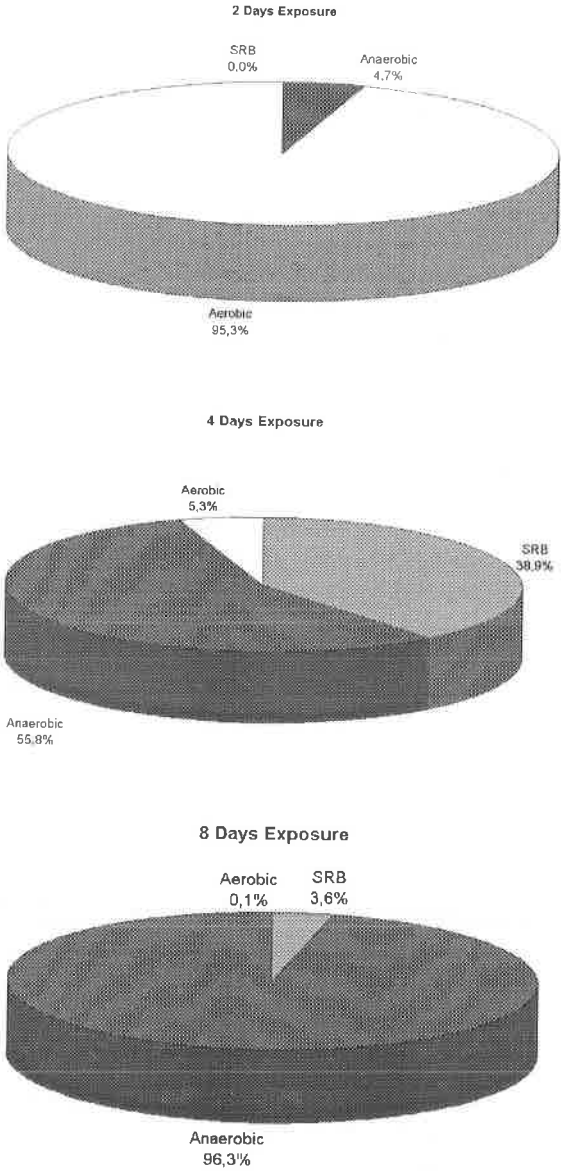

Figure 1 - Percentage variation of bacterial groups in the biofilms according to time of exposure

found in the literature, which relate practical cases of microbiological control programs and corrosion monitoring (5; 13).

No signs of corrosion were detected on the coupons of stainless steel type AISI-304 during this period, although some of the microorganisms cited in the literature as responsible for accelerating metal deterioration were detected in the biofilms, such as anaerobic and aerobic bacteria (6), *Gallionella* (12) and SRB (8).

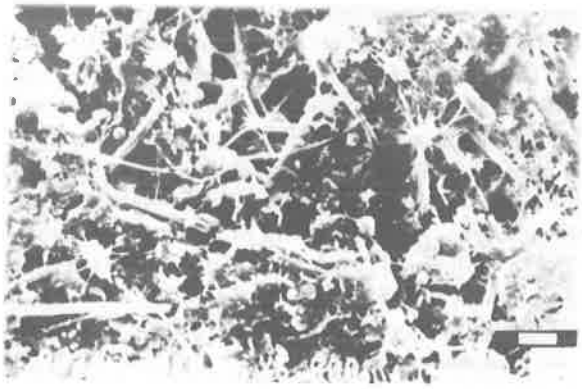

Figure 2 - SEM photomicrograph showing microfouling of stainless steel AISI - 304 after eight days of exposure (Bar = 1μm)

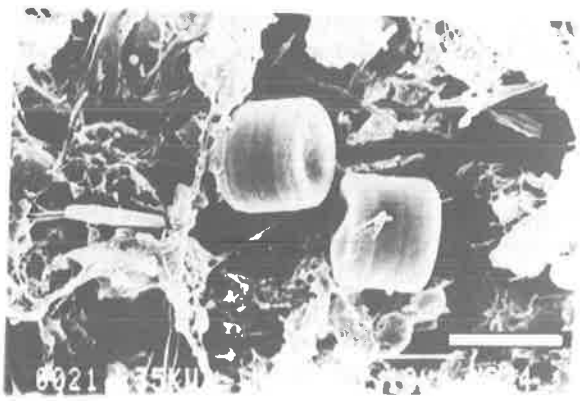

Figure 3 - SEM showing microfouling of stainless steel AISI - 304 with colonization by algae, other microorganisms and the presence of inorganic deposits, after eight days of exposure. (Bar = 10μm)

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RESUMO

Monitoramento de formação de biofilmes em água de refrigeração industrial em sistema aberto

Cupons metálicos de aço inoxidável AISI-304 foram instalados na bacia de captação de água salgada de uma planta industrial e retirados para análises em intervalos de 2, 4 e 8 dias com o objetivo de detectar os diferentes grupos de microrganismos integrantes dos biofilmes formados na superfície do metal.

Durante este período observou-se variação quantitativa dos grupos de microrganismos. As bactérias aeróbias, que tiveram uma proporção de 95,3% do total de microrganismos no início do experimento, no 8º dia de exposição representavam apenas 0,1% dos microrganismos. Já as bactérias anaeróbias que no início representavam apenas 4,7% dos microrganismos, com 8 dias de exposição corresponderam a 96,3% dos microrganismos totais integrantes do biofilme. As bactérias redutoras de sulfato estavam presentes e também sofreram variações, assim como as quantidades de sulfetos totais. Através da microscopia eletrônica de varredura, observou-se os microrganismos aderidos ao metal, inclusive presença de microalgas.

Palavras-chave: água de refrigeração; biocorrosão; biofilmes

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IN VITRO SUSCEPTIBILITY TESTS OF DERMATOPHYTES TO GRISEOFULVIN AND IMIDAZOLE DERIVATIVES

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ABSTRACT

The sensitivity of dermatophytes (89 "wild" and 38 "collections" strains) to griseofulvin and azole derivatives (clotrimazole, cetoconazole, tioconazole, miconazole, econazole, isoconazole and oxiconazole) was studied using "in vitro" determinations of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The broth dilution method was used; the antifungal drugs were used in concentrations between 0,05 to 100 μg/ml. The inoculum was (standardized adjusting the suspension to contain) 1,0 to 1,5 X 10^6 ufc/ml. The MIC and MFC values showed different results for many fungi strains and different concentrations of antifungal agents. The values for clotrimazole varied from 0,1 to 1,56 μg/ml in almost all strains. Higher values were obtained for *T. rubrum* (12,5 μg/ml) and *T. mentagrophytes* (25,0 μg/ml). Cetoconazole was the imidazole drug that showed the highest MIC and MFC values, as high as 100,0 μg/ml when tested against the species of *T. rubrum*, *T. mentagrophytes*, *T. tonsurans* and *M. gypseum*. The antifungal activities of the other imidazole drugs were comparable, the MIC and MFC values varied from 0,10 to 12,5 μg/ml. In relation to griseofulvin, the fungi used in this study, presented two different behaviours: higher MIC and MFC values (12,5 to 100,0 μg/ml) for *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *T. soudanense*, *M. canis*, *M. gypseum* and *M. persicolor* and lower values (0,39 to 3,12 μg/ml) for *T. schoenleinii*, *T. violaceum*, *E. floccosum*, and *M. audouinii*. *E. floccosum*, *M. canis* and *T. schoenleinii* had higher susceptibilitis to the drugs, (MIC and MFC values varying from 0,05 to 0,20 μg/ml), when compared to *T. mentagrophytes*, *T. rubrum*, *M. gypseum* and *T. soudanense*. No important differences were observed in the MIC and MFC values for the "wild" and the "collection" strains.

Key words: Dermatophytes, antifungal susceptibility

INTRODUCTION

Superficial mycotic infections caused by dermatophytes affect the epidermis, hair follicles and nails.

The term "dermatophyte" literally means "skin plant" but the exact origin of this term is not known (1,9).

This fungi group consists of the genera *Trichophyton*, *Microsporum* and *Epidermophyton* that cause infections in men and animals.

According to the cutaneous area that is affected, they are categorised as: tinea capitis, tinea corporis, tinea inguinalis, **tinea pedis, tinea barbae** and **onix** or **onicomicosis** (9,17).

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Depending on their natural habitat, dermatophytes are classified in three categories: anthropophilic, zoophilic and geophilic. These classifications indicate the relative stages of their evolution, adaptation to specific hosts and their ability to infect and cause diseases (1,9,13).

The study of superficial mycotic infections is important because of the high frequency and also because sometimes they cause epidemic outbreaks such as **tinea pedis** in military recruits, and **tinea capitis** in scholars (2,7,8,18).

For a long time, the treatment of superficial mycosis has been restricted to the use of compounds with a not fully defined antifungal action such as anhydrotic, antiperspirants,organic acids and dyes. These compounds are useful and have been widely used. Recently, more specific antifungal substances have been gradually introduced mainly because of the limited effect of the compounds mentioned before and skin discolouring. The evolution of technology, the development of new antifungal agents and the knowledge that fungi are capable of developing resistance to drugs during or after treatment, made the improvement of "in vitro" techniques, like antifungal susceptibility tests, necessary. These "in vitro" methods are indispensable for therapeutic control and to enable selection of more specific and effective antifungals for each mycosis infection.

The aim of this study was to verify the "in vitro" behaviour of dermatophytes towards the antifungals imidazole and griseofulvin, through the determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC).

MATERIALS AND METHODS

The dermatophytes "in vitro" sensitivity tests were carried out with 127 strains, 89 of them were "wild" and 38 were from "collections". The wild strains were obtained at Departamento de Dermatologia do Hospital das Clínicas (SP) (55 strains), Departamento de Dermatologia da Escola Paulista de Medicina (19 strains) and the Departamento de Análises Clínicas da Faculdade de Ciências Farmacêuticas (USP) (15 strains). The species of the wild fungi strains are shown in Table 1.

The collection strains were obtained at Departamento de Micologia Médica da Faculdade de Medicina (USP) (33 strains) and Departamento de Micologia Médica do Instituto de Ciências Biomédicas (USP) (5 strains). The species of these strains are shown in Table 2. These strains were been kept in mineral oil from 3 up 33 years.

The antifungal agents were supplied by Janssen Pharmaceutical Ltd, Squibb Ltd and Pfizer Ltd, Pharmaceutical Industries, and were used in a micropulverized formula.

The experiments were carried out as described in the literature, using the Sabouraud Broth yeast extract (0,125%) dilution method. Imidazole was dissolved in

Table 1 - Origin of wild strains studied with the imidazole and griseofulvin antifungics (MIC and MFC determination)

Species	Origin			
	HC	EPM	FCF	TOTAL
E. floccosum	6	2	0	8
M. canis	10	4	2	16
M. gypseum	2	1	1	4
T. mentagrophytes	5	7	6	18
T. rubrum	29	5	6	40
T. schoenleinii	2	0	0	2
T. tonsurans	1	0	0	1
TOTAL	55	19	15	89

E = Epidermophyton
M = Microsporum
T = Trichophyton
HC = Hospital das Clínicas de São Paulo - SP
EPM = Escola Paulista de Medicina - SP
FCF = Faculdade de Ciências Farmacêuticas (USP-SP)

Table 2 - Origin of collection strains studied with the imidazole and griseofulvin antifungics (MIC and MFC determination)

Species	Origin		
	ICB	FMUSP	TOTAL
E. floccosum	0	4	4
M. audouinii	0	1	1
M. canis	2	3	5
M. gypseum	0	4	4
M. persicolor	0	2	2
T. mentagrophytes	1	5	6
T. rubrum	2	7	9
T. schoenleinii	0	2	2
T. soudanense	0	1	1
T. tonsurans	0	3	3
T. violaceum	0	1	1
TOTAL	5	33	38

E = Epidermophyton
M = Microsporum
T = Trichophyton
ICB = Instituto de Ciências Biomédicas (USP-SP)
FMUSP = Faculdade de Medicina (USP-SP)

dimethyl sulfoxide (DMSO) and griseofulvin in acetone to obtain a concentration of 10.000 μg/ml. These solutions were transfered to screw caps-tubes (1 ml /tube) and stored at -70°C. The stock solution was diluted 1:100 in liquid medium, stored at 4°C and used in 15 days. Twelve 1:2 serial dilutions were performed in order to obtain concentrations that varied from 100 μg/ml to 0,05 μg/ml. The cultures were grown in Sabouraud Agar for 8 to 15 days (10,11).

In order to obtain a homogeneous innoculation, fungal growth was suspended in distilled water and washed twice. In order to separate mycelia from conidia, the final suspension was vortexed and filtered through a double layer of sterile gauze. The number of conidia/ml was determined by counting in a hematocytometer. A standard suspension of conidia (1 to 1,5 X 10^6 ufc/ml) was used to innoculate 100 μL for each test tube (in duplicate). These tests were performed at 30°C and interpreted after 5 to 15 days.

Under standard conditions, the MIC was considered to be the lowest concentration that inhibited fungal growth. Positive tubes were those in which growth could be seen without a microscope.

For the determination of the MFC, the tubes with no growth were reinnoculated in test tubes aining new medium without the antifungal agent. This new series was kept at 30°C and read after 5 to 15 days.

RESULTS AND DISCUSSION

A large diversity of MIC and MFC values was observed for individual strains of the same species. Figs. 1, 2 and 3 represent the range of sensitivity of the species *Microsporum*, *Trichophyton* and *Epidermophyton*, groups I and II, to griseofulvin and azole derivatives. *E. flocosum*, *M. canis* and *T. schoenleinii* were sensitive to the lowest concentrations of the antifungal agents (0,10 μg/ml to 0,20 μg/ml). The highest frequency of sensitivity of strains of each species converged to concentrations from 0,20 μg/ml to 6,25 μg/ml; a low percentage of strains deviated from MIC medium levels and tended to higher concentrations (12,5 μg/ml to 100,0 μg/ml). These species were *T. rubrum*, *T. mentagrophytes*, *M. gypseum* and *T. soudanense*. Based on medium values of MIC and MFC, the antifungi azole group except of ketoconazole, presented good results. Lower sensitivity to clotrimazole (0,10 μg/ml - 1,56 μg/ml), miconazole and oxiconazole (0,10 μg/ml - 3,12 μg/ml), econazole and isoconazole (0,10 μg/ml -

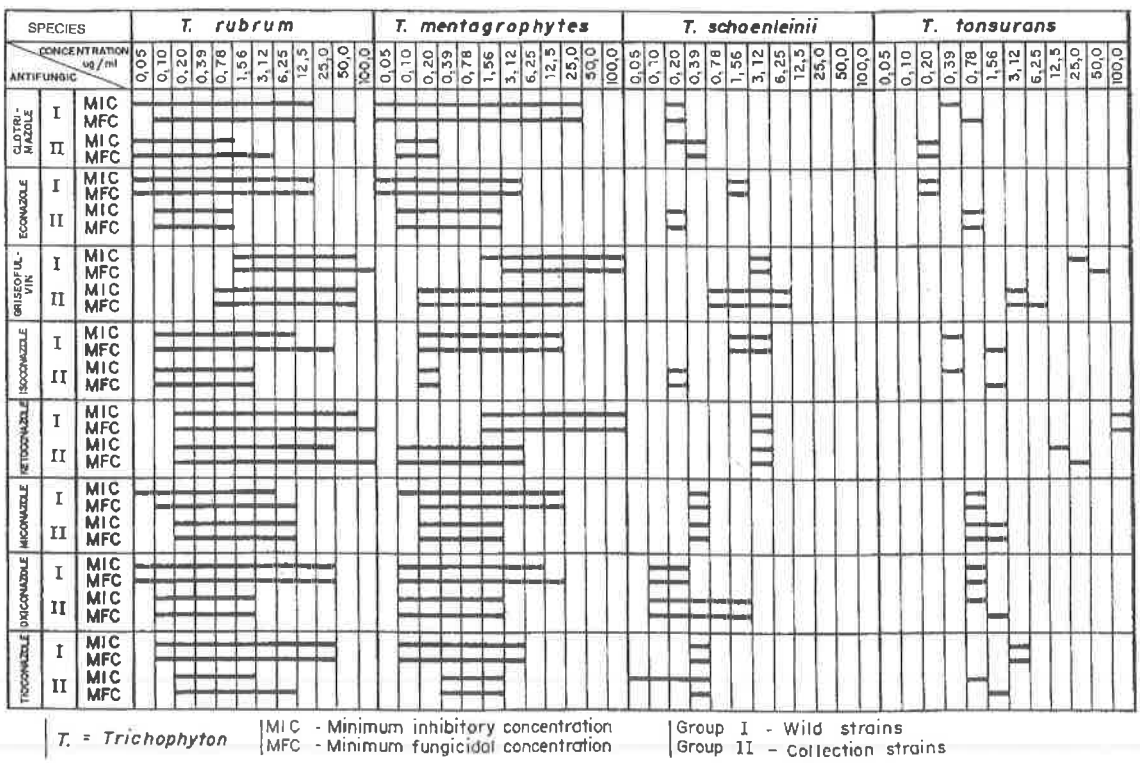

Figure 1. MIC and MFC values of *Trichophyton* species (groups I and II) for griseofulvin and imidazole derivatives.

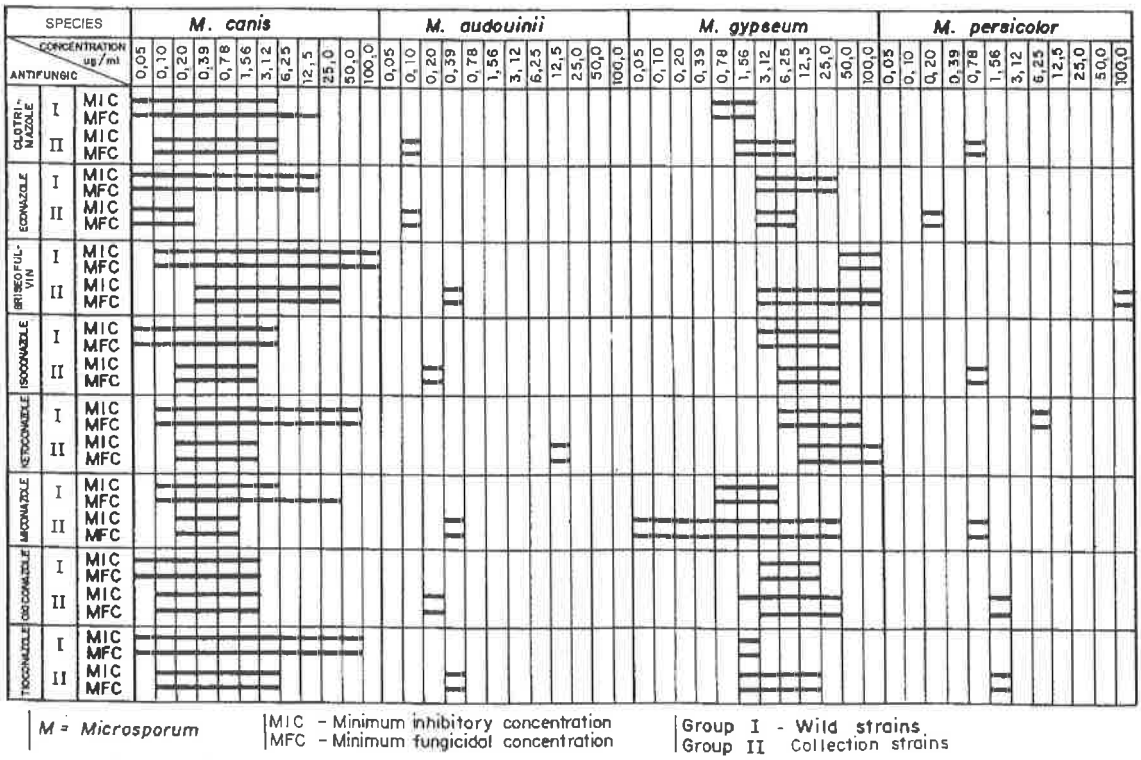

Figure 2. MIC and MFC values of *Trichophyton* and *Epidermophytos* species (group I and II) for griseofulvin and imidazole derivatives.

6,25 µg/ml), tioconazole (0,10 µg/ml - 12,5 µg/ml) was observed. Ketoconazole was the azole group with the highest indices of MIC and MFC (0,78 µg/ml - 50,0 µg/ml).

The activity of griseofulvin demonstrated two behaviours: for the *T. schoenleinii*, *T. violaceum*, *E. floccosum* and *M. audouinii*, the levels of MIC and MFC were between 0,30 µg/ml and 3,12 µg/ml and for the species *M. gypseum* and *M. persicolor* they were between 12,5 µg/ml and 100,0 µg/ml.

No exceptional differences in behaviour between wild and collection strains were observed in the "in vitro" tests.

The definition of a strain as sensitive or resistant based on the concentration of the antifungal agent is a complex task because the results vary according to the technique used in the determination.

For the analysis of the susceptibility belts obtained in this study we focused on one belt which had the highest susceptibility situated between the concentrations of 0,05 µg/ml to 6,25 µg/ml. The concentration of 6,25 µg/ml can be considered as a medium susceptibility or a reference index of real MIC, because the majority of the strains converged at this concentration. As index of lower susceptibility were between 12,5 µg/ml to 100,0 µg/ml that corresponded to standard maximum indexes; these strains can be seen as those with a greater probability of developing resistance "in vivo".

From the behaviour of the strains in relation with the antifungal, there was an assumption that the variation in levels of susceptibility show characteristics inherent to the individual strains, more so than within a group of species. These could be attributed to intrinsic factors not yet well established.

These findings led us to the hyphothesis that the strains belonging to the same species could differ in enzyme potential or other factor that have the ability to develop defense or repair systems against the harm caused by antifungal in specific areas of the strain and that could vary. The results obtained in this research are supported by previous reports that some microrganisms belonging to the same species are naturally resistant, while, some others appear to be more susceptible (3,4,5).

Studies on the development of resistance mechanisms to antifungals are being carried out. An amount of *Candida albicans* strains (isolated from Candidiases chronic mucocutaneous) was documented as clinically resistant.It has been studied

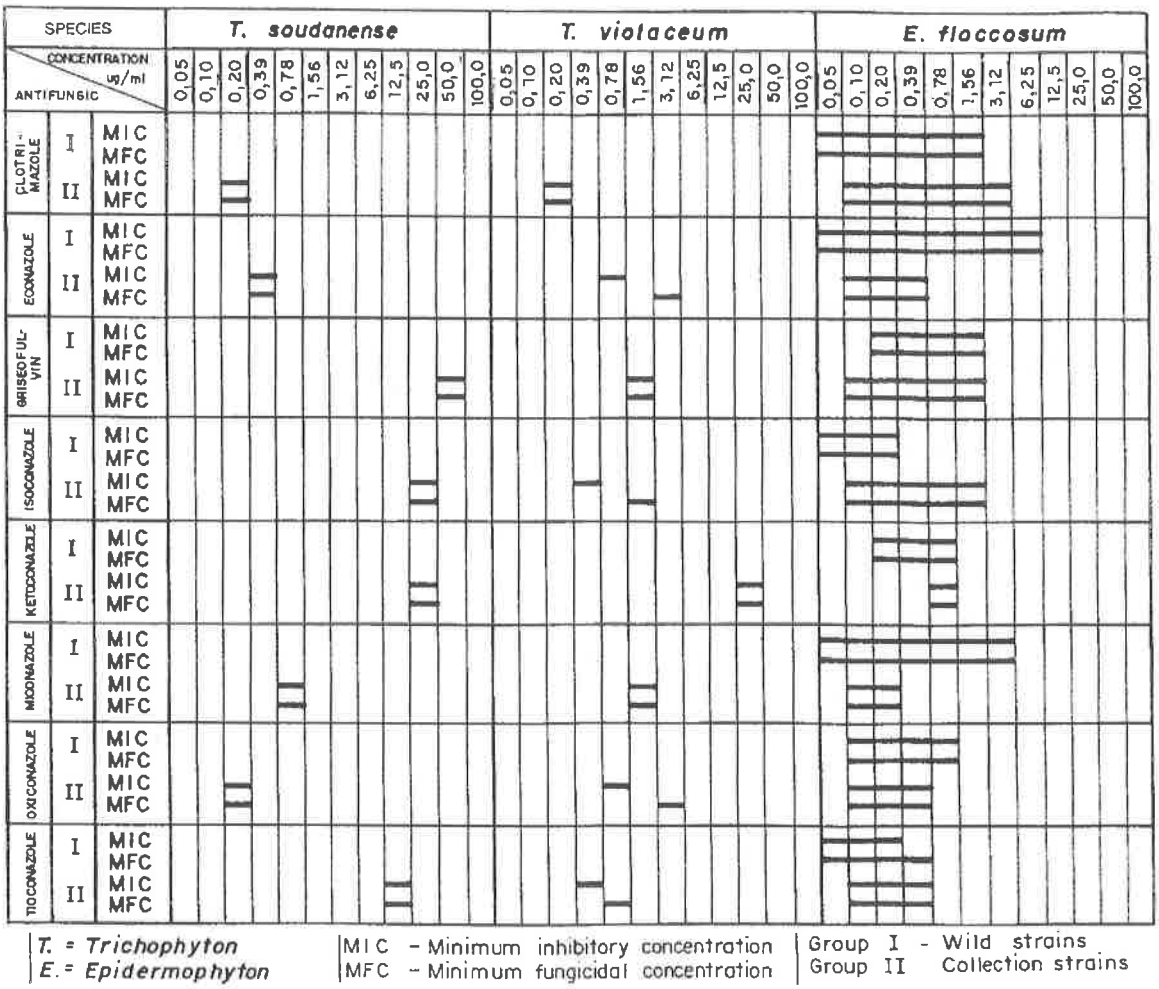

Figure 3. MIC and MFC values of *Microsporum* species (groups I and II) for griseofulvin and imidazole derivatives.

by using the polymorphous restriction genetic analysis with the objective to identify "in vitro" its resistance mechanism. The results demonstrated that some variant strains resistance, after repeated subcultures there were differences in the sterol composition (12).

Sensibility tests "in vitro" are clinically important as they allow us to estimate the probability of resistance developing. There is evidence that defense mechanisms emerge only during the clinical stage. The tests should be carried out before therapy starts, with the aim of finding the MIC, and should repeated in be order to evaluate the appearance of resistance strains (6,14).

Some considerations need to be made on treatment failures observed in dermatological clinics. The favorable or unfavorable effects of a dermatophyte infection depends on the activity of the drug to inhibit the emaciate and the ability of the host to destroy it. In chronic dermatophyte infections immunity defects are relevant factors and in similar cases there could be a deficiency in the defense mechanism of the host, affecting the organisms capacity to fight infection. One question is, if the dermatophyte anthropophilic, *Trichophyton rubrum* often related relapses, is different in relation to other species in its ability to escape immunological defense of the host. The pathogenicity of *T. rubrum* and its capacity to invade internal organs, occasionally causing death, has been documented in some studies (15,16).

From the above results we conclude that the susceptibility tests "in vitro" with fungous is in evolution but show some obstacles mainly because technical limitations.

RESUMO

Testes de sensibilidade "in vitro" de dermatófitos a griseofulvina e derivados imidazólicos

Estudou-se a sensibilidade "in vitro" de dermatófitos (89 cepas "selvagens" e 38 "de coleção") frente à griseofulvina e derivados imidazólicos (clotrimazol, miconazol, cetoconazol, tioconazol, isoconazol, econazol e oxiconazol),pelas determinações da concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM). Empregou-se o método de diluição em meio líquido; os antifúngicos foram usados em concentrações entre 0,05 a 100,0 μg/ml. O inóculo foi padronizado ajustando-se a suspensão para conter 1,0 a 1,5 X 10^6 ufc/ml. Os valores encontrados para CIM e CFM mostraram diferentes resultados para as diversas cepas fúngicas e diferentes concentrações de agentes antifúngicos. Os valores para clotrimazol variaram de 0,10 a 1,56 μg/ml para a maioria das cepas. Valores mais elevados foram obtidos para *T. rubrum* (12,5 μg/ml) e *T. mentagrophytes* (25,0 μg/ml). Cetoconazol foi o imidazólico que mostrou os valores de CIM e CFM mais elevados atingindo 100 μg/ml quando testado contra as espécies de *T. rubrum*, *T. mentagrophytes*, *T. tonsurans* e *M. gypseum*. As atividades antifúngicas dos demais imidazólicos foram comparáveis, variando os valores de CIM e CFM de 0,10 a 12,5 μg/ml. Com relação a griseofulvina, os fungos usados neste estudo, apresentaram dois comportamentos: valores altos de CIM e CFM (12,5 a 100,0 μg/ml) para *T. rubrum*, *T. mentagrophytes*, *T. tonsurans*, *T. soudanense*, *M. canis*, *M. gypseum*, *M. persicolor* e valores mais baixos (0,39 a 3,12 μg/ml) para *T. schoenleinii*, *T. violaceum*, *E. floccosum* e *M. audouinii*. *E. floccosum*, *M. canis* e *T. schoenleinii*, tiveram altas sensibilidades para as drogas (CIM e CFM variando de 0,05 a 0,20 μg/ml) quando comparadas com *T. mentagrophytes*, *T. rubrum*, *M. gypseum* e *T. soudanense*. Não foram observadas diferenças marcantes nos valores de CIM e CFM para as cepas selvagens e as cepas de coleção.

Palavras-chave: dermatófitos, sensibilidade a antifúngicos.

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PRODUCTION AND REGENERATION OF *PENICILLIUM EXPANSUM* AND *PENICILLIUM GRISEOROSEUM* PROTOPLASTS

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ABSTRACT

The best conditions for the production and regeneration of *Penicillium expansum* and *P. griseoroseum* protoplasts were the following: 0.5 M KCl, pH 5.5, as an osmotic stabilizer; Novozym 234 at 1.7 mg/ml for *P. expansum* and 3.3 or 6.7 mg/ml for *P. griseoroseum*; 1 to 2 hours of digestion at 25°C; shaking at 130 rpm for 1-hour digestion times and 100 rpm for longer periods. These conditions were defined based on the results of protoplast production and regeneration, and resulted in a protoplast yield in the range of 10^7 protoplasts/ml. For protoplast regeneration, 0.56 M sucrose was selected as an osmotic stabilizer for the regeneration medium with an incubation temperature of 25°C. Under these conditions, maximum percentages of protoplast regeneration, based on the means for all experiments, were 46.5% for *P. expansum* and 56.9% for *P. griseoroseum*.

Key words: *Penicillium*, protoplasts, regeneration

INTRODUCTION

Several species of the genus *Penicillium* are important producers of antibiotics, enzymes and other substances of industrial interest, particularly pectolytic enzymes (7, 12, 13, 14). *Penicillium* spp. has been investigated to determine the best conditions for growth and maximum pectolytic enzyme production (2, 3, 4, 5). Genetic manipulation of *Penicillium* spp. is a new approach, with an enormous potential for the development of strains with higher enzyme production.

The conditions for production and regeneration of protoplast represent important steps to be established in *Penicillium* spp.. It is reported that each species requires specific conditions for protoplast production and regeneration (6, 11). For several fungal species ideal conditions have already been determined. Factors, such as mycelium age, mycelium mass, osmotic stabilizer, temperature, pH, and digestion time strongly affect the production and regeneration of protoplasts (6, 11, 15).

MATERIALS AND METHODS

Fungi. *Penicillium expansum* and *Penicillium griseoroseum* isolated from forest tree seeds at Universidade Federal de Viçosa, Viçosa, MG, Brazil, were used throughout the experiments.

Culture media. The oatmeal-agar medium for spore production contained 30 g oatmeal, 15 g agar and water to 1000 ml, pH 7.0.

The MS (minimal supplemented) medium used for mycelial growth contained 2 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2 SO_4$, 18 ml 5% $MgSO_4.7H_2O$, 50 ml 20% glucose, 20 ml 6% yeast extract, and water to 1000 ml, pH 7.0.

The MR (regeneration medium) was used for protoplast regeneration and was made by addition of an osmotic stabilizer (0.56 M sucrose) to the MS medium.

Evaluation of fungal growth in the presence of different osmotic stabilizers. *P. expansum* and *P. griseoroseum* growth was measured using the

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following osmotic stabilizers at 0.5 M: sucrose, mannitol, sorbitol, $MgSO_4$, KCl, and NH_4Cl. Plates containing osmotically stabilized solid medium were inoculated in two ways: a) with a spore suspension to a final concentration of 100 spores/plate; b) With agar disc/plate containing sporulated mycelium. The inoculated plates were incubated at 25°C for one week.

Protoplast production. Suspension of 10^6 spores were inoculated into 125-ml flasks containing 50 ml MS medium and incubated at 25°C at 130 rpm for 16-24 h. The mycelium produced was washed twice with an osmotic stabilizer by centrifugation at 10,000 x g at 4°C for 10 min. The mycelial mass was weighted and digested at 25°C; shaken at 50 and 130 rpm and incubated at 1 to 5 h with NOVOZYM 234 solution prepared with the osmotic stabilizers, pH 5.5. The flasks were shaken at 130 rpm for one hour or at 100 rpm for longer times. Ten milliliters of enzyme solution were added to 3 g fresh mycelium. Protoplast production was monitored under the microscope by counting their number with a Neubauer chamber.

The following solutions were tested as osmotic stabilizers: 0.56 M sucrose, 0.63 M mannitol, 0.37 M NaCl, 0.37 M KCl, and 0.62 M $MgSO_4$. The enzyme concentrations and the digestion times tested were 1.7, 3.3 and 6.7 mg/ml, and 1 to 5 h, respectively.

Protoplast regeneration. Protoplasts were purified by filtering the digestion mixture through a slightly compacted cotton wool layer in a 5-cm^3 syringe followed by washing two times with the osmotic stabilizer by centrifugation at 2,000 x g for 10 min at 4°C. The pellet was resuspended in 1 ml of the osmotic stabilizer. The following osmotic stabilizers were tested: 0.56 M sucrose, 0.63 M mannitol, 0.63 M sorbitol, 0.37 M KCl, 0.37 M NaCl, 0.62 M $MgSO_4$, and 0.6 M NH_4Cl. Samples with different dilutions of the protoplast suspensions were spread with the aid of glass beads onto plates containing osmotically stabilized medium. All plates were incubated at 25°C.

RESULTS AND DISCUSSION

$MgSO_4$, KCl, NaCl, sucrose and mannitol were tested as osmotic stabilizers for protoplasts production. The best results were obtained with the salts (Figure 1). Protoplasts produced in presence of $MgSO_4$ were hard to pellet during centrifugation, possibly due to the formation of large vacuoles (11). Larger vacuoles and larger protoplasts have been related to lower stabilizer concentrations, while smaller vacuoles and smaller protoplasts have been related to higher stabilizer concentrations (15). In the presence of $MgSO_4$, protoplasts tend to agglutinate (1), becoming difficult to count and to dilute for plating. Consequently, KCl was chosen as the osmotic stabilizer for further protoplast production. No difference in protoplast production was observed when the concentrations of 0.37 M or 0.5 M KCl were tested. At both concentrations, the yield was in the range of 10^7 protoplasts/ml.

Protoplast release was affected by poor cell wall digestion when mycelium was grown in shaker for more than 24 h. Therefore the maximum mycelium age for protoplast production was limited to 16 to 24 h in all experiments. Mycelial age has been cited as a determinant in the process of protoplast release (8, 9, 10), however the mecanism has not been elucidated, but probably it is associated with changes in hyphal cell wall composition (11). According to these authors, α-1,3-glycan deposition as an outer layer on the cell wall of older hyphae may be the determinant factor for such effect in *Aspergillus nidulans*.

When the digestion mixture was shaken at 130 rpm for 1-2 h protoplast release was greater and faster yielding 1.3 x 10^7 protoplasts/ml. To obtain an equivalent amount of protoplast at 50 rpm shaking, at least 3 h of incubation were required. Without shaking less than 10^7 protoplasts/ml were obtained after 5 h of digestion. Increase of Novozym 234 concentration to 6.7 mg/ml resulted in higher protoplast release (Figure 2).

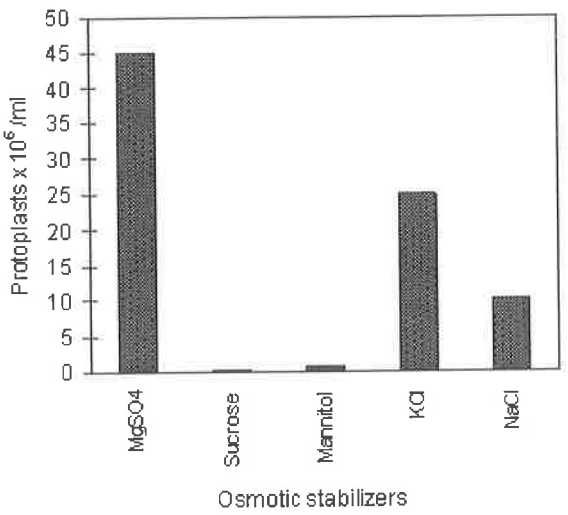

Figure 1. Production of *Penicillium expansum* protoplasts in the presence of different osmotic stabilizers at a molar concentration equivaent to -14.49 bars. Enzyme concentration: 3.3 mg/ml. Digestion time: 2 hours.

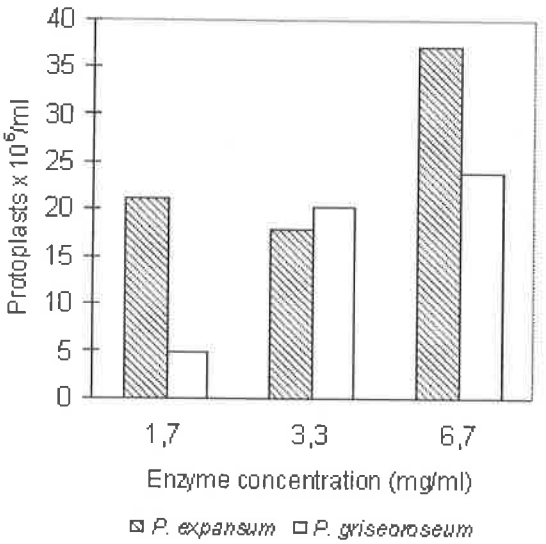

Figure 2. Protuction of *Penicillium expansum* and *P. griseoroseum* protoplasts as a function of different concentrations of NOVOZYM 234 in the digestion mixture. Osmotic stabilizer: 0.5 M KCl. Digestion time: 2 hours.

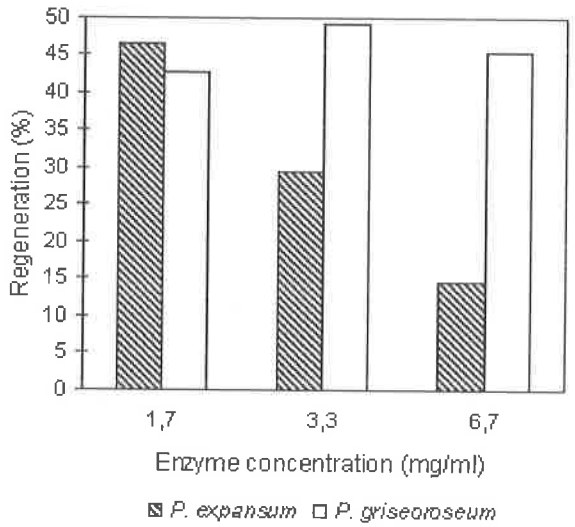

Figure 3. Percent regeneration of *Penicillium expansum* and *P. griseoroseum* protoplasts obtained as a function of NOVOZYM 234 concentration in the digestion mixture.

Shaking the digestion mixture during protoplast production did not affect protoplast regeneration. *P. expansum* protoplasts produced without shaking regenerated at rate of 45.7% and *P. griseoroseum* at 61.5%, after 1 h incubation. When the protoplasts were produced at 130 rpm, the regeneration rates were 46.5% for *P. expansum* and 56.9% for *P. griseoroseum*. Shaking at 130 rpm was done only in the first hour of digestion. For longer digestion times up to 5 h, shaking was reduced to 100 rpm. Under these conditions, protoplast regeneration remained around 50%.

Regeneration of protoplasts of *P. expansum* and *P. griseoroseum were* not affected by digestion time between 1 to 5 h, as specified above. This may be particularly important when the enzymatic treatment of the mycelium has to be lengthen. For *P. expansum*, regeneration percentages were 45.1 and 46.5% for digestion times of one and 5 h, respectively. For *P. griseoroseum*, regeneration percentages were 45.4% for one-hour digestions and 44.9% for 5-h digestions.

Higher enzyme concentration in the digestion mixture had a negative effect on protoplast regeneration for *P. expansum*, but not for *P. griseoroseum* (Figure 3). Digestion mixture with Novozym 234 at 1.7 mg/ml was the most adequate for protoplast regeneration in *P. expansum*, whereas for *P griseoroseum* the enzyme concentrations can vary from 1.7 to 6.7 mg/ml without affecting the regeneration. These results may be related to differences in cell wall composition in the two *Penicillium* species and indicate the importance of evaluating enzyme concentration in protoplast production and regeneration. It was not found in the literature information about the best enzyme concentration for protoplast regeneration. Fixed enzyme concentrations have been normally used, 5 and 10 mg/ml being the commonest values. Regeneration percentage could be higher than 90% for some fungal species (6) and lower than 10% for others (9,10). Enzyme concentration for digestion should be investigated when regeneration percentage is below the desired values.

We have observed that high osmotic stabilizer concentration greatly affect the growth of some filamentous fungi, depending the species and the stabilizer used. The growth of *P. expansum* and *P. griseoroseum* was evaluated in the presence of different osmotic stabilizers at 0.5 M (data not shown). Sucrose promoted the best mycelial growth for *P. expansum* and *P. griseoroseum*, followed by $MgSO_4$, KCl, and NaCl. Mannitol, sorbitol, and NH_4Cl limited or fully inhibited fungal growth. NH_4Cl had the greatest inhibitory effect on fungal growth. This effect is particularly important when a stabilizer is efficient for protoplast production but impairs cell wall regeneration in the culture medium.

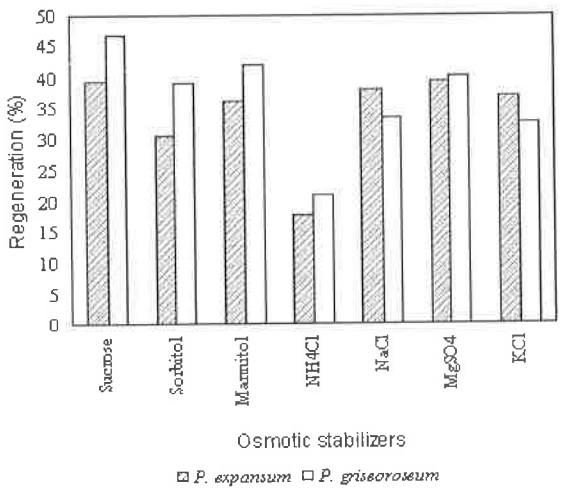

Figure 4. Regeneration percentage of *Penicillium expansum* and *P. griseoroseum* protoplasts at different osmotic stabilizers at a molar concentration equivalent to -14.59 bars.

For protoplast regeneration, except for NH_4Cl, no statistically significant difference was observed for the other osmotic stabilizers (Figure 4). NH_4Cl promoted the lowest protoplast regeneration percentage.

Sugars were good stabilizers for protoplast regeneration but inefficient for protoplast production. May be the sugars favors the osmotic stability for protoplast viability but somehow it affects negatively the enzimatic digestion of the cell wall. $MgSO_4$ as stabilizer promoted good results for production and regeneration of protoplasts. But it was not chosen because at high temperatures it precipitates in the regeneration medium.

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RESUMO

Obtenção e regeneração de protoplastos de *Penicillium expansum* e *Penicillium griseoroseum*

As condições mais adequadas para a obtenção e regeneração de protoplastos de *P. expansum* e *P. griseoroseum*, foram: KCl 0,5 M como estabilizador osmótico, pH 5,5; Novozym 234 a 1,7 mg/ml para *P. expansum* e 3,3 ou 6,7 mg/ml para *P. griseoroseum*; tempo de digestão de 1 a 2 horas e temperatura de 25°C, com agitação de 130 rpm para 1 hora de digestão e de 100 rpm para períodos mais prolongados. Estas condições foram definidas considerando os resultados tanto da obtenção quanto da regeneração dos protoplastos, e permitiram a liberação de protoplastos na ordem de 10^7/ml. Para a regeneração de protoplastos, sacarose 0,56 M foi escolhida como estabilizador osmótico para o meio de regeneração, com uma temperatura de incubação de 25°C. Nestas condições, considerando as médias de todos os experimentos, obteve-se uma percentagem máxima de regeneração de 46,5% para *Penicillium expansum* e 56,9% para *P. griseoroseum*.

Palavras-chave: *Penicillium*, protoplastos, regeneração

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OBTENTION AND EVALUATION OF PATHOGENICITY OF ULTRA VIOLET RESISTANT MUTANTS IN THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA*

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ABSTRACT

UV-light resistant mutants of the entomopathogenic fungus *Beauveria bassiana* were obtained and their pathogenicity and conidial production were evaluated. Two mutants were selected, which produced conidia at a lower rate than the wild strains. In terms of pathogenicity, one of them maintained the pathogenicity and other was less effective in causing the death of *Diatraea saccharalis* larvae. Since pathogenicity is a complex trait, these results may be attributed to pleiotropic effects of the gene(s) conferring resistance to UV-light, or to the accumulation of mutations that affect pathogenicity as the result of the various irradiations performed to obtain mutants.

Key words: *Beauveria bassiana*; UV-light resistant mutants; pathogenicity; biological control

INTRODUCTION

The Deuteromycete *Beauveria bassiana*, which causes white muscardine disease in several insect species of agricultural interest, has been studied for several years because of its potential as a biocontrol agent against several pests (2, 9, 11, 14). However there are several problems related to the viability of fungal propagules in environment. The conidia are exposed to sunlight and the direct incidence of ultraviolet rays may impair their stability and viability (1, 6, 7, 10, 12, 13). The derivation of strains with higher resistance to UV-light would be of interest for the use of the fungus for biological control because there could be more prolonged conidial exposure to sunlight. Genetic studies are essential (but very scarce) to try to solve these problems (17, 18). Thus the objective of the present study was to obtain UV-light resistant mutants of *B. bassiana*, to evaluate the survival rates, the production of conidia and their pathogenicity against *Diatraea saccharalis* larvae.

MATERIALS AND METHODS

Strains: *Beauveria bassiana* 196 and 256 strains isolated respectively from *Diatraea saccharalis* and *Euchistus heros* were used (kindly provided by Dr. Sergio Batista Alves, Department of Entomology, Escola Superior de Agricultura "Luiz de Queiroz". University of São Paulo. SP. Brazil).

Media: Complete medium (21) containing yeast extract (0,5 g), hydrolized casein (1,5 g), vitamins (1 ml), $NaNO_3$ (6,0 g), KH_2PO_4 (1,5 g), $MgSO_4 . 7H_2O$ (0,5 g), $FeSO_4$ (0,01 g), $ZnSO_4$ (0,01 g), Glicose (10,00 g), Ágar (15,00 g), destilated water (100 ml), pH 6.8.

Survival after exposure to UV-light: Conidial suspensions were prepared in a 0,1% (v/v) aqueous of Tween 80 and the number of conidia was estimated in a Neübauer chamber. The suspensions were diluted in 0,85% saline solution to a concentration of 10^6 conidia ml^{-1} and irradiated in the dark with short-wave UV-light (5 joules/m^2/s) using a Mineralight lamp

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model UUSL-25, 115 volts, 50-60 CVC, 60 amps. Samples were removed after different exposure periods, appropriately diluted, and 0,1 ml of the dilution was placed on Petri dishes containing complete medium. The material was incubated for 10 days at 28°C and the number of colonies formed was counted. The number of colonies obtained at time zero of irradiation was considered to be 100% of survival.

Production of UV-light resistant mutants: A suspension containing 10^6 conidia ml^{-1} was irradiated with a dose of UV-light sufficient to render practically all conidia no viable (6 minutes of exposure) and 1 ml of the irradiated suspension was placed on Petri dishes containing complete medium and incubated for 10 days at 28°C. The colonies that developed after treatment with this dose were isolated and submitted to longer exposure to UV-light (10 and 15 minutes of exposure). After three successive exposures, two resistant mutants were isolated and designated 196/R_1 and 256/R_1.

Conidial production: Production of conidia by mutants and by the strains that gave origin to them was estimated by removing a 0,50 cm diameter sample from the center of the colony. The conidia were dispersed in Tween suspension and counted in a Neübauer chamber.

Bioassays test: Suspensions of mutant and wild strains conidia were prepared in a 0,1% aqueous solution of Tween 80 and adjusted to a final concentration of 10^7 conidia ml^{-1}. *Diatraea saccharalis* larvae obtained from laboratory production of insects on an artificial diet were washed with sterilized distilled water and immersed in the following preparations for 10 seconds: 1) conidial suspension of strain 196; 2) conidial suspension of strain 256; 3) conidial suspension of strain 196/R_1; 4) conidial suspension of strain 256/R_1 and 5) solution containing no pathogen (control). The statistical lay out was a completely randomized design. The treatments had 5 repetitions with 10 larvae each.

The treated larvae were placed on Petri dishes containing pieces of sterilized maize stalks for food and maintained at 28°C and 70 ± 10% relative humidity. Mortality readings were taken daily and the experiment was terminated at the end of 20 days. The larvae showing exteriorization of fungal structures were considered to be parasitized.

The mortality percentage of were transformed in $\sqrt{x+0,5}$, and the means compared by the Tukey test ($p<0,05$) (23).

RESULTS AND DISCUSSION

Table 1 shows the survival percentage of wild and mutant strains after different doses of UV-light. Comparison of the survival percentages showed that the two mutants were more resistant than the strains gave origin to them, especially at higher radiation doses.

The mutants were also evaluated for conidial production (Table 2). The rate of conidial production for the strains 196/R_1 and 256/R_1, was lower than that of the wild strains, 196 and 256 after 12 days of growth.

The result indicated that, even though the UV-light resistant mutants may present potential advantages when compared with the wild strains that gave origin to them, the mutant 196/R_1 was less effective in causing larval mortality in the bioassay (Table 3). In addition, the UV resistant mutants produced a reduced number of conidia compared with the others. The effectiveness of an entomopathogenic fungus in attacking insects is determined by various factors from the time of fungal penetration to the time of fungal colonization of insect. Studies by Pekrul and Grula (20) on mutants of high and low pathogenicity infecting *Heliothis zea* and by Paris and Ferron (19) on *B. brongniartii* revealed that this is a complex process. The infection occurs by hyphal penetration through the

Table 1: Survival percentage, after UV-light irradiation of *Beauveria bassiana* conidia from wild and UV-light resistant strains

Time (min.)	Wild strains		resistant strains	
	196	256	196/R_1	256/R_1
0,0	100%	100%	100%	100%
3,0	3%	9,6%	7%	33,1%
6,0	0%	0%	2%	0,6%
10,0	0%	0%	0,15%	0,04%
15,0	0%	0%	0,07%	0,01%

Means of 3 replications

Table 2. Conidial production by wild strains and UV-light resistant mutants of *Beauveria bassiana*

STRAINS	MEAN NUMBER OF CONIDIA X 10^8*
256	1,523 a
196	1,130 a
256/R_1	0,952 b
196/R_1	0,010 c

Mean of 3 replications.
* Means followel by the same letter did not differ statistically by the Tukey test ($p<0,01$).

Table 3 Mortality of *Diatraea saccharallis* larvae caused by wild strains and UV-light resistant mutants of *Beauveria bassiana* twenty days after inoculation

STRAINS	MORTALITY MEAN (%)
256	46.00 a
196	48.00 a
256/R_1	36.00 a
196/R_1	6.00 b
CONTROL	0.00 b
CV	34.23

Mean of 5 replications, 10 larvae per replications.
Means followel by the same letter did not differ statistically by the Tukey test ($p \leq 0.05$).

tegument (9, 15,20), digestive tract (4) and respiratory system (5, 11). In this last case it is possible owing to the relatively small size of the conidia (mean diameter of 2,5 µm, 20, 22), which can cross the spiracle (5,3 µm diameter) to germinate there (20). According to Vining *et al.*(24), and Basyouni Sohair *et al.*(3) some strains of the *Beauveria* fungi synthesize a red pigment, oosporein, which diffuses through the culture medium and can be observed in the tissues killed by fungus. This pigment is known to have bactericidal action (8). In the present study, red pigmentation was observed only during the growth of mutant 196/R_1. Paris and Ferron (19) observed that only virulent strains of *Beauveria brongniartii* synthesized this pigment in Sabouraud medium, but the role of the pigment in the pathogenicity is still obscure. When the pathogenicity of the present mutants and wild strain was tested on *D. saccharalis* larvae (Table 3), the strain 196/R_1, which produced the red pigment, was found to cause a reduced mortality rate. In this case the pigment oosporein was produced by a avirulent strain of *Beauveria bassiana* different to that observed in *B. brongniartii* by Paris and Ferron (19).

In the present study, the lower efficiency of the UV-light resistant mutant 196/R_1 in attacking insect larvae may have been due to a pleotropic effect of the gene or genes that code for this resistance or to an accumulation of mutations caused by successive irradiations, which may have altered other traits linked to pathogenicity. If this is the case crosses by parasexual cycle or protoplast fusion (17, 18) between resistant and sensitive strains exhibiting greater pathogenicity may lead to the production of more efficient and UV-light resistant segregants that may be of value for biological control at the field level. This mutant could also be used to study the genetic factors linked to the pathogenicity of *Beauveria bassiana*.

The results of this work show that the mutant 256/R_1 can be used in the field for control of insect pests with greater effectiveness.

RESUMO

Obtenção e Avaliação da Patogenicidade de Mutantes Resistentes à Luz Ultravioleta no Fungo Entomopatogênico *Beauveria bassiana*

Mutantes resistentes à luz ultravioleta do fungo entomopatogênico *Beauveria bassiana*, foram obtidos e avaliada a patogenicidade e a produção conidial dos mesmos. Dois mutantes foram selecionados, os quais apresentam uma taxa de produção conidial menor que das linhagens selvatens. Um dos mutantes manteve a patogenicidade enquanto o outro apresentou menor virulência contra larvas de *Diatraea saccharalis*. Uma vez que a patogenicidade é um mecanismo complexo, estes resultados podem ser atribuídos aos efeitos pleiotrópicos do(s) gene(s) que conferem resistência á luz ultravioleta, ou a um acúmulo de mutações resultantes das sucessivas irradiações sofridas pelas linhagens selvagens para a obtenção dos mutantes.

Palavras-chave: *Beauveria bassiana*, mutantes resistentes a luz UV, patogenicidade, controle biológico

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ISOLATION OF *CAMPYLOBACTER JEJUNI* FROM VISCERA AND BILE SECRETION OF BROILER CHICKENS WITH DIARRHEA

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ABSTRACT

Since chickens are important reservoirs of *Campylobacter jejuni* and their meat is the most frequent route of transmission for human campylobacteriosis, the purpouse of the present study was to investigate the presence of *Campylobacter* in viscera of chickens with diarrhea, evaluating the frequency of isolation of this microorganism from organs considered to be preferential for its isolation. A total of 107 visceral samples from chickens with diarrhea from different farms in the Ribeirão Preto region, SP, were examined for the presence of *Campylobacter jejuni*. The material consisted of 73 livers and 34 spleens, plus 29 bile secretion samples. The frequency of *Campylobacter jejuni* isolation was 54.79% for the liver samples, 35,29% for the spleens and 6,89% for the bile secretion samples. The data suggest that, under the conditions of the present study, the liver may be the organ of choice for the isolation of *Campylobacter* in the presence of diarrhea and liver involvement in chickens.

Key words: *Campylobacter*, viscera, diarrhea, fowl, broiler chickens

INTRODUCTION

Over the last few years, chickens have been considered to be responsible for several outbreaks of food-induced enteritis caused by *Campylobacter* sp. Some investigators (3, 10) have demonstrated the presence of *Campylobacter jejuni* in 80 to 100% of chickens destined to human consumption, whereas others have shown that the agent is quite frequent in the gastrointestinal tracts of chickens sent to the slaughterhouse and can survive the slaughtering and processing routine and still be detected in the viscera of these animals (7, 9, 16).

Campylobacter jejuni is widely distributed in the gastrointestinal tract of domestic and wild fowl as a normal inhabitant (4). However, according to Newell (13) and Luechtefeld and Wang (11), not all fowl present *Campylobacter jejuni* as a normal constituent of their intestinal flora, and some strains present may be pathogenic and cause chicken hepatitis and enteritis (12).

According to Blaser *et al.* (4), in many cases infection by *Campylobacter jejuni* is not accompanied by obvious signs of disease. However, several studied carried out in the 1950's and in the mid-1960's related avian vibrionic hepatitis to a clinical entity that affected adult matrices and laying hens and whose causal agent was *Campylobacter jejuni* (9). Affected chickens may present mortality rates ranging from a few deaths to 100% of the lot. The major lesion occurs in the liver, which becomes discolored and yellowish. Petechiae and diffuse inflammation occur in the intestine, and inflammation occurs in the gallbladder, so that, according to Peckman (14), bile becomes the most appropriate specimen for the isolation of *Campylobacter*.

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In this respect, Bertchinger (2) reported the isolation of *Campylobacter* spp. from the bile of 21% of chickens with necrotizing hepatitis, whereas Acevedo *et al.* (1) isolated the agent from 2 (1.24%) of 161 bile samples studied.

Acevedo *et al.* (1) isolated *Campylobacter coli* from only 1 of 161 viscera studied (0.62%), whereas Franco (8), in Rio de Janeiro, isolated *Campylobacter jejuni* from 100% of the liver samples analyzed. In Portugal, Veloso (16), in a study of 12 viscera ready for consumption, including liver, gizzards and heart, isolated *Campylobacter jejuni* from 7 samples (58.3%) and *Campylobacter coli* from 5 (41.7%).

Christopher *et al.* (7) studied 20 liver and gizzard samples obtained from chickens immediately after evisceration and detected *Campylobacter jejuni* in 85% of the liver samples and in 89% of the gizzard samples.

Chickens are considered to be important reservoirs of *Campylobacter jejuni*, so that their meat, when insufficiently treated by heat or poorly manipulated in terms of hygiene, becomes a frequent cause of human campylobacteriosis, a zoonosis of interest in terms of public health. Thus, the objective of the present study was to determine the presence of *Campylobacter* in the viscera of chickens with diarrhea in order to evaluate the frequency of isolation of this microrganisms from organs of choice for its isolation.

MATERIAL AND METHODS

Samples. A total of 107 samples of viscera and 29 samples of bile secretion were studied. The samples were collected weekly from chickens with diarrhea whose body weight appeared to be below the mean for the lot, corresponding to a total number of 73 animals at the end of the sampling period. Animals were selected one day before the study at different chicken farms in the Ribeirão Preto region, SP, and sacrificed at the time of visceral sampling.

Preparation of samples for the isolation of *Campylobacter*. The chickens were sacrificed with electric current and plucked in the ventral region. The viscera were collected as a whole (liver, spleen and gallbladder) from each animal. However, after evisceration not all sets of viscera remained complete or were suitable for processing. Thus, the samples studied consisted of 73 livers, 34 spleens and 29 gallbladders, which were placed in sterilized plastic bags and carried to the laboratory immediately after collection.

Bile samples were obtained from each gallbladder with a sterilized syringe and one drop was directly inoculated into the culture medium. A portion of the liver (one half) was cut and ground with the aid of sterilized sand and sterile physiological saline, and the spleen was fully ground. The samples were then inoculated into plates with culture medium.

Isolation of *Campylobacter*. The samples were inoculated into the selective medium described by Skirrow (15) using 7% defibrinated sheep blood and the plates were incubated under microaerophilic conditions obtained by a mixture of gases (5% O_2, 10% CO_2 and 85% N_2) for 48 hours at 42°C. Colonies of morphology similar to that of the genus *Campylobacter* were selected for biochemical identification.

Biochemical identification of *Campylobacter*. The morphology of the selected colonies was determined by microscopy and those that were Gram-negative and spiral or curved in shape were selected. Motility was observed by the technique of Bryner and Frank (5) in which microrganisms of the genus *Campylobacter* present characteristic sergipitiform movements. The following tests were performed to characterize *Campylobacter* species: oxidase, catalase, H_2S production, growth in 1% glycine and 3% sodium chloride, sensitivity to nalidixic acid, resistance to cephalothin, and hippurate hydrolysis (6).

RESULTS AND DISCUSSION

The frequency of *Campylobacter jejuni* in the 136 materials analyzed was 39.7% (54 positive samples), 40 (54,79%) liver samples, 12 (35,29%) spleen samples and 2 (6,89%) bile samples were positive for *Campylobacter jejuni* (Table 1)

Table 1 Frequency of *Campylobacter jejuni* isolation from the 136 samples of viscera and bile obtained from chickens with diarrhea

Samples	Samples analysed	isolations	
		nº	%
Bile	29	2	6,89
Spleen	34	12	35,29
Liver	73	40	54,79
Total	136	54	39,70

Although Blaser *et al.* (4) have reported that *Campylobacter jejuni* infection frequently occurs without clear signs of disease, some studies have demonstrated a relationship between infection and vibrionic hepatitis and enteritis in chickens, as well as *Campylobacter jejuni* isolation from different organs, in agreement with the present findings. All chickens studied here presented hemorrhagic petechiae in at least one of the three intestinal portions, most of the times in the first portion, in agreement with the data reported by Peckman (14) who considered hemorrhagic petechiae in the intestine to be characteristic lesions of *Campylobacter* infection in chickens.

When lesions due to *Campylobacter* infection appear, the major damage occurs in the liver, which becomes discolored and shows yellowish zones (a fact observed in many of the samples studied here) although the liver has not been reported to be the organ of choice for isolation of the agent. Although the frequency of *Campylobacter* isolation is higher in liver tham in bile, the last is more often mentioned in scientific reports as the material of choice for the isolation of this agent (14).

The frequency of *Campylobacter jejuni* isolation from liver samples was higher than that reported by Acevedo *et al.* (1), lower than those reported by Franco (8) and Christopher *et al.* (7), but similar to that reported by Veloso (16). We believe that the frequency of isolation observed in the present study may be related to both characteristics of *Campylobacter* infection in birds, i.e., the onset of diarrhea and liver involvement, which increase the chance of isolating the microorganism from the liver.

The gallbladders studied did not present any macroscopic alteration and the frequency of *Campylobacter* isolation from bile was low, i.e., 6.9% (2 samples), although higher than that reported by Acevedo *et al.* (1.24%) (1).

However, Bertchinger (2) recovered *Campylobacter* spp. from 21% of the bile samples obtained from chickens with necrotizing hepatitis. With respect to the spleen, we found no reports in the literature about involvement of this organ, a fact that prevents a comparative discussion of our data. Although the studies cited in the present report did not mention the conditions of the liver or gallbladder from which the agents were isolated, the present results suggest that the liver, especially in cases of involvement of the organ, is the best source for the isolation of *Campylobacter* spp.

RESUMO

Isolamento de *Campylobacter jejuni* em vísceras e secreção biliar de frangos de exploração comercial com diarréia

Sendo as aves consideradas importantes reservatórios do *Campylobacter jejuni*, e sua carne a mais frequente via de transmissão da campilobacteriose humana, objetivou-se no presente trabalho pesquisar a presença deste agente em vísceras de aves com diarréia, avaliando-se a frequência de isolamento deste microrganismo nos órgãos considerados como de eleição para esta finalidade. Desta maneira, foram estudadas 107 amostras de vísceras de frangos com diarréia procedentes de granjas da região de Ribeirão Preto-SP, representadas por 73 fígados e 34 baços, além de 29 amostras de secreção biliar. A frequência de isolamento do *Campylobacter jejuni* foi de 54,79%, para as amostras de fígado, 35,29% para as de baço e 6,89% para as de vesícula biliar. Os dados sugerem, nas condições do presente estudo, a possibilidade de ser o fígado o órgão de eleição para o isolamento de *Campylobacter*, quando associado a quadros diarréicos e comprometimento hepático em aves.

Palavras-chave: *Campylobacter*, víscera, diarréia, frangos, aves

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OPTIMIZATION OF SOME ENVIRONMENTAL CONDITIONS TO ENHANCE GASOLINE BIODEGRADATION IN SOIL MICROCOSMS BIOAUGMENTED WITH *PSEUDOMONAS PUTIDA*

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ABSTRACT

The contamination of soil and groundwater by gasoline as a result of accidental spills and leaking of underground storage tanks is a serious environmental problem. The *in situ* bioremediation is a promising technology for cleaning these toxic gasoline constituents up and involves the application of biological activity, inorganic nutrients and oxygen to enhance the in place gasoline degradation. The aim of this study was to optimize some environmental conditions in batches of non sterilized soil microcosms contaminated with gasoline and inoculated with *Pseudomonas putida* (ATCC 12633). The gasoline degradation was evaluated by measuring some toxic constituents such as toluene, ethylbenzene, nonane, undecane, dodecane and tridecane, by gas chromatography. The best degradation results of some gasoline components (toluene - 13.2%, ethylbenzene - 41.8%, n-nonane - 59.2%, n-undecane - 53.7%, n-dodecane - 24.9% and n-tridecane - 8,7%) were reached using NH_4NO_3 and H_2O_2 in concentrations of 30 μmol/g of soil and 0.1 mM, respectively. A lack of significant results using phosphorus as an inorganic nutrient source was considered a result of the inherent fertility of the soil used.

Key words: bioremediation, biodegradation, *Pseudomonas putida*, gasoline.

INTRODUCTION

Several environmental problems can be caused by leaking of gasoline underground storage tanks. Besides the eminent explosion risk, gasoline can percolate the soil and reach the groundwater, compromising the water quality and becoming a public health problem (7,5).

A portion of gasoline remains trapped in the soil, specially the hydrophobic aromatic fraction, making the physical extraction difficult. These compounds can dessorb slowly and reach the water table, providing a considerable long term contamination (11).

Some gasoline constituents, specially the aromatic fractions as benzene, toluene, ethylbenzene and xylene, can represent a serious risk for human health because they are considered carcinogens. Their concentrations in water table must be controlled (8).

Several methods can be employed to remove gasoline from the soil and from the groundwater. The biological treatments comprise processes which use microorganisms to degrade or decompose organic compounds considered toxic or pollutant (6).

There are in nature, in soil in particular, a great variety of microorganisms that have the capacity to degrade partially or totally the gasoline hydrocarbon constituents (2). The microbial community can interact, supplying proper conditions to the environmental establishment after a disturbance caused by an accidental leakage.

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After an accidental spill of gasoline into the soil, the native microflora is ready to adjust the changes trying to recompose the environment. This adjustment, however, can be very slow and sometimes an optimization of the environmental conditions to accelerate the process can be necessary. The compensation of some inorganic nutrients as nitrogen and phosphorus, important to the cellular metabolism, and proper oxygen supply, stimulating the aerobic degradation, are essential conditions for the bioremediation application (1,3).

The aim of this work was to optimize some environmental conditions such as inorganic nutrients and oxygen, to enhance gasoline biodegradation in soil microcosms bioaugmented with *Pseudomonas putida* ATCC 12633.

MATERIALS AND METHODS

Gasoline. The gasoline was obtained from PETROBRÁS (Petróleo Brasileiro S.A.) gas station (Rio de Janeiro, Brazil). Its characteristics, under the described test conditions, were preserved and confirmed by regular chromatographic analysis.

Soil. The soil utilized in this work was collected to a depth of 30 cm. The quantity was sufficient to storage for a maximum period of a month (10). The soil was sieved through screens with 2 mm diameter openings to remove stones, rot materials and other debris. The water holding capacity was evaluated as suggested by Watwood *et al.* (14). Table 1 summarizes the soil characteristics performed by CNPS (Centro de Pesquisas de Solos) - EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária).

Microorganism. *Pseudomonas putida* (ATCC 12633).

Media and growth conditions

Inoculant production. The inoculum was prepared using *P. putida* cells transferred from the storage culture to a test tube containing 10 ml of organic medium. This medium consisted of 3 g of beef extract and 5 g of peptone in 1 liter of distilled water. The pH was adjusted to 7.0 with NaOH 1 N. After incubation at 30°C for 14 h, the inoculum was propagated to a 500 ml flask containing 100 ml of the same organic medium, and also incubated at 30°C, with shaking (150 rpm) for 2 hours (mid-log phase).

Environmental conditions optimization. The tests were realized in 50 ml flasks containing 5 g dry weight of unsterile soil. A total of 10^6 cells was added per gram of soil. The gasoline concentration was 5% (V/W) and the nutrients and oxygen fonts analyzed were:

Nitrogen source. The fonts of nitrogen used were ammonium nitrate, in four concentrations (15, 30, 60 and 120 $\mu mol.g^{-1}$ of soil) and ammonium sulfate, in the same nitrogen concentrations.

Phosphorus source. The font of phosphorus utilized was (K_2HPO_4) in the following concentrations: 1.25, 2.5, 5.0 and 10.0 $\mu mol.g^{-1}$ of soil.

Oxygen source. To supply sufficient electron acceptor to the biodegradation process, hydrogen peroxide (H_2O_2) was used in three different concentrations: 0.1, 1.0 and 10.0 mM with perhidrol 32,7%.

In all tests, the water content was adjusted to 100% of the water holding capacity, considering the inoculum volume and nutrients added. The vials were crimp-sealed with rubber cap and aluminum seal and incubated for 72 h at 30°C. For viable cell counts, the pour plate method was used. The numbers of colonies were counted after 24 hours of incubation at 30°C. The gasoline degradation was measured at the end of the experiments by gas chromatography. To differentiate evaporative losses from biodegaradative ones, untreated controls without inoculation were performed. The results of all degradative tests realized were calculated in relation to the untreated controls, non inoculated, containing sterile soil and gasoline, at the same described conditions (4).

All tests were performed in replicates. The evaluated concentrations were obtained from searched literature.

Associated optimization. The tests were conducted using optimum concentration defined for nitrogen font in optimization tests. The objective was to evaluate some fluctuation in the phosphorus and oxygen optimization in the presence of nitrogen. The font of phosphorus utilized was K_2HPO_4 in the following concentrations: 2.5 and 5.0 $\mu mol.g^{-1}$ of soil. The oxygen font was hydrogen peroxide in two different concentrations: 0.1 and 1.0 mM, both in the presence of ammonium nitrate in the concentration of

Table 1. Characteristics of the soil

Chemical analysis		Texture (%)	
pH	6.80	Sand	74
Carbon (%)	0.82	Silt	16
Total Nitrogen (%)	0.07	Clay	10
Available Phosphorus (ppm)	93.00		

30 μmol.g^{-1} of soil. The procedure was the same as the optimization tests.

Gasoline quantification. The gasoline was extracted from soil with CS_2 (carbon disulfide) in the following proportion: 3 ml of CS_2/ 5 g of soil, maintaining 24 h as a contact time at 10°C. The extracts were analyzed by gas chromatography using an instrument (model 37-D; Instrumentos Científicos CG Ltda) equipped with a flame-ionization detector (FID) and a 25 m x 0.25 mm (Diameter) fused-silica capillary column using an immobilized (OV-101) phase. The hydrogen carrier flow rate was 30 ml. min^{-1} (4). The gasoline components analyzed were toluene, ethylbenzene, n-nonane, n-undecane, n-dodecane and n-tridecane.

RESULTS AND DISCUSSION

The microorganisms need for survival and growth adequate environmental conditions. Their unfeasibility can be related with the lack of essential metabolic factors. The introduction of organic matter (fuel) to the soil can cause an unbalance in the nitrogen, phosphorus and oxygen availability. To accelerate the biodegradation process, nutrients must be added in sufficient amount to balance the organic matter available (5). The degradation results of some gasoline components utilizing NH_4NO_3 as nitrogen source are shown in Table 2. It is possible to note that the best values of n-C_{11} degradation was reached using the concentrations of 30 and 60 μmol.g^{-1} of soil. Considering that the difference was not significant for both concentrations and that the cellular growth was only evidenced in 30 μmol.g^{-1} of soil concentration, the latter was selected as optimum.

According to Song *et al.* (12), the aliphatic components are more easily biodegraded than aromatics. The C_{10} to C_{11} constituents of gasoline, under the conditions of these experiments (presence of NH_4^+ and PO_4), were more rapidly biodegraded, agreeing with the results in this work.

The degradation values obtained with ammonium sulfate utilization (Table 3) were not significant for all concentrations tested. and were smaller than that obtained with ammonium nitrate (Table 2). It's probable that ammonium nitrate can be used as a nitrogen source as well as a final electron acceptor.

It is possible to conclude that ammonium nitrate was the best font of nitrogen and the optimum concentration was 30 μmol.g^{-1} of soil for the biodegradation experiments.

The Table 4 shows the degradation values and cellular growth obtained with phosphorus utilization as K_2HPO_4. For all phosphorus concentrations tested, the degradation values were not significant compared to that one obtained in ammonium nitrate optimization. The cellular growth for all concentrations was not observed either. Considering

Table 2. Biodegradation rate of some gasoline constituents and cellular growth utilizing NH_4NO_3 as nitrogen source.

NH_4NO_3 (μmol/g of soil)	DEGRADATION RATE(%)						CFU/g of soil (0 h)	CFU/g of soil (72 h)
	TOL	ETB	n-C_9	n-C_{11}	n-C_{12}	n-C_{13}		
15	3,9	1,8	3,9	8,6	12,1	17,9	9,9X10^5	4.0 X10^5
30	0,0	0,0	0,0	77,5	7,5	7,1	9,9X10^5	5,0 X10^6
60	0,0	0,0	0,0	78,4	8,7	10,4	9,9X10^5	2.7 X10^5
120	0,0	0,0	11,0	0,0	14,6	22,3	9,9X10^5	4,8 X10^5

gasoline constituents: TOL - toluene, ETB - ethylbenzene, n-C_9 - n-nonane, n-C_{11} - n-undecane, n-C_{12} - n-dodecane, n-C_{13} - n-tridecane

Table 3. Biodegradation rate of some gasoline constituents and cellular growth utilizing $(NH_4)_2SO_4$ as nitrogen source.

$(NH_4)_2SO_4$ (μmol/g of soil)	DEGRADATION RATE(%)						CFU/g of soil (0 h)	CFU/g of soil (72 h)
	TOL	ETB	n-C_9	n-C_{11}	n-C_{12}	n-C_{13}		
15	0,0	0,0	0,0	0,0	0,0	0,0	9,3 x10^5	1,4 x10^6
30	0,0	2,2	4,4	0,4	6,3	0,0	9,3 x10^5	1,7 x10^5
60	0,0	1,9	6,0	3,0	7,0	4,2	9,3 x10^5	2,1 x105
120	17,2	12,0	15,7	10,4	14,5	11,8	9,3 x10^5	2,7 x10^5

gasoline constituents: TOL - toluene, ETB - ethylbenzene, n-C_9 - n-nonane, n-C_{11} - n-undecane, n-C_{12} - n-dodecane, n-C_{13} - n-tridecane

Table 4. Biodegradation rate of some gasoline constituents and cellular growth utilizing K_2HPO_4 as phosphorous source.

K_2HPO_4 (μmol/g of soil)	DEGRADATION RATE(%)						CFU/g of soil (0 h)	CFU/g of soil (72 h)
	TOL	ETB	n-C_9	n-C_{11}	n-C_{12}	n-C_{13}		
1.25	6.2	0.0	1.1	9.8	9.2	12.6	1.4×10^6	2.2×10^5
2.5	6.8	1.2	0.0	0.0	0.0	0.0	1.4×10^6	2.5×10^6
5	17.2	4.2	1.3	2.7	0.0	2.0	1.4×10^6	5.7×10^5
10	13.4	6.0	1.3	0.0	0.0	1.0	1.4×10^6	4.2×10^5

gasoline constituents: TOL - toluene, ETB - ethylbenzene, n-C_9 - n-nonane, n-C_{11} - n-undecane, n-C_{12} - n-dodecane, n-C_{13} - n-tridecane

Table 5. Biodegradation rate of some gasoline constituents and cellular growth utilizing H_2O_2 as oxygen source.

H_2O_2 (μmol/g of soil)	DEGRADATION RATE(%)						CFU/g of soil (0 h)	CFU/g of soil (72 h)
	TOL	ETB	n-C_9	n-C_{11}	n-C_{12}	n-C_{13}		
0.1	28.0	18.1	7.9	41.3	9.0	13.0	8.2×10^5	1.5×10^6
1	33.5	4.4	0.0	41.8	9.6	7.4	8.2×10^5	1.2×106
10	13.4	9.6	4.6	47.0	15.7	18.5	8.2×10^5	9.9×10^5

gasoline constituents: TOL - toluene, ETB - ethylbenzene, n-C_9 - n-nonane, n-C_{11} - n-undecane, n-C_{12} - n-dodecane, n-C_{13} - n-tridecane

the high rate of phosphorus in this soil (Table 1), it is possible to suppose that the addition of K_2HPO_4, even in the smallest concentration tested, has caused an inhibitory effect and so it was not selected for the future experiments.

According to Odu (9), the lack of significant degradation results with the utilization of 1% of crude oil, without inorganic nutrients utilization, is related with the inherent fertility of the utilized soil.

Oxygen is the more critical metabolic factor in a biodegradation process. It is utilized in bioremediation of polluted subsuperficial environments as a final electron acceptor in the microbial metabolism. It is also utilized as molecular oxygen in many degradation reactions of aliphatic and aromatic hydrocarbons (13).

In relation to the degradation results (Table 5) obtained with the utilization of hydrogen peroxide as oxygen font using three concentrations tested (0.1, 1.0 and 10.0 mM), it is possible to verify that H_2O_2, in the concentration of 10 mM, showed the highest degradation values of undecane, dodecane and tridecane, while for the smallest one were detected considerable values for all gasoline constituents tested.

Although the values obtained with the highest H_2O_2 concentration (10 mM) had been slightly superior to others, the smallest one (0.1 mM) showed highest total percentage of toluene and ethylbenzene degradation. These gasoline constituents are considered to be carcinogens and so the highest toxicity content. Furthermore, 0.1 mM concentration showed the highest values of cellular growth in 72 hours compared to other concentrations. Considering also the high application cost of hydrogen peroxide in *in-situ* bioremediation treatments, the choice of a concentration hundred times bigger would not be appropriate. So, the 0.1 mM concentration was selected for the biodegradation tests.

The results obtained with phosphorus optimization as K_2HPO_4 in the presence of ammonium nitrate at a concentration of 30 $\mu mol.g^{-1}$ of soil are shown in Table 6. The degradation values obtained were not significant for all analyzed components in the concentrations tested.

A light inhibition can be observed with the utilization of K_2HPO_4 and NH_4NO_3 in the concentrations of 5 and 30 $\mu mol.g^{-1}$ of soil. The optimization of nitrogen as ammonium nitrate (Table 2) showed a superior total degradation percentage than that obtained in the presence of phosphorus (Table 6). It can be justified by the high rate of phosphorus in soil (Table 1).

The results obtained from the associated optimization of nitrogen and oxygen showed that 0.1 mM hydrogen peroxide concentration had the best degradation values for all gasoline components analyzed. It did not occur with nitrogen optimization on his own (Table 2). The microbial growth value (Table 6) confirmed a stimulus caused by the presence

Table 6. Biodegradation rate of some gasoline constituents and cellular growth utilizing associated optimization of phosphorous and oxygen.

NH_4NO_3 (μmol/g of soil)	K_2HPO_4 (μmol/g of soil)	H_2O_2 (mM)	DEGRADATION RATE(%)						CFU/g of soil (0 h)	CFU/g of soil (72 h)
			TOL	ETB	n-C_9	n-C_{11}	n-C_{12}	n-C_{13}		
30	**2.5**	-	8.8	6.4	0.0	0.0	0.0	0.0	1.2×10^6	1.9×10^6
30	5.0	-	8.7	0.0	0.0	2.1	8.4	0.0	1.2×10^6	2.3×10^5
30	-	0.1	13.2	41.8	59.2	53.7	24.9	8.7	7.0×10^5	1.3×10^7
30	-	1.0	7.0	8.6	7.2	0.0	0.0	0.0	7.0×10^5	7.0×10^5

gasoline constituents: TOL - toluene, ETB - ethylbenzene, n-C_9 - n-nonane, n-C_{11} - n-undecane, n-C_{12} - n-dodecane, n-C_{13} - n-tridecane

of nitrogen and oxygen in the mentioned concentration.

The results show that NH_4NO_3 in the concentration of 30 μmol per gram of soil and H_2O_2 in the concentration of 0.1 mM can be considered important environmental factors to the bioremediation of this soil contaminated by gasoline (5% V/W) and utilizing inoculum of *P.putida.*

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RESUMO

Otimização de algumas condições ambientais para aumentar a biodegradação de gasolina em microcosmos constituídos de solo bioaumentados com *Pseudomonas putida*

A contaminação do solo e águas subterrâneas com gasolina em conseqüência de derramamentos acidentais e vazamentos de tanques subterrâneos de estocagem acarreta sérios problemas ambientais. A biorremediação *in situ* é uma tecnologia promissora para remoção dos constituintes tóxicos da gasolina envolvendo a ação biológica, acompanhada de condições nutricionais inorgânicas e tensão de oxigênio adequados para melhorar a biodegradação de gasolina no local. O objetivo deste trabalho foi a otimização destes nutrientes e do oxigênio em microcosmos constituídos de solo não esterilizado, contaminado com gasolina e inoculado com *Pseudomonas putida* (ATCC 12633). A degradação da gasolina foi avaliada medindo-se alguns constituintes tais como tolueno, etilbenzeno, n-nonano e n-tridecano por cromatografia gasosa. As melhores taxas de degradação de alguns componentes da gasolina foram alcançadas utilizando NH_4NO_3 e H_2O_2 nas concentrações de 30 μmol/g de solo e 0,1 mM, respectivamente. A falta de resultados significativos utilizando fósforo como fonte de nutriente inorgânico pode ser considerada como resultado da fertilidade inerente ao solo utilizado.

Palavras-chave: biorremediação, biodegradação, *Pseudomonas putida*, gasolina.

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ERRATUM

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01- pg. 23-24

Utilization of *spirillum volutans* for monitoring the toxicity of effluents of a cellulose and paper industry

Pedro Magalhães Lacava* and Marcos Rogério Ortolono

1 - The correct name of the second author is **Marcos Rogério Ortolano**

2 - Include Table 1 as follows:

Table 1. Results of COD, BOD and MEC_{90}, 30 min in effluents from a cellulose and paper industry

Effluent	COD (Kg/day)	BOD (Kg/day)	MEC_{90} 30min sampling n° 1	MEC_{90} 30min sampling n° 2
A	414	104	90%	95%
B	30.004	13.976	1%	1%
C	4.809	1.677	10%	10%
D	428	231	10%	4%
E	5.810	3.088	90%	20%
F	146	60	95%	10%
G	nd	nd	nt	nt
H	32	14	0.8%	0.8%
I	3.838	1.412	1%	2%
J	47.328	20.269	10%	20%
K	11.832	2.027	nt	nt

(**A**)-washing and wood preparing; (**B**)-chlorination; (**C**)-oxidative extraction; (**D**)-hypochlorination; (**E**)-effluent from machinery; (**F**)-liquid of condensed gases from evaporation step; (**G**)-gases water washing; (**H**)-sludge pond-1; (**I**)-sludge pond-2; (**J**)-primary decanter (initial treatment); (**K**)-supernatant effluent from biological treatment basin-2 (final effluent); (**nt**)-non toxic; (**nd**)-non determined.

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

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

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

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

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