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EXTREMELY THERMOPHILIC MICROORGANISMS AND THEIR POLYMER-HYDROLYTIC ENZYMES

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MINI-REVIEW

ABSTRACT

Thermophilic and hyperthermophilic microorganisms are found as normal inhabitants of continental and submarine volcanic areas, geothermally heated sea-sediments and hydrothermal vents and thus are considered extremophiles. Several present or potential applications of extremophilic enzymes are reviewed, especially polymer-hydrolysing enzymes, such as amylolytic and hemicellulolytic enzymes. The purpose of this review is to present the range of morphological and metabolic features among those microorganisms growing from 70°C to 100°C and to indicate potential opportunities for useful applications derived from these features.

Key words: Archaea, extremophiles, amylases, xylanases, pullulanases, thermostability

INTRODUCTION

In recent years it became obvious that extremophilic microorganisms differ from eucaryotic cells because they have adapted to grow under extreme conditions such as high temperature (>100°C), high salinity (saturated NaCl), extremes of pH (<2.0, >10.0), and substrate stress. These kinds of extreme microbial growth conditions are found in exotic environments which were more widespread on primitive Earth. Extreme environments include also high pressure (> 50 MPa) and the presence of organic solvents (e.g. > 1% toluene) or heavy metals.

The evolution and taxonomy of extremophiles, especially the thermophiles, is an area that is receiving increasing attention. In general, moderate thermophiles are primarily bacteria and display optimal growth temperature between 60°C and 80°C. Hyperthermophiles are primarily archaea and growth optimally at 80°C or above, being unable to grow below 60°C (47).

The hyperthermophiles are now well characterised taxonomically at the DNA-DNA hybridisation level, and their evolutionary relatedness has been examined. By using 16S rRNA sequence comparison, an archaeal phylogenetic tree has been proposed (54), with a tripartite division of the living world consisting of the domains Eucarya, Bacteria, and Archaea. In this

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division *Sulfolobales* and *Thermoproteales* form one branch (the Crenarchaeota) and the remaining thermophiles form another branch containing methanogens, and extreme halophiles (the Euryarchaeota). Currently, the only hyperthermophilic organisms within the Bacterial domain are members of the genus *Thermotoga* and *Aquifex* (47). Until now, no hyperthermophilic microorganisms in the domain Eucarya have been reported.

Hyperthermophiles are represented at the deepest and shortest lineages, including both genera of hyperthermophilic bacteria and the genus *Pyrodictium*, *Pyrobaculum*, *Desulfurococcus*, *Sulfolobus*, *Methanopyrus*, *Thermococcus*, *Methanothermus*, *Archaeoglobus* within the Archaea. Recently, genetic elements, e.g. viruses and plasmids (excluding IS elements and transposons) have been described in the kingdom Crenarcheota (*Thermoproteales* and *Sulfolobales*) and in the kingdom Euryarchaeota (*Thermococcales* and *Thermoplasmales*) of the archaeal domain (57). Some similarities between the archaeal virus FH and the bacterial phage P1 strongly indicate that this temperate phage type already existed before the separation of the Archaea from the Bacteria, which was the first documented lineage diversion in cellular evolution (57). Based on these observations, hyperthermophiles may still be rather primitive and the last common ancestor, the progenota, may have been a hyperthermophile (47, 54).

In the last decades thermophilic and hyperthermophilic anaerobes have been isolated from continental and submarine volcanic areas, such as solfatar fields, geothermal power plants, geothermally heated sea sediments and hydrothermal vents (14, 18, 47, 50). Sites from which hyperthermophilic organisms have been isolated comprises solfataric fields; steam-heated soils, mud holes, surface waters; deep hot springs; geothermal power plants as well as submarine hot springs and fumaroles; hot sediments and vents,"black smokers" or "chimneys"; and active sea-mounts.

It is interesting that some organisms have been isolated from areas with temperatures much higher than their maximum growth temperature, e.g., *Hyperthermus butilicus* (56) and *Pyrococcus abyssi* (18), which suggests that in these environments the organisms may not be actively growing. The same could be true for the organisms isolated from temperatures much below their growth temperature optimum, such as *Archaeoglobus profundus* (7).

Thermophiles and hyperthermophiles: physiological and morphological aspects. Most of the anaerobic thermophilic bacteria are chemoorganotrophic in their metabolism. The bacterial thermophilic thermoanaerobes, for example, belong to nearly the same range of nutritional categories as do mesophilic bacteria. The hyperthermophilic bacteria *Thermotoga* are able to ferment various carbohydrates like glucose, starch and xylans, forming acetate, L-lactate, H_2 and CO_2 as end product (47), while the hyperthermophilic *Aquifex* is strictly chemolithoautotrophic, using molecular hydrogen, thiosulfate and elemental sulphur as electron donors and oxygen (at low concentrations) and nitrate as electron acceptors (22).

In general, the physiological processes for adaptation to environmental stress in anaerobic bacteria seem to have involved different factors from those in aerobic bacteria. First, anaerobes are energy limited during the chemoorganotrophic growth because they can not couple dehydrogenation reaction to oxygen reduction and gain a high level of chemical free energy. Second, growth of most chemoorganotrophic anaerobes (except for methanogens) is naturally associated with the generation of toxic end products (e.g., organic acids or alcohol's, HS^-), which requires that anaerobic species develop some sort of dynamic adaptation mechanism or tolerance to their catabolic end products.

The most interesting group of thermophiles is the hyperthermophiles, since the isolation of these organisms has caused a revaluation of the possible habitats for microorganisms and has increased the high-temperature limits at which life is known to exist. The hyperthermophilic anaerobic archaea have almost the same size as one typical procaryotic cell, about 0.5 - 2.0μm, although some of them have unusual morphological features (47). Hyperthermophiles are rather diverse with respect to their metabolism, since they include methanogens, sulphate-reducers, nitrate-reducers and also the aerobic respirers. However the majority of the species know at the present are strictly anaerobic heterotrophic S^0 reducers (24). Among the terrestrial Archaea, three groups can be distinguished. Acidophilic extremethermophiles, which are found exclusively within continental solfataric fields. The organisms are coccoid-shaped, strict and facultative aerobes, and require acidic pH (opt. approx. pH 3.0) to grow. Phylogenetically, they belong to the archaeal

genera *Sulfolobus*, *Metallosphaera*, *Acidianus*, and *Desulfurolobus* (47). On the other hand, the slightly acidophilic and neutrophilic thermophiles are found both in continental solfataric fields and in submarine hydrothermal systems. All of them are strict anaerobes. Solfataric fields contain members of the genera *Thermoproteus*, *Pyrobaculum*, *Thermophilum*, *Desulfurococcus*, and *Methanothermus*. *Pyrobaculum islandicum* is able to grow autotrophically by anaerobic reduction of S^0 with H_2 as electron donor (35), but is also able to grow heterotrophically by sulphur respiration (47). Strains of *Thermophilum* and *Pyrobaculum organotrophum* are obligate heterotrophs. They grow by sulphur respiration using different organic substrates. Interestingly, *Thermophilum pendens* shows an obligate requirement for a lipid fraction of *Thermoproteux tenax* (117).

The variety of hyperthermophilic archaea that are adapted to the marine environment is represented by the crenarchaeal genera *Archaeoglobus*, *Pyrodictium*, *Thermodiscus*, *Staphylothermus*, *Hyperthermus*, *Methanopyrus*, *Pyrococcus*, *Thermococcus*, and some members of *Methanococcus*. From these organisms, Optimum growth temperatures range from 75° to 105°C, and the maximum temperature of growth can be as high as 113°C (*Pyrobolus*) or even up to 110°C (*Pyrodictium occultum).* They are so well adapted to high temperatures that they are unable to grow below 80°C (47).

Like all Archaea, Crenarchaeota are prokaryotic, and are bounded by ether-linked lipid membranes which contain isoprinoid side chains instead of fatty acids. Cells range in size from cocci <1μm in diameter to filaments over 100μm in length. Species display a wide range of cell shapes, including regular cocci clustered in grape-like aggregates (*Staphylothermus*), irregular, lobed cells (*Sulfolobus*), discs (*Thermodiscus*), very thin filaments (<0.5μm diameter; *Thermofilum*), and almost rectangular rods (*Thermoproteus*, *Pyrobaculum*). Most species possess flagella and are motile. A few members of the Crenarchaeota exhibit strange morphologies: *Pyrodictium occultum* and *Pyrodictium brockii* grow as a mold-like layer on sulphur, and have uncommon cells, which are irregularly disc shaped and dish shaped, with granules of sulphur frequently seen sticking to the fibbers, whose production may confer an adaptation advantage to the organism in trapping nutrients. The cells are connected by a network of ultra thin hollow tubules (47). Strains of *Pyrodictium* are usually chemolitoautotrophs gaining energy by reduction of S^0 by H_2. Although growth is stimulated by yeast extract, both species of *Pyrodictium* are strictly dependent upon H_2.

As an exception, *Pyrodictium abyssi* is a heterotroph growing by fermentation of peptides and is unable to grow chemolitotrophically on H_2/CO_2 either in the presence of S^0 or $S_2O_3^{-2}$. Similar to the other members of the genus, the cells of *Pyrodictium abyssi* are highly polymorphous, often disk-shaped, and display ultra flat areas. The cell envelope consists of the cytoplasmic membrane, a periplasmic space, and a surface layer protein. The ultra thin sections also reveal a zigzag structure of the S-layer (40). Usually S-layer proteins are highly stable, maintain the structural integrity of bacterial cells under extreme environmental conditions, and resist dissociation by high temperature, chemical treatment, or mechanical disruption (32). The existence of such a coat suggests an adaptative mechanism to the extreme environment in which these organisms live and could have a barrier function against both external and internal factors, that would affect the stability of the cells.

All of the hyperthermophilic heterotrophs can use complex peptide mixtures, like peptone, tryptone, or yeast extract, as carbon and energy source. Relatively few hyperthermophiles are, however, saccharolytic. Nevertheless, this number is increasing steadily, especially because several species that were originally described as growing solely on peptides, recently were shown to grow also on carbohydrates (24).

Biotechnological features of thermophiles and hyperthermophiles. In addition to the heterotrophic extremophiles, many autotrophic hyperthermophiles are able to grow by fermentation or respiration of organic matter too, and are, therefore opportunistic heterotrophs. They are able to synthesise heat stable molecules, including enzymes. The current biotechnological interest in enzymes from these microorganisms is motivated by their ability to work under conditions that are normally denaturing for mesophilic enzymes. Particular attention has been focused on enzymes from extremely thermophilic archaea (1, 30). A wide range of enzymes from hyperthermophilic archaea, both intracellular and extracellular, has been investigated and data on isolation, purification and

structural/functional characterization have been presented (Tables 1 and 2).

Whereas conventional enzymes are irreversibly inactivated by heat, the enzymes from these extremophiles show not only great thermostability, but also enhanced activity in the presence of common protein denaturants such as detergents, organic solvents and proteolytic enzymes (26, 30). Enzymes from thermophilic and extreme thermophilic microorganisms have received

Table 1. Hydrolases, Polymerases and Isomerases from thermophilic and extremely thermophilic Archaea belonging to the *Sulfolobales*, *Termoproteales*, *Methanopyrales* and *Pyrodictiales* groups, which have been isolated and/or investigated in some detail.

Species	T-opt. [°C]	Habitat	Enzyme	References
Sulfolobales				
Sulfolobus acidocaldarius	75	t	DNA polymerase	(17)
			Proteinase	(31)
			RNA polymerase	(3)
			Topoisomerase I	(20)
			Topoisomerase II	(34)
Sulfolobus solfataricus	80	t	DNA polymerase	(37)
			s-Adenosyl-homocysteine-hydrolase	(38)
Thermoproteales				
Thermoproteus tenax	88	t	RNA polymerase	(3)
Pyrobaculum aerophilum	100	m	Proteinase	(51)
Methanopyrales				
Methanopyrus kandleri	98	m	Topoisomerase type I	(46)
Pyrodictiales				
Pyrodictium occultum	105	m	DNA polymerase	(50)

m, marine; t, terrestrial

Table 2: Hydrolases, Polymerases and Isomerases from thermophilic and extremely thermophilic Archaea belonging to the *Thermococcales*, *Desulfurococcales* and *Thermoplasmales* groups, which have been isolated and/or investigated in some detail.

Species	T-opt. [°C]	Habitat	Enzyme	References
Thermococcales				
Pyrococcus furiosus	100	m	Proteinase(14)	
			DNA-polymerase	(50)
Pyrococcus woesei	100	m	Proteinase	(25)
Thermococcus stetteri	75	m	Proteinase	(26)
Thermococcus AN1	75-80	t	Proteinase	(25)
Thermococcus celer	87	m	Proteinase	(25)
Thermococcus litoralis	88	m	Proteinase	(25)
Desulfurococcales				
Desulfurococcus amylolyticus	90	t	Topoisomerase type I	(46)
			Proteinase	(13)
Desulfurococcus mucosus	88	t	RNA polymerase	(3)
Staphylothermus marinus	92	m	Proteinase	(25)
Thermoplasmales				
Thermoplasma acidophilum	55-60	T	DNA polymerase	(3)
			RNA polymerase	(3)
			Topoisomerase	(20)

m, marine; t, terrestrial

considerable attention from industry, because of their special characteristics such as high stability to changes in pH. Reasons for targeting these enzymes include their suitability as models for investigating protein thermostability and their potential as biocatalysts in modern biotechnology. Thus, these molecules have considerable industrial potentialities, giving better yields under extreme operational conditions.

For instance, the proteolytic archaea *Thermococcus litoralis* and *Thermococcus celer* showed good growth on starch. Also species belonging to the *Desulfurococcales* (*D. mucosus* and *D. mobilis*) which were thought to use only peptides, were found to grow on starch (8). Moreover, some species (*Thermophilum pendens*) were found to produce amylase or glucosidase, due their potential for growth on carbohydrates (4).

Hyperthermophiles, that are saccharolytic, either perform a complex oxidation to CO_2, and energy is gained from aerobic respiration or anaerobic S^0-respiration (*Sulfolobales*, *Archaeoglobales*, *Thermoproteales*), or they exhibit a fermentative metabolism, leading to acetate, alanine or lactate as predominant products, in addition to H_2 and CO_2 (members of the *Thermococcales*, the *Pyrodictiales*, the *Desulfurococcales* and the eubacterial *Thermotogales*). The latter incomplete oxidisers are mostly facultatively S^0-dependent, S^0 being used as a sink for reductant (24). However, the exact type of metabolism is often difficult to judge because of the limited information that is available on the end products formed. Beside *Pyrococcus furiosus* (88), *Sulfolobus* species (24), *Thermoproteus tenax* (91) and *Thermotoga maritima* (24), few organisms have been investigated in more detail. Therefore, little is know on the metabolism of a number of carbohydrate utilising hyperthermophiles.

Biopolymer degradation at high temperatures. Polysaccharides must be initially hydrolysed prior to transport into the periplasmic space, because of the size of substrate which can be transported into the cell is severely restricted; The size limit in most cases is a molecular weight (MW) of ~600 kDa (112). Extracellular enzymes are hence necessary for the degradation of macromolecules like cellulose, hemicellulose (xylan), pectin, pullulan and starch. In addition, polysaccharides have tertiary structures (ribbons, loops, coils), which may aid or impede enzymatic access to hydrolytic sites.

Enzymes from thermophiles and extremethermophiles can replace their mesophilic counterparts in different industrial processes and thereby reduce the need for cooling. For instance, a variety of industries employ microbial amylolytic enzymes in the enzymic conversion of starch into different sugar solutions, representing an important growth area of industrial enzyme usage. The bioprocessing of starch into malto-oligosaccharides is gaining importance because of their potential uses in food, pharmaceutical and fine chemical industry (50). A high value is placed on thermostable and thermoactive amylases in these processes, since the bioprocessing of starch at elevated temperature improves the solubility of starch, decreases its viscosity, limits microbial contamination, and reduces reaction times. Another hydrolytic enzyme, pullulanase, is used in combination with saccharifying amylases for the improved production of various sugar syrups (15). In addition, pullulanase has gained significant attention as a tool for structural studies of carbohydrates.

An additional application for themophilic enzymes is the development of new processes to reduce the release of environmentally harmful chemicals by replacement of existing chemical reactions with enzymatic reactions. A good example can be found in the paper-pulping industry. Kraft pulping, a process widely used in paper manufacture, removes about 95% of the lignin by alkaline sulphate cooking. The remaining lignin gives the pulp a brown colour which is removed in a multistage bleaching process with a variety of agents (35). Currently, there is concern about the environmental impact of some of the compounds used in the process, particularly chlorine and chlorine dioxide. The traditional chemical bleaching of paper pulp can be reduced, however, by introducing a biobleaching step using thermostable xylan-degrading enzymes from thermophilic organisms (35). By adding thermostable xylanases to the unbleached pulp it is possible to remove parts of the lignin by hydro lysing the bonds that link the lignin, via xylan, to the cellulose fibbers. The use of hemicellulases in bleaching is considered as one of the most important, new large scale industrial applications of enzymes (35). Indeed the mesophilic enzymes currently in use have limitations because of the high temperatures used in bleaching.The current prices of the enzymatic treatment, therefore, are expected to decrease as more efficient production strains and technologies are adopted.

Xylanases can also be used in clarification of

juices, preparation of dextran for use as food thickeners, production of fluids and juices from plant materials, in processes for the manufacture of liquid coffee, adjustment of wine characteristics and enhancement of astaxanthin extraction (19).

Xylanolytic enzymes from hyperthermophiles. A very large number of reports on the production, properties and applications of xylanases has been published in the last 25 years. The characteristics of these enzymes from bacterial and fungal sources have been dealt with detail in several review (19, 49). However the knowledge about the hemicellulases from extreme thermophilic bacteria (*Aquifex* sp. and *Thermotoga* sp.) are still limited and little is know about this enzyme in archaea (Crenarcheota and Euryarchaeota).

The first description about the occurrence of xylanases in extreme thermophilic bacteria was made by Bragger *et al.* (4). Screening was performed on solid media including 0.1% of polymer. All *Thermotoga* strains were able to degrade xylan forming clear zones on the plates against a red background, after staining with aqueous congo red and destaining with NaCl. The endoxylanase of *Thermotoga* sp. strain FjSS3B.1 exhibited maximum activity at 105°C and the main hydrolysis products of oatspelt xylan by the enzyme were xylobiose, xylotriose and medium-sized oligomers (45). The strain produces also a heat stable ß-D-xylosidase, which was largely cell-associated, probably associated with the "toga" structures of the organism (42). This might indicate that the substrate is hydrolysed at the toga prior to uptake of the carbohydrates into the cells. Furthermore, the gene expressing xylanase activity was isolated from a genomic library of *Thermotoga* sp. strain FjSS3-B1 (43). The sequence of the gene shows that it encodes a single domain, and belongs to family 10 of xylanases. The plasmid expression vector pJLA602 was used for overexpression of xyn A in *E. coli*. The temperature optimum of the recombinant enzyme of 85°C is the highest value reported for a recombinant xylanase to date.

Recently, two extremely thermostable endoxylanases designated Xyn A and Xyn B, were purified from another member of *Thermotogales*, *Thermotoga maritima* (52). The primary structure of Xyn A from *T. maritima* indicated that this enzyme is also a member of family 10 of glycosyl hydrolases, which corresponds to ß-glycanase family F (53). It is interesting to note that most of the highly thermostable xylanases investigated so far belong to this enzyme family. The gene that encodes the thermostable xylanase was cloned in *E. coli* by screening and expression library of *T. maritima* DNA (10). The enzyme was active at 100°C for several hours and efficient in releasing lignin from the kraft pulp, releasing reducing sugars and aromatic materials from the pulp suspensions over a pH range from 3.5-10.

Almost at the same time, two endoxylanases were purified and characterised from the enzyme complex of *Thermotoga thermarum* (100). While the crude xylanase from *T. thermarum* showed a half-life of 40 min at 90°C, the purified endoxylanase 1 showed a half-life of 16 min at 70°C and 80°C; the more thermostable endoxylanase 2 had a half-life of 18 min at 90°C.

Interestingly, in the last decade, xylanolytic enzymes in archaea (Table 3) have been reported only in two *Thermophilum* strains isolated in New Zealand, which grow at 88°C and pH 6.0 (4). Indeed no characterization or detailed studies were made on xylanases from these archaea. This may be partially due to the difficulties involved in growing thermophilic archaea, especially *Thermophilum* strains (55).

Recently, an archaeal xylanase has been detected in extracts of the hyperthermophilic archaeon *Pyrodictium abyssi* (2). The enzyme displays optimal activity at 110°C and pH 6.0, and is very thermostable, showing activity even after 100 min of incubation at 105°C. The analysis of hydrolysis products performed by HPLC showed as main product xylotriose and xylotetraose, indicating the presence of an endoxylanase.

Starch-hydrolysing enzymes from thermophiles and hyperthermophiles. Numerous microorganisms, including bacteria, fungi and yeasts are able to degrade starch and related polysaccharides by the action of enzymes that split α-1,4- or α-1,4- and/or α-1,6-linkages of α-glucan. Thermophilic and hyperthermophilic microorganisms have been found to grow on starch indicating that they posses starch-degrading enzymes (Tab. 3).

Amylolytic activity was detected in two *Sulfolobales* (*S. acidocaldarius* and *S. solfataricus*), and in strains of *Thermophilum*, *Desulfurococcus*, *Thermococcus* and in the thermophilic bacteria *Thermotoga* (4). After growth on starch, the thermophilic bacteria *Thermotoga maritima* produced amylolytic enzymes, which contained three different specificities, β-amylase, α-amylase and glucoamylase (44). The amylases from *T. maritima*

Table 3: Enzymes from thermophilic and extremely thermophilic Archaea involved in carbohydrate hydrolysis.

Species	T-opt. [°C]	Habitat	Enzyme	References
Sulfolobales				
Sulfolobus solfataricus	80	t	α-Amylase	(21), (24)
			β-Glycosidase	(33)
Thermoproteales				
Thermoproteus tenax	88	t	α-Amylase	(4), (24)
			Cellulase	(4), (24)
			Xylanase	(4), (24)
Pyrodictiales				
Pyrodictium abyssi	97	m	α-Amilase	(2)
			Pullulanase	(2)
			Xylanase	(2)
Desulfurococcales				
Desulfurococcus mobilis	88	t	α-Amylase	(8)
Desulfurococcus mucosus	88	t	α-Amylase	(8)
			Pullulanase	(8)
			Transglucosylase	(3)
Staphylothermus marinus	92	m	α-Amylase	(8)
Thermococcales				
Pyrococcus furiosus	100	m	α-Amylase	(5)
			α-Glucosidase	(12)
			β-Glucosidase	(28)
			β-Mannosidase	(24)
			Pullulanase type II	(6)
Pyrococcus abyssi	96	m	β-Glycosidase	(24)
Pyrococcus woesei	100	m	α-Amylase	(27)
			α-Glycosidase	(30)
			Pullulanase type II	(41)
Thermococcus celer	87	m	α-Amylase	(8)
			Pullulanase	(4)
			α-Glucosidase	(24)
			β-Glucosidase	(29)
Thermococcus hydrothermalis	80	t	α-Amylase	(29)
			Pullulanase	(29)
			α-Glucosidase	(29)
Thermococcus litoralis	88	m	α-Amylase	(6)
			Pullulanase type II	(6)
			α-Glucosidase	(24)
Thermococcus profundus	80	m	α-Amylase	(11)

m, marine; t, terrestrial

showed high thermal stability with an upper temperature limit at 95°C.

Extremely thermostable amylolytic enzymes were reported to be produced by the hyperthermophile *Pyrococcus woesei* and *P. furiosus* (11, 27). The amylolytic enzymes are produced by *P. furiosus* in response to the presence of complex carbohydrates in the growth medium (5). The very stable α-glucosidase from *P. furiosus* exhibited remarkable thermostability in the presence of various denaturing agents, like 100 mM dithiothreitol and 1.0 M urea (12). The α -amylase from *P. furiosus* was described as a homodimer with a subunit molecular mass of 66 kDa. The enzyme displayed optimal activity, with substantial thermal stability at 100°C. The gene encoding this highly thermostable

amylase was cloned and expressed in *E. coli* (28). The amylase expressed in *E.coli* exhibited the temperature-dependent activation characteristic of the original enzyme from *P. furiosus*, but a higher apparent molecular weight which was attributed to the improper formation of the native quaternary structure. It was not possible, however, to determine whether this improper assembly was due to translation at lower temperature or to unidentified aspects of production in *E. coli.* On the other hand, the α -amylase from *P. woesei* has showed catalytic activities at a temperature range between 40°C and 130°C. The purified enzyme consisted of a single subunit with a molecular mass of 70 kDa (27).

Most of the archaeal amylases from *Thermococcus celer*, *Desulfurococcus mucosus*, *Staphylothermus marinus* as well as in the two novel archaeal isolates from deep-sea hydrothermal vents (TY and TYS strains) displayed optimal activity at 100°C with the exception of *Thermococcus celer*, with an optimum at 90°C (8). One extracellular thermostable amylase from *Thermococcus profundus* exhibited maximal activity at pH 5.5 and was stable in the range of pH 5.9 to 9.8 (11). Recently, it has been described the production of α-glucosidase and α-amylase by *Thermococcus hydrothermalis* after growth on maltose or starch (29). α-Glucosidase seems to be the dominant amylolytic activity in the enzymatic extract and was capable of hydrolysing the α(1-4)linkages of oligosaccharides and maltose.

Another extracellular amylase has been isolated from culture supernatants of *Sulfolobus solfataricus* during growth on starch (21). The secreted protein has an apparent mass of 240 kDa, consisting of two identical subunits. Its levels in crude culture supernatants varied greatly in response to the carbon source used for growth of the organism.

Pullulanases from thermophilic and hyperthermophilic archaea. Since the discovery of *Klebsiella pneumoniae* pullulanase, a number of microbial pullulanases have been purified and characterised from thermophilic bacteria and archaea by many investigators (11, 12, 41, 85). However most enzymes from thermophilic bacteria belong to type II pullulanase. Among the several amylolytic enzymes produced by the hyperthermophilic archaeon *Pyrococcus furiosus*, pullulanase was characterised by temperature optimum of at least 100°C and a high degree of thermostability (5). The pullulanase from *P. furiosus* was purified and reported to be a glycoprotein with an optimum of activity at 100°C (6).

An extracellular amylopullulanase from *Thermococcus litoralis* (6) was optimally active at 110°C, but the presence of Ca^{+2} extended the range at which the activity could be measured (up to 130°C-140°C) Thermoactive pullulanases have been characterised in *Thermococcus celer*, *Desulfurococcus mucosus*, *Staphylothermus marinus*, and in the two novel archaeal strains (TYS and TY). The enzymes showed temperature optima between 90°C and 105°C and exhibit remarkable thermostability, even in the absence of substrate and calcium ions (8). An extracellular pullulanase has been found also in the culture medium after fermentation of starch by *Thermococcus hydrothermalis* (29), with an extracellular production represented almost 80% of the total pullulanase production.

The pullulanase from *Pyrococcus woesei* has been purified and the gene has been cloned and expressed in *E.coli* (41). The native and cloned enzymes are identical in their physiochemical properties, being optimally active at 100°C and pH 6.0. The high rigidity of the heat stable enzyme was demonstrated by fluorescence spectroscopy in the presence of denaturing agents (41).

Unlike all thermoactive pullulanase know so far, the pullulanase from *Pyrodictium abyssi* showed highest activity at alkaline pH, at pH 9.0, and very high optimal temperature (100°C). Preliminary results also indicate that *P. abyssi* forms a real debranching enzyme, i.e., pullulanase type I, which is very rare among bacteria and archaea (2).

Thermophily and thermostability. A consistent characteristic of all enzymes from hyperthermophilic microorganisms is their high level of thermostability. The positive correlation between the thermophily of the source organism and thermostability of both intra and extracellular proteins has been demonstrated frequently (16, 40, 51).

Different protein engineering studies, where diverse point mutations can enhance (or reduce) protein thermostability have shown, that there are consequences, both structural and functional, in artificially enhancing protein thermostability. One increase of the thermal stability of a protein may result in reduced conformational flexibility and depending on locality and extent of these changes, this may result in significant (and sometimes detrimental) consequences with respect to the biological function. There is some evidence from various sources including

proteolysis studies, ^{1}H-^{2}H exchange studies, and X-ray diffraction that at room temperature a thermophilic protein will be less flexible than its mesophilic equivalent. At their respective growth temperatures, similar proteins from both mesophilic and thermophilic sources will posses similar levels of molecular flexibility, a consequence that molecular flexibility is critical for function (3, 14).

Moreover, it is not clear what are the upper limits for the thermal stability of proteins. Studies with one protease from *Pyrobaculum aerophilum*, which exhibits strong proteolytic activities with a temperature range of 80°C-130°C, allowed identification of sites potentially contributing to the thermostability of the protein (51). Aspartic acids were found at the N-terminus of several surface helices, possibly increasing stability by interacting with the helix dipole. Several of the substitutions in regions expected to form surface loops were adjacent to each other in the tertiary structure model. A marked increase in glutamic acid residues in the hyperthermostable citrate synthase from *Pyrococcus furiosus* with respect to its mesophilic counterpart, may be related to the high concentration of compatible solutes present within the cell. The percent of aromatic amino acids is also one of the highest in the citrate synthases, which may lead to enhanced stabilising aromatic packing interactions (66).

The presence of mannosylglycerate, a compatible solute, in two unrelated thermophilic bacteria lead to the speculation that the accumulation of this compound could also be related to the thermophily of organisms. Indeed, until the physiological adaptation to temperature stress has been examined, no final conclusions can be drawn from the relationship between mannosylglycerate and thermophily (36).

Ion pairing also plays a role in protein stabilization (9). This was also confirmed by the determination of the structure of glutamate dehydrogenase (GDH) from *Pyrococcus furiosus*, which was compered with GDH from mesophilic origin. This comparison has revealed that the hyperthermophilic enzyme contains a striking series of networks of ion-pairs which are formed by regions of the protein which contain a high density of charged residues. The ion-pair networks are clustered at both inter domain and inter subunit interfaces. They may well represent a major stabilising feature associated with the adaptation of enzymes to extreme temperatures (39).

Therefore, a study of enzymes from extremely thermophilic archaea, may reveal the existence of enzymes with still greater thermostability. This suggests that enzyme stability does not need to confine the existence of life to 110°C or below. It has also implications for enzyme applications in the industry at high temperatures. At the present the industrial applications of thermostable enzymes are still limited to a few areas. Although the genetic engineering allows the design of "tailor-made" enzymes by altering their amino acid composition, the construction of thermostable enzymes are still highly empirical. This is because little is known concerning the molecular basis of protein thermostability. Enzymes of extremophiles are, however, good starting points for engineering "tailor-made" enzymes. Furthermore, enzymes from thermophiles and especially hyperthermophiles present physiological features and potential technological properties, which must be understood, before an industrial process can be designed or compared with those currently in use.

CONCLUSIONS

There are many existing applications in which more thermally stable versions of enzymes now used will be advantageous. This is especially true in the hydrolysis of corn starch to produce high fructose corn syrup. Amylolytic enzymes are now used at temperatures exceeding 100°C in some cases to hydrolyse liquified starch to oligosaccharides and eventually to glucose. Glucose is then partially isomerized to fructose using immobilized xylose (glucose) isomerase. Many of these same enzyme activities are available in extreme thermophiles. Given the preference of many of these organisms for saccharides, it should be possible to isolate a range of saccharidases for evaluation in starch processing.

There are other hydrolysis reactions that can be catalysed by high temperature enzymes. Cellulose and hemicellulose hydrolysis is important in the processing of renewable resources. Activities to these substrates have been detected among the thermophiles and extreme thermophiles. The isolation of new thermophilic strains on cellulosic substrates is at present an area of great interest.

On the other side, in food processing enzyme use has been limited because of the need to mantain asseptic conditions. However, if enzymes with

sufficient thermostability were available, applications involving modifying the fiber content of foods, perhaps during the baking process, could be considered. The treatment of complex wastes from food processing, such as lactose-laden streams, may also be facilited by decreasing the viscosity and increasing the solubility of lactose at high temperature.

To take advantage of the biotechnological potential of microorganisms growing at extremely high temperatures, there is still a great deal to be learned about their metabolic and genetic characteristics. An interaction between scientists and engineers will be require to assure that fundamental insights are used effectively for technology development.

RESUMO

Microrganismos extremotermofílicos e suas enzimas despolimerizantes

Microrganismos termofílicos e hipertermofílicos são encontrados como habitantes normais de áreas vulcânicas continentais e submarinas, sedimentos marinhos com aquecimento geotermal e ventos hidrotermais; portanto, são considerados extremofílicos. Diversas aplicações presentes ou potenciais de enzimas de extremofílicos são revisadas, especialmente enzimas que hidrolizam polímeros, tais como amilases e hemicelulases. São também apresentadas as variações das características morfológicas e metabólicas entre estes organismos que crescem entre 70°C e 100°C e indicadas as oportunidades potenciais de aplicações derivadas destas características.

Palavras-chave: Archaea, extremofílicos, amilases, xilanases, termoestabilidade.

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SCREENING OF BACTERIAL STRAINS FOR PECTINOLYTIC ACTIVITY: CHARACTERIZATION OF THE POLYGALACTURONASE PRODUCED BY *BACILLUS* SP

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ABSTRACT

One hundred sixty eight bacterial strains, isolated from soil and samples of vegetable in decomposition, were screened for the use of citrus pectin as the sole carbon source. 102 were positive for pectinase depolymerization in assay plates as evidenced by clear hydrolization halos. Among them, 30% presented considerable pectinolytic activity. The cultivation of these strains by submerged and semi-solid fermentation for polygalacturonase production indicated that five strains of *Bacillus* sp produced high quantities of the enzyme. The physico-chemical characteristics, such as optimum pH of 6.0 – 7.0, optimum temperatures between 45°C and 55°C, stability at temperatures above 40°C and in neutral and alkaline pH, were determined.

Key words: *Bacillus* sp, polygalacturonase, pectinolytic activity

INTRODUCTION

Many plant-pathogenic bacteria and fungi are known to produce pectolytic enzymes useful for invading host tissues. Moreover, theses enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere (2).

Pectinases include depolymerizing and demethoxylating enzymes. Depolymerizing enzymes are polygalacturonase (EC 3.2.1), which cleaves the α-1,4 glycosidic bonds between two galacturonic acid residues, and pectin-lyase (EC 4.2.2), which catalyses a β-elimination reaction between two methylated residues (3). De-esterifying enzymes include pectin-esterase (EC 3.1.1), which catalyses the demethoxylation of methylated pectin, producing methanol and pectin (20).

Preparations containing pectin-degrading enzymes have been extensively used to improve the stability of fruit and vegetable nectars and in the clarification of fruit juices and wines (5, 13, 21, 23, 24). Currently, they are widely used in industry for retting of natural fibers and extraction of oils from vegetable and citrus peels (4, 6).

The enzymes preparations used in the food industry are of fungal origin because fungi are potent producers of pectic enzymes and the optimal pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3,0 to 5,5 (26). Such preparations are not suited for production of vegetable purées or other preparations in which pH

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values are close to neutral (7). Furthermore, due to the relatively low temperature stability of the fungal enzyme preparations, maceration needs to be carried out at temperatures not exceeding 45°C, necessitating the incorporation of a pasteurization step to limit the growth of mesophilic microorganisms (22).

The present investigation was on pectinolytic activities of bacteria strains isolated from Brazilian soil and samples of vegetable in decomposition.

MATERIALS AND METHODS

Microorganisms: portions of 5g of samples of soil, from agricultural and vegetable wastes were pooled and homogenized in sterile medium with pH 6.0 containig 1% citrus pectin with 67% of metoxilation, 0.14% $(NH_4)_2SO_4$, 0.20% K_2HPO_4, 0.02% $MgSO_4$ $7H_2O$ and 0.10% nutrient solution (5 mg/L $FeSO_4.7H_2O$; 1.6 mg/L $MnSO_4$. H_2O; 1.4 mg/L $ZnSO_4$. $7H_2O$; 2.0 mg/L $CoCl_2$). A loop of the homogenate was then streaked onto nutrient medium and incubated at 30°C for 24 to 72h. All morphological contrasting colonies were purified by repeated streaking. Pure cultures were sub-cultured onto slants media and maintained for identification and enzymes studies. Identification of the genus was based on morphological and biochemical characteristics (15).

Plate assay of depolimerized pectin: The medium was the same used for isolation of cultures, supplemented with 2% agar. Pure culture was inoculated by puncture in the medium and incubated for 48h at 30°C. After the colonies reached around 3 mm, iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330ml H_2O) was added to detect clearance zones (11).

Production of pectic enzyme on solid-state (SSF) and submerged (SmF) fermentation: Strains presenting large clearing zones were used for enzyme production assays on liquid and solid medium. The liquid medium containing 1% citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.20% KH_2PO_4 and 0.01% $MgSO_4$ $7H_2O$, pH 6.0 was inoculated with a suspension containing 10^6 cells/ml. Cultures were grown in 125ml Erlenmeyers flasks with 25 ml of medium in a rotary shaker (150rpm) at 30°C. After 48h the biomass was separated by centrifugation at 1000xg for 20 min and the supernatant was used to evaluate polygalacturonase (PG) activity. The SSF was done using a 250 ml Erlenmeyer flask containing 5g of wheat bran and 10 ml of 1% $(NH_4)_2SO_4$ and 0.02% $MgSO_4$ (67% of moisture). 10^6 cells per gram of wheat bran were added to each flask and maintained at 30°C. After 72h, fermented material was mixed with 40 ml distilled water and filtered at vacuum and centrifuged. The supernatant was used to evaluate PG activity.

Assay of polygalacturonase (PG) activity: PG activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent DNS assay (18). The reaction mixture containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2M citrate-phosphate, pH 6.0 buffer and 0.2 ml of culture supernatant, was incubated at 40°C for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of galacturonic acid released per minute.

RESULTS

1- Selection of strains with pectinolytic activity:

168 bacterial strains able to grow on medium containing citrus pectin as the only carbon source were isolated. These strains were tested for pectin hydrolysis by plate assay, at pH 6.0. The strains were classified as very good producers of pectin depolymerizing enzymes when presented clear halos around colonies of at least 1.5 cm (14), good producers when the halos were of at least 1 cm (36), weak producers when halos were at least 0.5 cm (52) and poor producers when no pectinolytic activity and no clear lysis zones were observed (66).

2- Production of poligalacturonase from *Bacillus* sp isolated on liquid and semi-solid medium

Five *Bacillus* sp demonstrated high pectinolytic activity when assayed by the plate method. These strains were cultivated for polygalacturonase production using SSF and SmF. The data obtained are presented in Table 1.

Table 1. Production of polygalacturonase (U / ml) by *Bacillus* sp in solid-state (SSF) and submerged (SmF) fermentation

Microorganism	SSF	SmF
Bacillus sp Ar1.2	0.8	3.5
Bacillus sp B1.3	0.4	3.0
Bacillus sp M2.1	0.7	4.0
Bacillus sp P4.3	0.3	4.0
Bacillus sp P6.1	0.4	2.7

Extracts from SSF cultures showed higher pectionolytic activities than those obtained by SmF. Maximal polygalacturonase activity was obtained for strains M2.1 and P4.3.

Physico-chemical characteristics of PG

The effect of pH on the activity of PG produced by the *Bacillus* sp strains in SSF was determined by using reaction mixtures at pH values ranging between 3.0 and 10.0 (Fig.1). Pectinolytic activity was the highest at pH 6.0 for all strains except for *Bacillus* P4.3, which presented optimal pH between 6.5 and 7.0.

Assay for determination of the optimal temperature for PG activity indicated maximal activities at 40-50°C for AR1.2, B1.3 and P4.3 strains and 50-55°C for M2.1 and P6.1 strains (Fig 2). PG from AR1.2 showed the largest spectrum of activity as a function of temperature.

To determine the effect of temperature on the stability of PG, enzyme solutions were incubated for 60 min at temperatures between 10°C and 70°C and the residual activity was assayed after cooling. Practically no changes in pectinolytic activities were observed when the incubation temperature was lower than 50°C. The PG of P4.3 was stable for 1h at 60°C (Fig. 3), and the results indicated that the PG activity was similar to the previously obtained values.

Results of the incubation of enzyme solutions at pH values between 3.0 and 10.0 for up to 24 h at 25°C indicated that these PG were stable in the pH range 6-8. PG P4.3 was stable between pH 7 and 9 (Fig. 4).

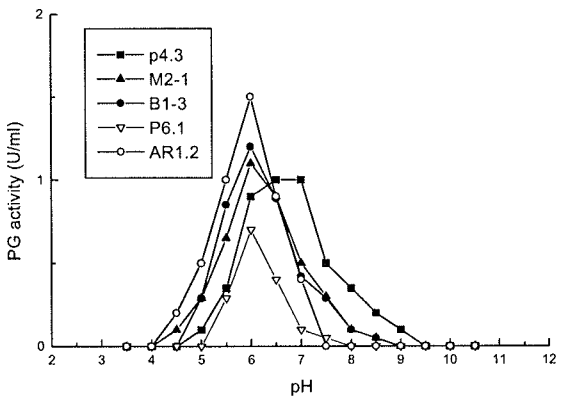

Figure 1: Effect of pH on the polygalacturonase activity produced by *Bacillus* sp on wheat bran. The buffers used were sodium acetate (pH 3.5-5.5), citrate-phosphate (pH 5.5-7.0), tris-HCl (pH 7.0-8.5) and Gly-NaOH (pH 8.5-10.5).

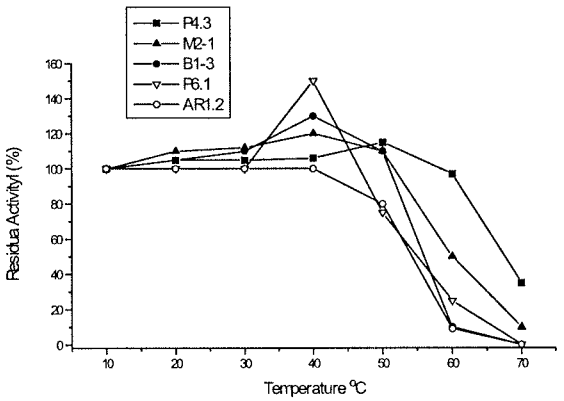

Figure 3: Stability of PG against temperature. The enzymatic solutions were maintained in the temperature range of 10 to 70°C, for 1h. 110% of activity (U/ml): P4.3=3.5; M2.1=2.5; B1.3=3.2; P6.1= 2.3; Ar1.2=3.3

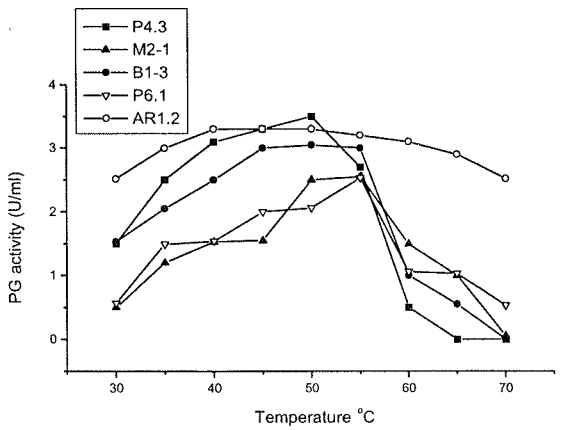

Figure 2: Effect of temperature on the polygalacturonase activity produced by *Bacillus* sp on wheat bran.

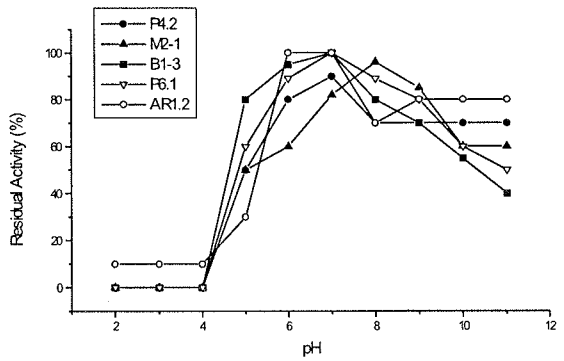

Figura 4: Stability of the PG against pH. The enzymatic solutions were dispersed on 0.1M buffers solutions at various pH values and maintained at 25°C for 24 h. An aliquot was assayed for the residual activity as described in the text. 110% of activity (U/ml): P4.3=3.5; M2.1=2.5; B1.3=3.2; P6.1= 2.3; Ar1.2=3.3

DISCUSSION

Production of pectinases in solid medium was higher than that produced in liquid medium. These results are similar to those obtained with endoglucanases and β- glucosidades (14), xylanases (8), α-amilases (17) and pectinases produced by *A. niger* (1, 25).

Activities of PG produced by *Bacillus* sp strains were higher than those produced by *Aspergillus niger* (19), *Aspergillus sp* and *A. niger* ATCC20107 (16), *Aureobasidium pullulans* (10) and *Tubercularia vulgaris* (12). However, they were lower than those reported using *Bacillus* GK-8 (9).

The pectinolytic activity of the crude solution has specific properties which may offer advantages over currently available pectinase preparations. The enzyme solution can be applied directly to vegetables without the need for pH modification. Furthermore, because of the temperature stability of the enzyme, it can be possibly used at processing temperature of 50°C, which is sufficient to limit the growth mesophilic contaminants (7) in the process.

RESUMO

Seleção de linhagens bacterianas para atividade pectinolítica: caracterização das poligalacturonases produzidas por *Bacillus* sp

A partir de amostras de solo e de material vegetal em decomposição, foram isoladas 168 linhagens bacterianas capazes de utilizar pectina de citrus como única fonte de carbono. Destas, 102 foram positivas para a despolimerização da pectina, através de ensaios em placa de Petri nos quais foram detectados halos claros ao redor das colônias. Entre essas, 30% (50) foram consideradas ótimas ou boas produtoras de poligalacturonase. O cultivo dessas linhagens através de fermentação submersa e semi-sólida para a quantificação das poligalacturonases (PG) produzidas indicou que 6 linhagens de *Bacillus* sp foram as melhores produtoras da enzima. As características físico-químicas, como pH ótimos entre 6,0 e 7,0 e temperaturas ótimas entre 45 e 55°C, estabilidade à temperaturas acima de 40°C e a pH neutros e alcalinos foram determinadas.

Palavras-chave: *Bacillus* sp, poligalacturonase, atividade pectinolítica

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PRODUCTION OF EXTRACELLULAR LIPASE BY THE PHYTOPATHOGENIC FUNGUS *FUSARIUM SOLANI* FS1

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ABSTRACT

A Brazilian strain of *Fusarium solani* was tested for extracellular lipase production in peptone-olive oil medium. The fungus produced 10,500 $U.L^{-1}$ of lipase after 72 hours of cultivation at 25°C in shake-flask at 120 rpm in a medium containing 3% (w/v) peptone plus 0.5% (v/v) olive oil. Glucose (1% w/v) was found to inhibit the inductive effect of olive oil. Peptone concentrations below 3% (w/v) resulted in a reduced lipase production while increased olive oil concentration (above 0.5%) did not further stimulate lipase production. The optimum lipase activity was achieved at pH 8.6 and 30°C and a good enzyme stability (80% activity retention) was observed at pH ranging from 7.6 to 8.6, and the activity rapidly dropped at temperatures above 50°C. Lipase activity was stimulated by the addition of n-hexane to the culture medium supernatants, in contrast to incubation with water-soluble solvents.

Key words: Enzyme kinetics, ester hydrolysis, extracellular lipase, *Fusarium solani*

INTRODUCTION

Lipases (triacylglycerol acylhydrolases E. C. 3.1.1.3) are enzymes that hydrolyse fatty acyl ester bonds of acylglycerols at the interface between oil and water (2). In organic medium, lipases are able to catalyse reactions of synthesis and transesterification of glycerides and phosphoglycerides, as well as a variety of non-glyceride, ester bonds (2,9,18,22). Lipases have been extensively studied because of their actual and potential applications in the detergent, oil and food industries. Recently, various strategies in the pharmaceutical and chemical industries have used lipases in the synthesis of optically pure drugs and agrochemicals that are more effective and produce fewer side effects compared with their racemates (11,22). Fungi are preferable lipase sources because fungal enzymes are usually excreted extracellularly, facilitating extraction from fermentation media. The selectivity of lipases has been exploited in industry in the synthesis of chiral compounds as well as in the resolution of racemic mixtures (19). The production of lipase by several *Fusarium oxysporum* strains has been studied in terms of enzyme production, protein properties and purification (7,8,11). The aim of this work was to describe the production of an extracellular lipase by a *Fusarium solani* FS1 isolated from *Carica papaya* fruit.

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MATERIALS AND METHODS

Microorganism: *Fusarium solani* FS1 was obtained from the culture collection of the Plant Health Department of the Federal Rural University of Pernambuco. The cultures were maintained at 4°C on potato dextrose agar plates.

Growth conditions: *Fusarium solani* FS1 was batch cultivated in a basal medium (BM) pH 6.0 consisting of ($g.L^{-1}$ of deionized water): $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1; $NaNO_3$, 3; peptone, 30. It was supplemented with different carbon sources (glucose, olive oil and peptone). Pre-inoculum consisted of 10^5 spores incubated in 10 ml of BM in a rotary shaker (120 rpm) for 2 days at 28°C. After this time, a 500 ml flask containing 90 ml of BM was inoculated with the pre-inoculum and cultivated as above. Aliquots of 10 ml of this culture were withdrawn at different periods of incubation and centrifuged. The supernatants were used to measure lipase activity, protease activity and pH. The pellet was washed in water, filtered and dried in pre-weighed filters for biomass determination. Peptone and oil concentrations varied as shown in results. The results were calculated from three independent cultures. Each measurement was carried out in triplicate.

Enzyme assay: Lipase activities were assayed in the enzyme-containing supernatants with p-nitrophenyl palmitate (pNPP) as substrate determined spectrophotometrically at 410nm (23). One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per minute. Protease in the supernatant was measured according to Ginther (5) using azocasein as substrate. One unit of protease activity is defined as the amount of enzyme that produces na increase in absorbance of 1.0 in 1h at 440 nm.

Effect of pH and temperature toward pNPP: The pH values of the reaction mixtures were varied from 5.4 to 7.8 (in 50 mM tris-maleate) and 8.0 to 8.8 (in 50 mM tris-HCl) and lipase activity was measured at 25°C. To determine the pH stability, lipase-containing supernatant was diluted 1:1 in 100 mM of the buffers described above and incubated for one hour at 25°C before assayed. Lipase activities were assayed in reaction mixtures of pNPP containing tris-HCl pH 8.0 at a range of temperatures from 15°C to 60°C to determine optimum lipase activity. For thermal stability, lipase-containing supernatants were pre-incubated for 60 minutes over a range of temperature from 25°C to 50°C in tris-HCl pH 8.0 buffer. After this time, the mixtures were left for 10 minutes at room temperature and lipase activity were measured using the standard procedure.

Effect of organic solvents: The supernatant was diluted with organic solvents in the concentrations of 0 to 50% (v/v). After 1 hour of incubation with constant shaking at 25°C, lipase activity was measured in the aqueous phase as described above.

RESULTS AND DISCUSSION

Effect of the medium composition on lipase activity

The activity of extracellular lipase of *F. solani* FS1 cultures was evaluated after 96 hours of incubation in medium containing 3% (w/v) peptone alone and supplemented with different carbon sources (Table 1). Highest lipase activity of 9,500 $U.L^{-1}$ was observed in the medium containing 1% olive oil.This represents an 8-fold lipase induction

Table 1. Effect of the carbon source on production of lipase activity by FS1 after 96h cultivation.

Additional carbon source [1% (w/v)]	Maximum lipase activity ($U.L^{-1}$)	Maximum mycelial dry weight ($g.L^{-1}$)	Maximum lipase activity/maximum mycelial dry weight: Specific lipase activity ($U.g^{-1}$)	Lipase Induction factor [a]
–	1240	6.2	200	1
Glucose	3800	6.7	567	3
Olive oil	9500	15.3	620	7.7
Gluc. + olive oil	4000	10.7	374	3.2

[a] Induction rate: ratio of specific activities with and without additional carbon source.

in relation to the basal medium (BM). The presence of 1% glucose, although stimulating a 3-fold lipase activity in relation to BM, abolished the enzyme induction produced by 1% olive oil. The results presented in Table 1 indicate a higher biomass production in 1% olive oil medium (15.3 $g.L^{-1}$) in relation to glucose or glucose/olive oil medium (6.7 and 10.7 $g.L^{-1}$, respectively). Our data shows similar lipase activities (in terms of Units per mg biomass) for both glucose and olive oil supplements. However, the presence of both supplements depresses lipase induction. Rapp (14) showed this effect with *Fusarium oxysporum* when mixtures of glucose and olive oil (both at 1%) resulted in a peak of biomass at approx 100h while lipase peaked at 220h. The effect of different concentrations of olive oil and peptone on lipase production was evaluated after 72 hours of cultivation (Fig. 1). At a peptone concentration of 3% (w/v), the highest lipase activity of 10,500 $U.L^{-1}$ was obtained in the media containing 0.5% (v/v) olive oil. Lower (0.25%) and higher (1%) oil concentrations decreased lipase production to 8,300 $U.L^{-1}$ and 7,800 $U.L^{-1}$, respectively (Fig. 1A). Decreases in lipase production with increases in inducer concentrations have been noted (11, 3) for *Aspergillus oryzae* (soyabean oil) and *Candida rugosa* (olive oil). However, others have reported (17, 13) increased lipase production with increasing inducer concentrations for *Penicillium citrinum* (olive oil) and *Candida rugosa* (oleic acid). Pokorny *et al.* (12) showed that the addition of olive oil in concentrations between 0.5% and 2% increased the lipase production by *Aspergillus niger* to a maximum at 72h. The addition of olive oil to the basal medium at a concentration of 0.2% increased lipase activity by *Torulopsis ernobii*, but amounts of olive oil greater than 0.8 % gave smaller increases (24). These authors suggest that the role of lipids in augmenting lipase production appears to be related to growth. These observation reinforce the constitutive and the biomass-linked lipase production by FS1 strain. Higher oil concentrations could be affecting the aeration rate of the culture and promoting a delay in mycelial growth and lipase production.

Further evidences for that biomass-linked lipase production are showed in the Fig. 1B, where a decrease in peptone concentration to half (1.5% w/v) promoted 2.5-times decrease in lipase activity after 72 hours of cultivation in BM containing 0.5% olive oil. Peptone concentration of 0.5% (w/v) almost abolished lipase activity (Fig. 1B). This peptone-dependence was demonstrated for the lipase production by *F. oxysporum* f. sp. *vasinfectum* (14).

Batch fermentations were carried out in basal medium supplemented with 0.5% (v/v) olive oil and 3% (w/v) peptone for lipase production by FS1 strain (Fig. 2). A low lipase activity was observed until 48 hours of cultivation, at the moment that the culture entered into stationary phase of growth. However, it increased exponentially up to 96h to a value of 9,500 $U.L^{-1}$. The production of lipase is accompanied by an increase in the supernatant pH up to value of 8.3. Such pH increase was also observed during lipase production by *Aspergillus oryzae* (11). In spite of the media containing peptone, the FS1 strain produced low quantity of protease during the period of fermentation, that reached maximal activity at 72 hours of cultivation (500 $U.L^{-1}$) and decreased thereafter (Fig. 2). Low protease activity should facilitate downstream process for lipase recovering (6).

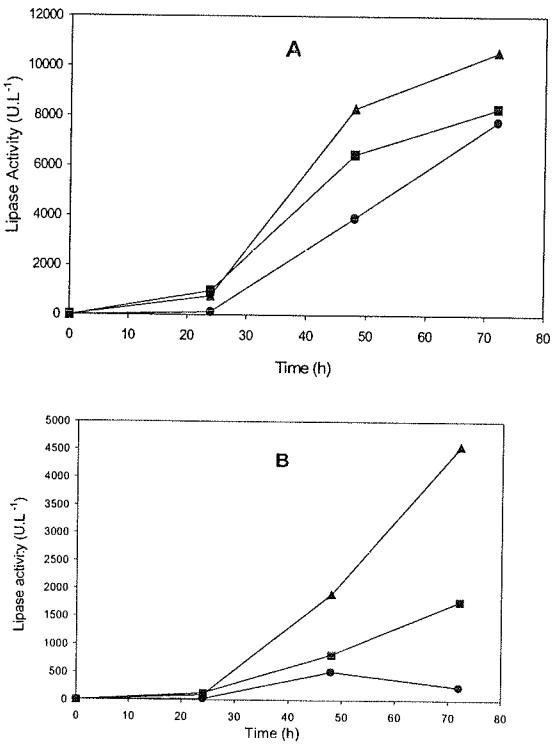

Figure 1. Effect of the olive oil and peptone concentration on lipase production by FS1 strain.**(A)** Medium containing 3% (w/v) peptone was supplemented with olive oil at concentrations (v/v) of 0.25% (■), 0.5% (▲) and 1% (●); **(B)** Medium containing 0.5% (v/v) olive oil was prepared with peptone at concentrations (w/v) of 0.5% (●), 1.5% (■) and 3% (●). Values are means of three experiments.

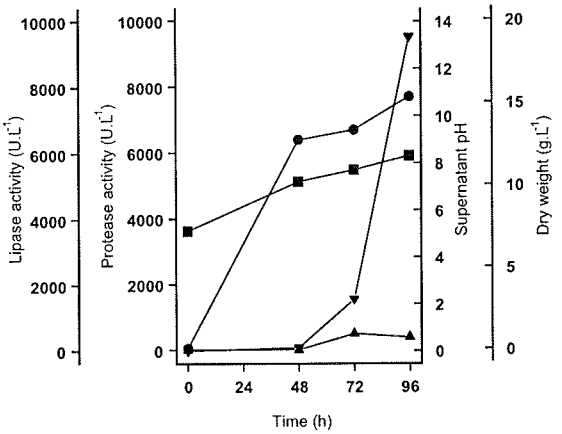

Figure 2. Batch fermentation of the FS1 strain in the medium containing 3% (w/v) peptone and 0.5% (v/v) olive oil (120 rpm at 28°C). Values of lipase activity, (▼) mycelial dry weight (●), supernatant pH (■) and protease activity (▲) were taken in different period of cultivation. Values are means of three experiments.

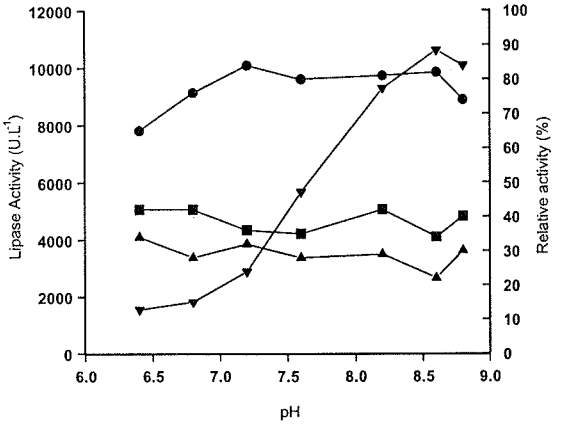

Figure 3. Effect of the pH on FS1 lipase activity and stability. Lipase activity was measured in reaction mixtures prepared with buffers at different pH values (▼) after previous incubation of the enzyme for one (●), two (■) and four (▲) hours (relative activity)at 25°C in the same buffers. Buffers were 50 mM tris-malcate (pH 5.4 to 7.8) and 50 mM tris-HCl (pH 8.0 to 8.8).

Effect of the pH and temperature on the lipase activity of *Fusarium solani* FS1 and stability

The lipase activity was evaluated at different pH values at 25°C using pNPP as substrate (Fig. 3). The optimum activity for pNPP hydrolysis of 10,620 $U.L^{-1}$ was reached at pH 8.6 in 50 mM tris-HCl buffer and pH values below 8.0 decreased lipase activity. Extracellular lipase of *F. oxysporum* f. sp. *line* and *F. oxysporum* f. sp. *vasinfectum* showed optimum activity at pH 7.0 (in 25 mM Tris-HCl buffer, 37°C) and at pH 5.8 (in 0.06 M sodium phosphate, 35°C), respectively (8,14). Lipase mantained around 80% its initial activity when incubated for one hour at pH values between 7.2 and 8.6, with an decrease in lipase activity at pH beyond this range. The enzyme was more stable at pH 7.2 in tris-maleate buffer, despite its optimum activity at pH8.6 in tris-HCl buffer (Fig. 3). The incubations of the enzyme for 2h and 4h at 25°C in the pH range of 6.4 to 8.8 decreased lipase activity for the values around 40% and 30%, respectively (Fig. 3). No lipase activity was observed after 24h incubation at any pH value tested. This decrease in enzyme stability should be due to some denaturation effect, as well as to the presence of protease in the samples. Extracellular lipase of *F. oxysporum* f. sp. *vasinfectum* showed stability over the pH range of 4.0 to 10 during 1h incubation at 30°C (14). On the other hand, others strains of *F. oxysporum* showed stability only at alkaline pH (15; 8).

Lipase presented two peaks of optimum temperature at 25°C and 45°C (Fig. 4). This behaviour of lipase activity from *Fusarium solani* FS1 may be indicative of the presence of isoenzymes. A considerable decrease in the lipolytic activity was observed for highest temperatures, reaching zero activity at 60°C. Maximal stability of the enzyme was observed in the temperature range of 25°C to 30°C after its thermal exposure for 1h (Fig. 4). Incubations above 40°C promoted inactivation of this enzyme. The lipase produced by *F. oxysporum* f. sp. *line* and *F. oxysporum* f. sp. *vasinfectum* showed optimum activity at 42°C and 45°C, respectively

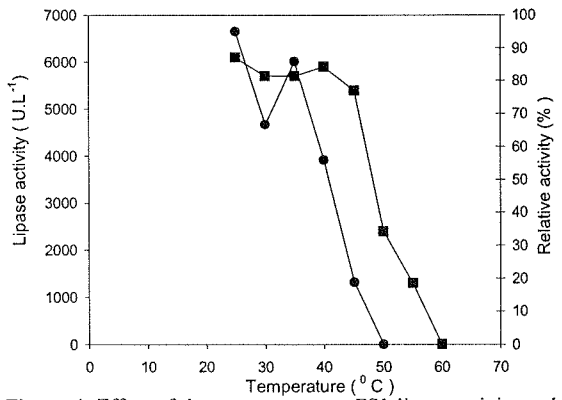

Figure 4. Effect of the temperature on FS1 lipase activity and thermal stability. Lipase activity was measured in 50 mM tris-HCl pH 8.0 over a range of temperature from 25°C to 60°C (■) and after previous incubation of the supernatant mixtures for one hour in the temperatures indicated (●).

(8,14). Moreover, the lipases produced by both of the strains above were more thermostable than the FS1 lipase. The optimun temperatures were 25°C for *A. oryzae* (16), 37°C for *C. cylindracea* (16), and 40°C for *Geotrichum candidum* (21).

Stability of the FS1 lipase in organic solvents

Experiments presented in Fig. 5 demonstrate that 1-pentanol completely abolished lipase activity at 10% (v/v), while acetone and n-propanol maintained 30% of activity under this condition. At 20% (v/v), the last two solvents completely inhibited lipase activity. In contrast, n-hexane increased lipase activity to 170% of its initial activity at 40% (v/v) solvent concentration (Fig. 5). A similar result was demonstrated when *F. Oxysporum* f. sp. *vasinfectum* lipase was incubated with n-hexane (14). On the other hand, lipase from *Bacillus sp* was inhibited in the presence of n-hexane (20). This effect has been discussed by Rapp (14) as an increase of the reaction mixture emulsification by n-hexane, an water-immiscible solvent, promoting the best contact between enzyme and substrate. The activity of lipase depends on the quality of an oil-water interface (4)

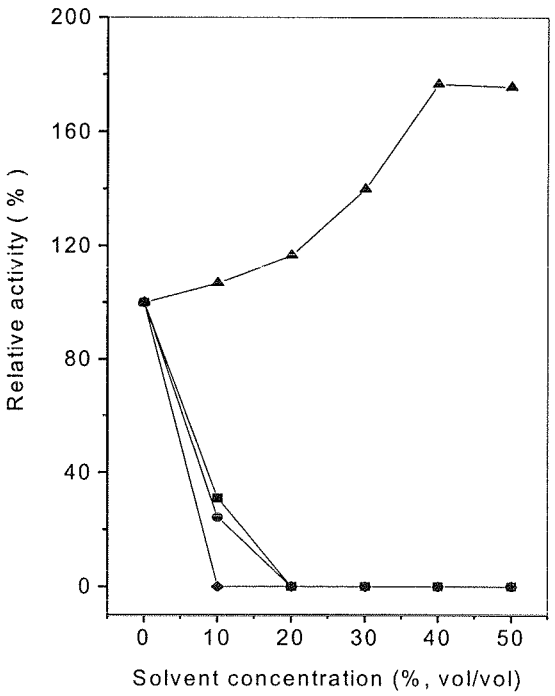

Figure 5. Effect of the organic solvent on activity of the FS1 lipase. The enzyme was incubated for one hour at 25°C with constant with different concentrations of acetone (■), n-propanol (●), n-hexane (▲),1-pentanol (♦).

and water-miscible organic solvents generally cause protein denaturation (1). In studies on the use of lipase for transesterification and synthesis of esters, the reactions have previously been carried out in media containing water-immiscible organic solvents and a small amount water, due to the fact that enzymes are less susceptible to denaturation in such systems (20). Since lipase from *Fusarium solani* FS1 has been shown to be stimulated by the addition of n-hexane, the enzyme might be useful for transesterification and ester synthesis. Experiments in flavour synthesis using FS1 lipase has been initiated in our laboratory with very promising results.

RESUMO

Produção de lipase extracelular pelo fungo fitopatogênico *Fusarium solani* FS1

Uma estirpe brasileira de *Fusarium solani* foi testada para produção de lipase em meio contendo peptona e óleo de oliva. O fungo produziu 10.500U.L^{-1} de lipase após 72h de incubação a 25°C e 120 rpm em meio contendo 3% (p/v) de peptona e 0,5% (v/v) de óleo de oliva. A glicose (1% w/v) parece inibir o efeito estimulador do óleo de oliva. Concentrações de peptona abaixo de 3% (w/v) reduziram a produção de lipase enquanto que o aumento na concentração de óleo de oliva acima de 0,5% (v/v) não estimulou a produção da enzima. Ótima atividade lipásica foi alcançada em pH 8,6 e 30°C e uma boa estabilidade enzimática foi observada em pH variando de 7,6 a 8,6, mas a atividade caiu rapidamente à temperaturas acima de 50°C. A atividade lipásica foi estimulada pela adição de n-hexano à mistura de incubação, em contraste com a incubação em solventes orgânicos solúveis em água.

Palavras-chave: cinética enzimática, hidrólise de ésteres, lipase extracelular, *Fusarium solani*, pNPP.

ACKNOWLEDGEMENTS

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GROWTH AND ENDOGLUCANASE ACTIVITY OF *ACETIVIBRIO CELLULOLYTICUS* GROWN IN THREE DIFFERENT CELLULOSIC SUBSTRATES

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ABSTRACT

The growth kinetics of *Acetivibrio cellulolyticus* grown in medium containing different carbon sources (cellobiose, amorphous or crystalline cellulose) was investigated. The specific growth rate was higher in cellobiose fed cultures than in the presence of the other two substrates. Endoglucanase production was greater in cultures grown on amorphous cellulose; enzyme activity increased during the stationary phase in cultures grown on crystalline cellulose.

Key words: *Acetivibrio cellulolyticus*, endoglucanase activity, cellulose

INTRODUCTION

Acetivibrio cellulolyticus is a mesophilic anaerobic bacterium able to degrade a wide variety of cellulosic materials (15) by producing enzymes capable of acting on native cellulose. Only a few organisms have the ability to hydrolyse native cellulose, such as *Clostridium cellobioparum* and *Ruminococcus albus* (6) isolated from soil and rumen, respectively. *A. cellulolyticus* plays an important role in the transformation of sewage sludge, which presents a cellulose content of around 13-15% (12).

Both the physiology and cellulolytic activity of aerobic and anaerobic bacteria and fungi have become subjects of studies because of the great interest on bioconversion of cellulose to fuels and chemical feedstocks (10). Anaerobic cellulolytic microorganisms are of great significance for large scale industrial applications; however, appropriate methods of cultivation are still at an early stage of development. Special attention should be given to biodigestion processes that aim at the fast transformation of cellulose rich pollutants.

The biological hydrolysis of cellulose is dependent upon a group of enzymes - endoglucanase, exoglucanase and β-glucosidase - which act synergistically on the substrates (2), generating low molecular weight reducing sugars (15).

The growth of *A. cellulolyticus* in the presence of different substrates has not been properly characterized yet, as well as the carbon sources that may provide both maximum cell protein and endoglucanase production.

Despite the fact that pure cultures do not mirror all the complex interactions taking place in a natural community, the potential action of certain microorganisms can only be verified in *in vitro* systems. The aim of the present investigation was to study the growth of a strain of *A. cellulolyticus*

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isolated from an established sewage sludge culture, in medium containing three different carbon and energy sources, establishing its growth phases and specific growth rates. Endoglucanase activity curves were also analysed under these conditions.

MATERIALS AND METHODS

The microorganism used was *A. cellulolyticus* (ATCC 33288) isolated and characterized by Patel *et al* (11). Cells were grown in synthetic medium previously described by Hatt and Gantt (3) and modified by lowering the concentration of $Na_2S \cdot 9H_2O$ (added as reducing agent) to 0.025 $g.L^{-1}$. This medium was prepared under an atmosphere of 80% N_2 - 20% CO_2. A volume of medium free of both carbon source and reducing solution was poured into a 1000 ml serum vial containing 3 $g.L^{-1}$ of either amorphous cellulose powder (Sigma C-6288) or filter paper (Whatman N° 1). Prior to being reduced and autoclaved at 121°C for 20 minutes, the pH of the medium was adjusted to 7.6. When D-(+)-cellobiose (Sigma C-7252) was used as substrate, the solution was filter sterilized using 0.22 µm pore size filters (Millipore) and added to the autoclaved medium, to give a final concentration of 3.0 $g.L^{-1}$. A 72 h culture grown in medium containing the same substrate tested in the assay was used as inoculum (10%, v/v). Each assay started from a lyophilised culture. The final volume of the culture was 350 ml. The headspace of the vials contained 80% N_2 – 20% CO_2. Cultures were incubated at 37°C in a rotary shaker at 200 rpm. Periodically, a 10 ml sample was removed from the growing culture and centrifuged for 20 min at 39,000 x g. The cell pellet was washed twice with distilled water and cell protein content was then measured by the method of Lowry (7), in order to monitor bacterial growth; bovine serum albumin (Sigma A-4378) was used as standard.

Endoglucanase activity was assayed in culture supernatants. Quantitation of endoglucanase activity was done by determining the amount of reducing sugar generated from carboxymethylcellulose (8). Briefly, 0.5 ml of culture supernatant was incubated with 0.5 ml of 0.05M citrate buffer, pH 4.8, containing 1% carboxymethylcellulose sodium salt (CMC, Sigma C-4888), 10 mM DL-dithiothreitol (Sigma D-0632) and 0.02% (w/v) NaN_3. The assay was performed aerobically at 37°C for 30 minutes. Reducing sugars were assayed colorimetrically using dinitrosalicylic acid reagent (9). Specific growth rates were determined using a software devised for that purpose (18). With respect to culture preservation, two methods were employed: freeze drying (17) modified for anaerobic bacteria and weekly subculture in fresh media. To determine the best substrate for subculturing of *A. cellulolyticus,* two cultures were maintained during 77 days. The cells were weekly subcultured in medium containing either cellulose or cellobiose and cell protein content was checked on each occasion.

RESULTS AND DISCUSSION

Based on the growth curves obtained for the three substrates studied (Fig.1), it was possible to determine the most appropriated one for growth of *A. cellulolyticus.* A clear difference was observed among the lag phases, which were longer in the cellulose containing cultures (29 h and 17 h for amorphous and crystalline cellulose, respectively) as compared to that grown on cellobiose (7h). Different results in the literature show that a lag phase may or may not be present when *A. cellulolyticus* grows on cellobiose, even considering an inoculum of 72 h (11, 15). Patel and Mackenzie (10) obtained growth curves where the lag phase extended for 8 h, a feature that was confirmed by this paper.

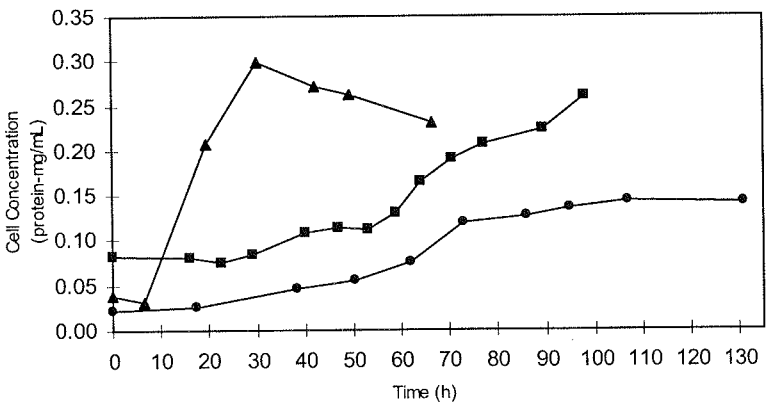

Figure 1. *A. cellulolyticus* growth curves obtained in the presence of cellobiose (▲) amorphous cellulose (■) and crystalline cellulose (●) expressed through the cellular protein concentration (average of three assays).

In the present study, the specific growth rate of *A. cellulolyticus* grown on cellobiose (0.130 h^{-1}) was 4.4 times higher than that detected when using either amorphous or crystalline cellulose as substrate (0.027 h^{-1} and 0.026 h^{-1}, respectively). A value of 0.170 h^{-1} was found by Patel and Mackenzie (10) for cultures of this microorganism in medium containing cellobiose. The low growth rates obtained with cellulose as substrate could be explained by the fact that its complex structure impose a constraint on solubilization (10). Cellulases are not characterized by fast hydrolysis of the substrate (20) and therefore do not promote a swift availability of soluble sugar; this, in turn, may affect bacterial proliferation. Although growth was slower in the presence of celluloses, the highest protein content obtained for amorphous cellulose (0.24 $mg.ml^{-1}$) was similar to that observed for cellobiose (0.27 $mg.ml^{-1}$), which establishes a very long exponential phase (Fig. 1). Cultures grown on crystalline cellulose had about 40% less protein than those grown on amorphous cellulose. Saddler and Khan (15) obtained different results, since their cultures grown on crystalline cellulose (avicel) and on cellobiose reached maximum protein values of 0.22 $mg.ml^{-1}$ and 0.20 $mg.ml^{-1}$, respectively. Saddler *et al.* (14) obtained lower values than these: 0.072 $mg.ml^{-1}$ for cellobiose, 0.060 $mg.ml^{-1}$ for amorphous cellulose, 0.035 $mg.ml^{-1}$ for crystalline cellulose and 0.084 $mg.ml^{-1}$ for avicel. Therefore, taken together, the data in the literature show that the cell protein content of the cellulolytic populations may differ considerably under similar culture conditions. On the other hand, it should be noted that the values obtained for cellulose cultures are probably underestimated because of the adsorption of the cells in insoluble substrate (1).

Culture preservation by sub-culturing (data not shown) showed that cellobiose does not sustain *A. cellulolyticus* viability for long periods of time. Cellulose, on the other hand, was a good substrate for this purpose. Despite having established which was the best substrate for preservation by subculturing, all the experiments started with freeze dryed *A. cellulolyticus* to ensure that the microorganisms were obtained from the same source.

Enzyme activity curves obtained for the three substrates studied indicated the most appropriated source to produce endoglucanase.

Fig. 2a shows that endoglucanase activity in cultures grown on cellobiose was detected during the exponential phase, that is, earlier than on both crystalline and amorphous cellulose, probably because growth in the presence of that sugar also took place earlier. Cultures on amorphous cellulose presented enzymatic activity during the lag phase (Fig. 1). Contrary to cellobiose fed cultures, endoglucanase activity in cultures grown on cellulose was always on the increase until the end of the tests. Fig. 2b shows a trend towards stability of specific activity during the stationary phase in the presence of amorphous cellulose, indicating that a greater enzymatic production does happen during the exponential growth phase. However, differing from other cultures, a raise in endoglucanase activity at the end of the exponential phase was observed in cultures grown on crystalline cellulose. Similar results were obtained for enzyme specific activity suggesting that the bacterial population continued to produce endoglucanase even during the stationary phase (almost half of the total measured activity). When analyzing the growth phases at which extracellular bacterial enzymes are released, Priest (13) noticed that, generally, there is little or no enzymatic secretion during the lag phase, except when they act on substrates that are their main or sole carbon and energy source. This was observed in the present study, where cultures grown on amorphous cellulose were active during the lag phase. The production of endoglucanase during the stationary phase when bacteria were grown on crystalline cellulose was an exception.

Endoglucanase activity of cultures grown on amorphous cellulose was 2.8 times higher than that of cultures grown on cellobiose (Fig 2a). The specific activity when using amorphous cellulose was 3.6 times higher compared to cellobiose containing cultures (Fig 2b). These results indicate that the low cell content observed on amorphous cellulose was able to produce the higher endoglucanase activity. Maximum endoglucanase activity measured in supernatants of cultures grown on crystaline cellulose was 1.2 times higher than that of cultures grown on cellobiose. Similar results were obtained by Saddler and Khan (15). Endoglucanase activity in culture supernatants of *A. cellulolyticus* grown on amorphous cellulose was 2.4 times higher than that detected when medium contained crystalline cellulose. These values do not agree with other data on endoglucanase activity in cultures grown on crystalline cellulose, which was reported to be about 1.4 times (14) or 1.1 times (16) higher than that on amorphous cellulose; also, in such studies, the specific activities were the same for both

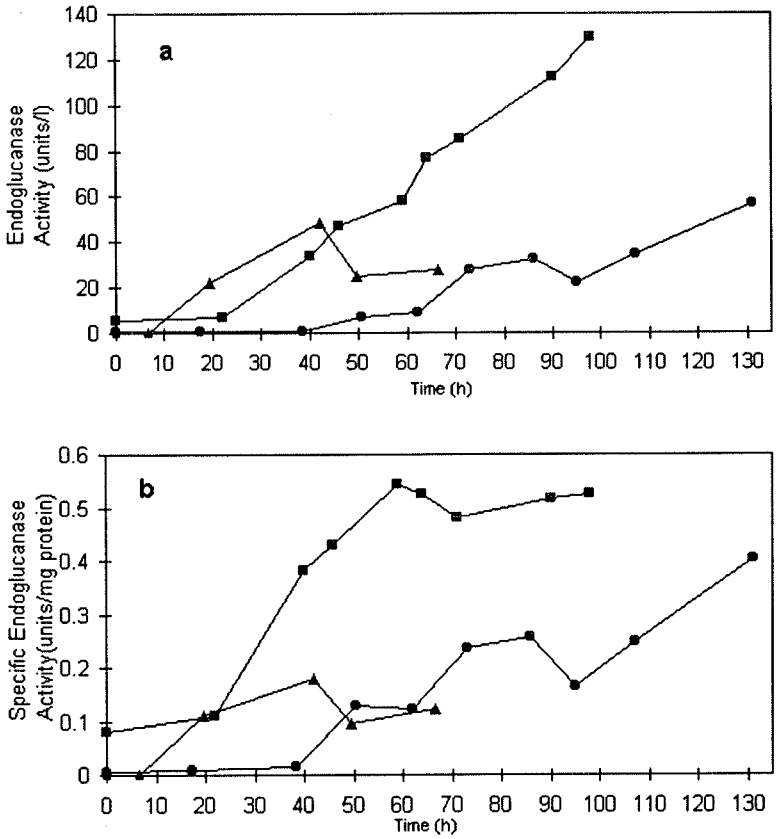

Figure 2. Endoglucanase activity curves of *A. cellulolyticus* grown on cellobiose (▲) amorphous cellulose (■) and crystalline cellulose (●)in culture supernatant (a) and specific activity (b) (the results represent the average of three assays).

substrates. Likely, the results presented in this report show similar maximum values for endoglucanase specific activity in both cultures grown on amorphous and on crystalline cellulose; however the curves showed that the enzyme production was delayed in cultures grown on crystalline cellulose.

Endoglucanase production in cultures grown on amorphous cellulose was, in this study, always higher compared to cultures grown on cellobiose and crystalline cellulose (Fig. 2a), indicating that amorphous cellulose is the best substrate for its production.

Taking together the results obtained with the different substrates presently used, it might be possible to infer some features of *A. cellulolyticus* endoglucanase regulation. As with other cellulolytic microorganisms, *A. cellulolyticus* endoglucanase synthesis could be viewed as an adaptive phenomenon. Up to now, no metabolic inducer has been described for this enzyme in *A. cellulolyticus*. In *Clostridium termocellum*, phylogenetically related to *A. cellulolyticus* (5), substrates that are metabolized more readily may lead to repressed cellulase synthesis and enzyme induction may only be triggered by low substrate concentration (4). This is confirmed by the present study, which shows that, when using cellulose as substrate, *A. cellulolyticus* growth rate was low but enzyme production was higher. The cellobiose concentration presently used (3.0 $g.L^{-1}$) was very close to that employed by Stoppok *et al.* (19) for growth of *Cellulomonas uda* (2.9 $g.L^{-1}$) and the results obtained were also similar, that is, a lower production of endoglucanase was found in the presence of cellobiose as compared to cultures grown on amorphous cellulose. The authors suggested inhibition of endoglucanase activity by a high cellobiose concentration and also proposed a mechanism of induction of enzyme production.

The results presented herein indicate that cellulose is the best carbon source for endoglucanase production as well as preservation of *A. cellulolyticus*. Nevertheless, greater amounts of cell protein were produced at a fast rate when cellobiose was used as carbon source.

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RESUMO

Crescimento e atividade de endoglicanase de *Acetivibrio cellulolyticus* cultivado em três diferentes substratos celulósicos

O estudo da cinética de crescimento de *Acetivibrio cellulolyticus* cultivado em três diferentes substratos celulósicos (celulose amorfa, celulose cristalina e celobiose) revelou que em celobiose, a velocidade específica de crescimento foi maior que nos dois tipos de celulose. A atividade de endoglicanase foi maior em celulose amorfa; em celulose cristalina a atividade enzimática, aumentou durante a fase estacionária.

Palavras-chave: *Acetivibrio cellulolyticus*, atividade de endoglicanase, celulose

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SOME ENZYMATIC PROPERTIES OF CHOLESTEROL OXIDASE PRODUCED BY *BREVIBACTERIUM* SP

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ABSTRACT

In this study we determined some properties of the cholesterol oxidase from a *Brevibacterium* strain isolated from buffalo's milk and identified the cholesterol degradation products by the bacterial cell. A small fraction of the enzyme synthesized by cells cultured in liquid medium for 7days was released into the medium whereas a larger fraction remained bound to the cell membrane. The extraction of this fraction was efficiently accomplished in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100. The enzyme stability under freezing and at 45°C was improved by addition of 20% glycerol. The optimum temperature and pH for the enzyme activity were 53°C and 7.5, respectively. The only steroidal product from cholesterol oxidation by the microbial cell and by the crude extract of the membrane-bound enzyme was 4-colesten-3-one. Chromatographic analysis showed that minor no steroidal compounds as well as 4-colesten-3-one found in the reaction media arose during fermentation process and were extracted together with the enzyme in the buffer solution. Cholesterol oxidation by the membrane-bound enzyme was a first order reaction type.

Key words: Cholesterol oxidase, *Brevibacterium* sp, cholesterol, 3β-hydroxysteroid oxidase

INTRODUCTION

Cholesterol decomposition ability is widespread among microorganisms that have been explored as free and immobilized cells (15, 29) or as enzyme source (12) in steroid biotransformations. Cholesterol may be completely oxidized by microbial cells to carbon dioxide and water by the action of a complex enzyme system in which cholesterol oxidase is the first enzyme involved. Cell-free enzymes and microbial cells have been investigated for reduction of cholesterol level in foods (2, 3, 14, 10, 26, 28), for precursors production in manufacturing pharmaceutical steroids from cheap sterols (15) and for clinical assay of serum cholesterol (7, 25). Microbial cholesterol oxidases have received much attention in recent years, mainly due to its large use in medical practice for determination of free and bound cholesterol.

Cholesterol oxidase (3β-hydroxysteroid oxidase,

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EC 1.1.3.6) is a bifunctional enzyme catalyzing the oxidation of Δ^5-3β-hydroxysteroids to the corresponding Δ^5-3-ketosteroid and also the isomerization of this compound to the Δ^4-3-ketosteroid, with reduction of oxygen to hydrogen peroxide (21, 26, 25). Although the enzyme exhibits a broad range of steroid specificities, the presence of a 3β-hydroxyl group, as in cholesterol (5-cholesten-3β-ol), 5α-cholestan-3β-ol, pregn-5-en-3β-ol-20-one, androst-5-en-3β-ol-17-one and 5α-androstan-3β-ol-7-one, is essential for activity.

In the sequence of the cholesterol oxidation by microbial cells, 4-cholesten-3-one (Δ^4-3-ketosteroid) maybe oxidized with accumulation of the steroids 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) (4), which are important precursors of chemically synthesized hormones, or may be transformed to no steroid intermediates (1).

Cholesterol oxidases can be intrinsic membrane-bound enzymes located on the outside of the cell, as produced by *Nocardia rhodochrous* (8, 7, 27), *Nocardia erythropolis* (19), and *Mycobacterium* species (22), or can be isolated from broth filtrate as in cultivation of *Streptomyces violascens* (23), *Brevibacterium sterolicum* (24), *Streptoverticilium cholesterolicum* (15), *Rhodococcus equi* nº 23 (26), *Mycobacterium* ATCC 19652 (9) and *R. erythropolis* (22).

The aim of this work was to identify the cholesterol degradation products by *Brevibacterium* sp. cell isolated from buffalo's milk and to define some properties of the cholesterol oxidase produced by the microorganism.

MATERIALS AND METHODS

Strain identification - bacterial strain used throughout this work was isolated from buffalo's milk in a liquid medium containing cholesterol and mineral salts as described in Menezes *et al.* (16). Taxonomic studies were carried out by the classic methodology according to Collins (11) and Balows *et al.* (6), complemented by chemiotaxonomic tests. The phenotypic characterization using the API-50 CH system was conducted in accordance to the analytical profile of bioMerieux *API index* (50CH, 50CHB, 50CHL) and the identification of diaminopimelic acid (LL-/meso-DAP) was done by thin-layer chromatography with ninhydrin development.

Microorganism maintenance and enzyme production - stock culture of isolated strain, identified as *Brevibacterium* sp., was maintained on nutrient agar slants and stock culture of *Rhodococcus equi* ATCC 25729 (ATCC 6939), used as type microorganism, was maintained in glucose-yeast extract medium (GY). Organisms were subcultured at monthly intervals. Before flasks inoculation, cultures were transferred from maintenance medium to agar slants consisting of 1.0% peptone (Difco, Detroit, MI, USA), 1.0% meat extract (Difco, Detroit, MI, USA), 0.5% NaCl and 2.0% agar (Difco, Detroit, MI, USA), and incubated for 24 hours at 30°C. A loopful of the 24 hours bacterial cultures was inoculated in 500 ml Erlenmeyer flasks containing 225 ml of a medium composed of: 0.1% NH_4NO_3, 0.025% K_2HPO_4, 0.025% $MgSO_4.7H_2O$, 0.0001% $FeSO_4.7H_2O$, 0.5% yeast extract (Difco, Detroit, MI, USA) and 0.1% cholesterol (Sigma Chemical Co., USA), pH 7.0 (medium I) (5). After sterilization, the medium was homogenized by sonication at room temperature to disperse cholesterol. Cultivation was carried out on a rotary shaker at 37°C for 7 days and at agitation speed of 150 rpm.

Enzyme isolation - the supernatant from centrifuged broth at 10,000 rpm for 10 minutes at 5°C was used as extracellular cholesterol oxidase solution. Harvested cells were washed twice with ethyl acetate and frozen at -18°C. Membrane-bound enzyme was extracted from frozen cells in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100 (v/v), for 18 hours at 5°C under stirring. The extract obtained by centrifugation of the cell suspension was used as membrane-bound enzyme solution.

Enzyme assay - the activity of the extracellular and membrane-bound enzymes was determined according to the method described in Inouye *et al.* (13). To 0.4 ml of 125 mM Tris-HCl buffer, pH 7.5, was added 0.1 ml of enzyme solution, and the mixture was incubated in water bath at 37°C. After 3 minutes, 25 µl of 12 mM of cholesterol in isopropanol solution were added to the mixture. After 30 minutes, 2.5 ml of absolute ethanol were added to the reaction medium and the amount of 4-cholesten-3-one was determined by measurement of the absorbance at 240 nm. Reaction blanks were prepared by replacing 25 µl of the cholesterol solution for 25 µl of isopropanol. One unit of cholesterol oxidase activity (U) was defined as that which brings about the formation of 1 µmol of 4-colesten-3-one in 30 minutes at 37°C.

The quantity of 4-colesten-3-one was obtained from a standard curve prepared with solutions containing 10 to 100 μg of 4-colesten-3-one dissolved in isopropanol (ε=2.32x10^4 M^{-1}. cm^{-1}).

Identification of the cholesterol oxidation products by thin-layer chromatography - in order to know the cholesterol degradation products accumulated in the culture medium due to the action of bacterial cells, 10 ml of medium I in 50 ml Erlenmeyer flasks were inoculated with a loopful of 24 hours cultures of *Brevibacterium* sp. or *Rhodococcus equi* ATCC 25729 and incubated for 7 days at 30°C and at agitation speed 150 rpm. After incubation time, cells were harvested by centrifugation and 10 ml of ethyl acetate were added to the supernatant. The organic phase was recovered in a funnel and evaporated at 60°C in a rotary evaporator until a white residue was formed. The dry residue was dissolved in 1ml of methylene chloride and analyzed by thin-layer chromatography. Blank of culture medium refers to no inoculated flask incubated and treated as the other ones.

Cholesterol degradation products resulted from the action of membrane-bound enzyme were also identified by thin-layer chromatography. The reaction medium and procedure used for cholesterol oxidation in this experiment were as those used to determine the enzyme activity but ten fold volumes of buffer, enzyme and substrate were actually employed and reaction were stopped by addition of 2 ml of methylene chloride at time intervals of 30, 60 and 90 minutes. The methylene chloride fractions were recovered and analyzed by thin layer chromatography. Reaction blank, where no cholesterol was added to the reaction mixture, was prepared for each time interval and treated in the same way.

Thin-layer chromatographies were carried out on pre-coated silica gel 60 F_{254} plate (0.25 mm thick, 20 cm x 20 cm) (Merck) at room temperature. Plates were activated at 110°C for 1 hour and spotted with 25 μl of samples. Benzene:ethyl acetate 9:1 (v:v) was used as solvent system and the development was carried out by spraying H_2SO_4: methanol 5:95 (v:v) solution followed by heating at 90°C until visualization of the spots. Twenty microlitres of 5.0 mg/ml solutions of cholesterol (5-cholesten-3-β-ol), cholestenone (4-colesten-3-one), 1,4-androstadiene-3,17-dione (ADD) and 4-androsten-3,17-dione (AD), from Sigma Chemical Co., USA, were spotted as standards.

Identification of the of cholesterol oxidation products by gas chromatography - a mixture of 20 ml of the membrane-bound enzyme solution (3.86 U/ml) and 80 ml of 12 mM Tris-HCl buffer, pH 7.5, was heated at 37°C. After reaching the reaction temperature, 5.0 ml of 12 mM of cholesterol in isopropanol solution were added to the mixture. Samples of 8.5 ml of the reaction medium were collected at 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes, and also at 24, 48 and 72 hours. The enzyme reaction was stopped by adding 20 ml of ethyl acetate to each sample. After saturation of the aqueous phase with NaCl, the organic phase was separated in a funnel and dried over anhydrous sodium sulfate. Ethyl acetate phase was evaporated to dryness at 60°C in a rotary evaporator. The dry residue was dissolved in ethyl acetate:methanol 9:1 (v:v). A blank, where no cholesterol was added to the reaction mixture, was prepared for each time interval and treated in the same way. Extracted compounds were quantified by gas chromatography performed with a Pye-Unicam 4600 gas chromatograph equipped with a flame ionization detector. Samples of 1 μl were injected in a poly-dimethylsiloxane column (25 m x 0.53 mm). Operating parameters were as follows: column temperature was kept at 100°C for 2 minutes and then raised, at a rate of 25°C/min, up to 250°C followed by a heating rate of 1°C/min up to 280°C, at which temperature was kept for 3 minutes; injector temperature 280°C; detector temperature 300°C; inlet pressure 14 psi and hydrogen as carrier gas. Cholesterol, cholestenone, 4-androstene-3,17-dione, 1,4-androstadiene-3,17-dione, 25-hydroxycholesterol, 5α,6α-epoxycholesterol, 3β,5α,6β-tri-hydroxycholestan, from Sigma Chemical Co. (USA), and 5α-cholestane were used as standards.

Protein concentration - protein concentration in enzyme solution, used for electrophoresis, was estimated according to the LOWRY *et al.* method (22), after extraction of Triton X-100 with Amberlite XAD-2 according to Cheethan (8). Bovine serum albumin (Merck) was used as standard protein.

Electrophoresis - a sample of the membrane-bound enzyme solution in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100 was concentrated to 2 mg/ml of protein by polyethileneglycol (PEG) 20,000, dialyzed for 18 hours against the same buffer free of detergent, and analyzed in polyacrylamide gel. Electrophoresis was performed in gradient 8-25% gel (Phast gel gradient

8-25) with native buffer strip (Pharmacia LKB-Biotechnology, Uppsala-Sweden) in PhastSystem equipment (Pharmacia LKB-Biotechnology, Uppsala-Sweden). Proteins were silver stained according to File nº 210 from equipment users manual.

Enzyme properties - the effect of temperature on the crude membrane-bound enzyme activity was estimated by the enzyme assay method, lightly modified, in the temperature range from 35°C to 65°C. The enzyme was added to the reaction medium after the mixture of buffer and substrate solutions had reached the reaction temperature. The same procedure was used to evaluate the effect of pH on the enzyme activity at 37°C, using 100 mM phosphate buffer for pH range from 5.0 to 7.5 and 50 mM Tris-HCl buffer for pH between 8.0 and 8.8. Thermal stability of the enzyme at 45°C was investigated in the presence of glycerol or not according to the standard method, only that before the enzyme activity measurement it was maintained at 45°C for different time intervals.

RESULTS AND DISCUSSION

The isolated microorganism was identified as a strain of *Brevibacterium* sp., with characteristics shown in Table 1.

When cultured in liquid medium the isolated strain produced both membrane-bound and extracellular cholesterol oxidase as observed for three *Rhodococcus* strains studied by Aihara *et al.* (1). The membrane-bound enzyme from frozen cells was easily extracted in 1 mM phosphate buffer solution containing 0.7% Triton X-100. According to Cheetham *et al.* (8) the enzyme can be extracted in these conditions because it is composed by a water-soluble enzymically active part and a predominant hydrophobic anchor that allows the incorporation of the enzyme into the cell membrane and detergent micelles. During extraction probably occurs the breakage of hydrophobic bonds between the enzyme and membrane lipids of the cell membrane. According to the authors, frozen cells would lead to higher yields of enzyme extraction due to the weakening of lipid-protein bonding caused by taking the membrane lipids below their thermotropic transition temperature.

We studied the effect of the ratio wet cell mass: buffer volume on the effectiveness of the enzyme solubilization in two extraction steps. In these experiments the same buffer volume was used in the first and second extraction and the ratios tried out were 15, 20, 30 and 60 mg/ml. The amount of the enzyme extracted in the first buffer volume varied between 68.5% and 77.7% of the total extracted in both steps. The highest enzyme concentration, 1.93 U/ml, was observed in the first extraction solution from 60 mg/ml ratio, where the enzyme concentration was three times that in the supernatant of the culture broth.

The activity of the enzyme from *Brevibacterium* sp. was dependent of Triton X-100 concentration so that it lost completely activity at 0% detergent concentration. Similarly to the enzyme from *Nocardia rhodochrous* (8), the activity in buffer solution with 0.7% Triton X-100 (0.35 U/ml) was lower than in solution containing 0.15% detergent (0.51 U/ml). Studies on the enzyme from *Nocardia rhodochrous* activity showed that the amount of the enzyme extracted increased with detergent concentrations up to 10% (v/v) and that more enzyme was solubilized using detergent concentrations higher than 0.5% (v/v). However, as the enzyme activity was dependent of detergent concentration used, the additional activity was masked by detergent inhibition (8). This information should be considered when comparing the amount of the enzyme produced by the isolated *Brevibacterium* strain with others from literature, since it may show a similar behavior.

The eletrophoretic analysis of the crude membrane-bound enzyme showed 10 protein components (Fig. 1), showing that the enzyme was not the unique protein extracted in the detergent solution. This profile was similar to the cholesterol oxidase of *Actinomyces lavendulae* extract in Tween 80, that showed 7 components (19).

The membrane-bound enzyme in 1 mM phosphate buffer, pH 7.0, containing 0.7% Triton X-100, gradually lost its activity when stored at -18°C. Addition of 20% glycerol to the enzyme solution stabilized the enzyme activity up to 15 days of storage and diminished the loss of activity at 30 days (Fig. 2), so that, at this time the loss of activity in the presence of the compound was around 24%, whereas in the absence was around 80%.

The temperature profile of the cholesterol oxidase from *Brevibacterium* sp. pointed out optimum temperature for enzyme activity at around 53°C (Fig. 3). This value is higher than the optimum temperature for the purified enzyme from *Rhodoccocus equi* and from *Corynebacterium cholesterolicum*, with

Table 1 - Bacteriological characteristics of the isolated strain.

Morphological:
Cells: isolated rods
Colonies: small, smoth and yellowish colored on nutrient agar
Motility: negative
Gram-reaction: positive
Spore: absent

Physiological:
Oxygen requirement: strict aerobe
Temperature relations: little growth at 5°C, good growth at 30°C
NaCl broth: growth at 4.5%, growth at 8% after 7 days, growth at 12% after 12 days
Nitrate reduction: negative
Catalase: positive
Oxidase: negative
Urease: negative
Prodution of H_2S: negative
Indol: negative
TDA: negative
Arginin dehydrolase, lisine decarboxilase, ornitine decarboxilase: negative
Casein, starch: not hydrolysed
Cell wall type: LL-/meso DAP
Gelatin hydrolysis: negative

Acid production from carbohydrates:

Control: negative	Inositol: negative	Melezitose: negative	Adonitol: negative
Glycerol: negative	Manitol: negative	D-Raffinose: negative	Dulcitol: negative
Erythritol: negative	Sorbitol: negative	Starch: negative	Galactose: negative
D-Arabinose: negative	α-m-D-Manoside: negative	Glycogen: negative	D-Glucose: positive
L-Arabinose: negative	α-m-D-Glycoside: negative	Xylitol: negative	D-Fructose: negative
Ribose: negative	N-Acetyl-glycosamine: negative	β-Gentiobiose: negative	D-Manose: negative
D-Xylose: negative	Amidaline: negative	D-Turanose: positive	L-Sorbose: negative
L-Xylose: negative	Arbutine: negative	D-Tugatose: negative	Ramnose: negative
Gluconate: negative	Esculin: negative	D-Fucose: negative	Salicin: negative
2-Keto-gluconate: negative	Melibiose: negative	L-Fucose: negative	Celobiose: negative
5-Keto-gluconate: positive	Sucrose: negative	D-Arabitol: negative	Maltose: positive
Inulin: negative	Trehalose: negative	L-Arabitol: negative	Lactose: negative
β-Methyl-xyloside: negative			

maximum activity at approximately 47°C (26) and 40°C (20), respectively. It is closer to that for the purified enzyme from *Streptomyces violascens,* with optimum temperature at around 50°C, with a plateau between 40°C and 60°C (23). 20% glycerol stabilized the enzyme at 45°C, so that after 30 minutes at this temperature the enzyme lost around 1% of the activity against a loss of 20% when the compound was not added (Fig. 4).

The enzyme was pH dependent as shown in Fig. 5, with maximum activity at around 7.5. Enzyme activity was at least 80% of the maximum value at pH range between 6.0 and 8.7. The optimum pH for cholesterol oxidase from *Brevibacterium* sp. activity was similar to that found in literature for the same enzyme from other microorganisms. Usually the optimum pH for the enzyme activity is between 7.0 and 8.0, as can be seen for the enzymes from *Actnomyces lavendulae* mycelium (19), *Corynebacterium cholesterolicum* (20), *Streptoverticillium cholesterolicum* (13), *Rhodococcus equi* nº 23 (26) and from *Streptomyces violascens* (23).

Thin-layer chromatography of the products accumulated in the culture medium fermented by *Brevibacterium* sp. and *Rhodococcus equi* ATCC

Figure 1: Electrophoresis of crude membrane-bound enzyme in gradient polyacrilamide gel 8-25%. 2 mg/ml protein in 1 mM phosphate buffer containing 0.7% Triton X-100. Silver stained proteins.

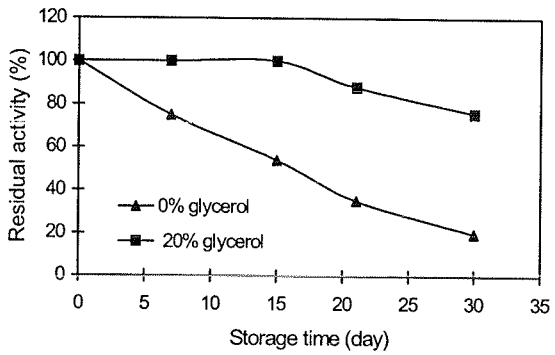

Figure 2: Stability of cholesterol oxidase at -18°C in 1 mM phosphate buffer solution containing 0.7% Triton X-100 added by 0% and 20% glycerol.

25729 cells during 7 days is shown in Fig. 6. During fermentation, cholesterol was oxidized by both strains to 4-colesten-3-one (spots 6 and 7) with very small amounts of the no oxidized compound still remaining in the culture medium. Although not visible in the photography, the samples showed a very faint spot with R_f value greater than for 4-colesten-3-one. The compound also appeared, although in lower intensity, in the control sample,

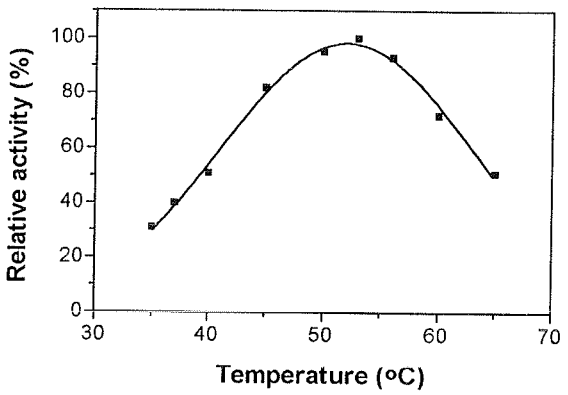

Figure 3: Effect of temperature on the cholesterol oxidase activity at pH 7.5.

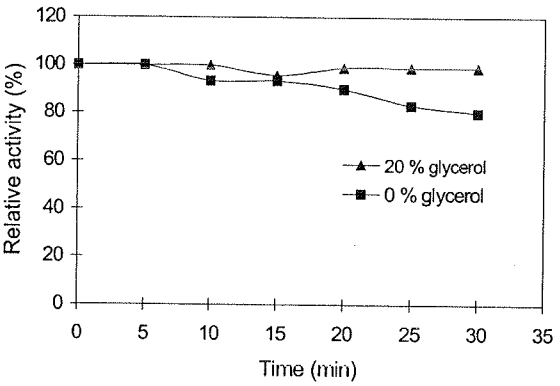

Figure 4: Thermal stability of cholesterol oxidase at 45°C in 1 mM phosphate buffer solution containing 0.7% Triton X-100 added by 0% and 20% glycerol.

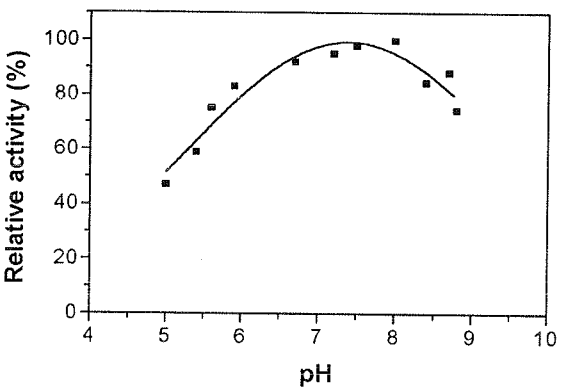

Figure 5: Effect of pH on the cholesterol oxidase activity at 37°C. 100 mM phosphate buffer was used for pH 5.0-7.5 and 50 mM Tris-HCl buffer was used for pH 8.0-8.8.

where no microorganism was inoculated, revealing that its presence was not due to the action of the microorganisms or to their enzymes on cholesterol, but probably to a metabolic compound excreted in the broth, or to a component of the medium formulation.

Cholesterol oxidation by the membrane-bound enzyme from *Brevibacterium* sp. resulted in 4-colesten-3-one as main product (Fig. 7, spots 6, 8 and 10) even after 90 minutes of reaction. Both reaction media (spots 6, 8 and 10) and controls, where no cholesterol was added, (spots 5, 7 and 9), showed very faint spots (not visible on the photography) on thin layer chromatography corresponding to a product with an R_f value higher than for 4-cholesten-3-one, the same spot detected on the chromatography of the fermented media (spots 6 and 7 in Fig. 6). As the compound was also detected in the controls of the reaction media, it was supposed to be extracted in the enzyme solution. Chromatography of the controls of the reaction media revealed the presence of 4-cholesten-3-one, probably adhered to the cell during fermentation course and extracted in the enzyme solution.

Gas chromatography analysis of the samples of reaction media and controls detected 7 to 16 peaks, most of them with retention time lower than for 5α-cholestane. Almost invariably 5 peaks, with retention times: 25.12 ± 0.06 min. (cholestenone), 11.89 ± 0.07 min., 18.78 ± 0.05 min., 26.45 ± 0.06 min. and 34.18 ± 0.07 min., were detected in the controls and in the reaction media. These four late compounds were at unchanged concentrations in all samples and were assumed to come from the membrane-bound enzyme extract. None of these compounds were identified as 25-hydroxycholesterol or 3β,5α,6β-trihydroxycholestane, both of which recognized as inhibitor of cholesterol biosynthesis and/or angiotoxic (17) or 5α,6α-epoxycholesterol, a potentially carcinogenic compound (18). 4-androsten-3,17-dione or 1,4-androstadien-3,17-dione were not detected in the samples at all. A peak at 21.91 ± 0.01 min., corresponding to cholesterol, was detected in the reaction medium at 105 minutes, showing that the conversion of the product to cholestenone was not complete until that time. The only time dependent concentration compounds in the reaction media were cholesterol and cholestenone and the rate of oxidation of that compound by the enzyme from *Brevibacterium* sp followed a first order kinetic, in which the total conversion occurred in

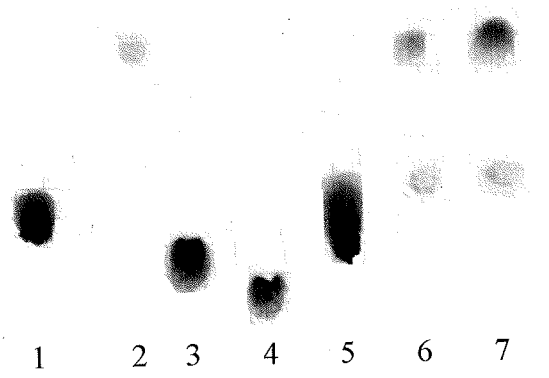

Figure 6: Thin-layer chromatography of medium I fermented by *Brevibacterium* sp. (spot 5) and *Rhodococcus equi* 25729 (spot 6) for 7 days at 37°C at agitation speed 150 rpm. 1-cholesterol, 2-cholestenone, 3-AD, 4-ADD, 5-no inoculated medium. Running solvent: benzene : ethyl acetate 9:1 (v/v). Development system: H_2SO_4 : methanol 5:95 (v/v).

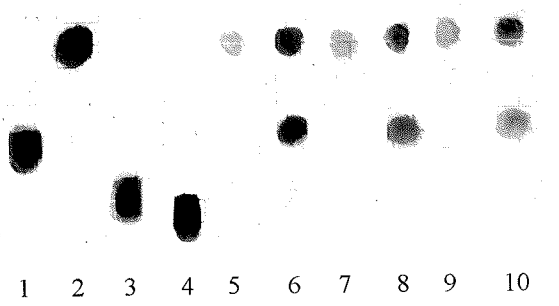

Figure 7: Thin-layer chromatography of reaction media and controls at different reaction times. 1- cholesterol, 2-cholestenone, 3-AD, 4-ADD, 5-control of reaction medium at 30 min., 6-reaction medium at 30 min., 7-control of reaction medium at 60 min., 8-reaction medium at 60 min., 9-control of reaction medium at 90 min., 10-reaction medium at 90 min. Running solvent: benzene : ethyl acetate 9:1 (v/v). Development system: H_2SO_4 : methanol 5:95 (v/v).

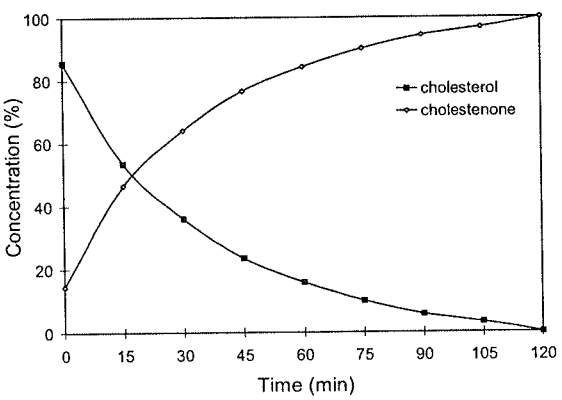

Figure 8: Cholesterol consumption and cholestenone production by membrane-bound cholesterol oxidase from *Brevibacterium* sp.

120 minutes (Fig. 8). An increase in the time reaction until 72 hours did not give rise to other compounds than cholestenone. These results show that, on the contrary of the extracellular enzyme from *Rhodococcus equi* nº 23 (2), the crude extract of the membrane-bound enzyme do not have enzymes for successive degradation of cholesterol.

ACKNOWLEDGMENTS

This work was supported by grants from RHAE/ CNPq

RESUMO

Algumas propriedades enzimáticas da colesterol oxidase produzida por *Brevibacterium* sp.

Neste trabalho foram definidas algumas propriedades da enzima colesterol oxidase produzida por uma linhagem de *Brevibacterium* sp. isolada de leite de búfala e foram identificados os compostos resultantes da degradação do colesterol pela bactéria. Uma pequena fração da enzima sintetizada pelas células cultivadas em meio líquido por 7 dias foi liberada no meio de cultura e uma fração maior permaneceu ligada à membrana celular. A extração desta fração foi eficientemente efetuada em tampão fosfato 1mM, pH 7,0, contendo 0,7% de triton X-100. A estabilidade da enzima congelada e a 45ºC foi aumentada pela adição de 20% de glicerol. A temperatura ótima para a atividade enzimática esteve ao redor de 53ºC e o pH ótimo esteve ao redor de 7,5. O único produto da degradação do colesterol, causada pela ação da célula bacteriana e pela colesterol oxidase ligada à membrana, acumulado no meio de cultura foi o 4-colesteno-3-ona. Análise cromatográfica revelou que uma pequena quantidade de compostos não esteroídicos, além de 4-colesteno-3-ona, formados durante a fermentação, permanecem aderidos à célula e são extraídos juntamente com a enzima na solução com detergente. A oxidação do colesterol pela enzima bruta ocorreu segundo uma equação de primeira ordem.

Palavras-chave: colesterol oxidase, *Brevibacterium* sp., colesterol, 3β-hidroxiesteroide oxidase

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PURIFICATION OF MICROBIAL β-GALACTOSIDASE FROM *KLUYVEROMYCES FRAGILIS* BY BIOAFFINITY PARTITIONING

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ABSTRACT

This work investigated the partitioning of β-galactosidase from *Kluyveromyces fragilis* in aqueous two-phase systems (ATPS) by bioaffinity. PEG 4000 was chemically activated with thresyl chloride, and the biospecific ligand p-aminophenyl 1-thio-β-D-galactopyranoside (APGP) was attached to the activated PEG 4000. A new two-step method for extraction and purification of the enzyme β-galactosidase from *Kluyveromyces fragilis* was developed. In the first step, a system composed of 6% PEG 4000-APGP and 8% dextran 505 was used, where β-galactosidase was strongly partitioned to the top phase (K = 2,330). In the second step, a system formed of 13% PEG-APGP and 9% phosphate salt was used to revert the value of the partition coefficient of β-galactosidase ($K = 2 \times 10^{-5}$) in order to provide the purification and recovery of 39% of the enzyme in the bottom salt-rich phase.

Key words: β-galactosidase, aqueous two-phase systems, protein purification, downstream-processing, affinity

INTRODUCTION

Partitioning of biomaterials in aqueous two-phase systems (ATPS) is a selective method for purification and for analytical studies of cellular components of several sizes, including proteins, nucleic acids, membranes and cellular organelles. An ATPS is formed by the addition of aqueous solutions of two polymers, such as PEG and dextran, or a polymer and a lyotropic salt, such as PEG and potassium phosphate. The extraction and separation process in ATPS can be used as a substitute for the initial steps of purification and of preparative chromatography of biomaterials, can be scaled-up without a significant loss of efficiency and can be accomplished in the absence of sophisticated equipment (2, 16).

The choice of a non-aggressive method such as ATPS partitioning maintains the biological properties of biomolecules. An ATPS contains a large amount of water in both phases, constituting an excellent mild biological method to recover cells, organelles or active proteins (2). Methods described in the literature, such as liquid-liquid extraction, are gaining prominence in meeting the basic demand due to the viability of their industrial applications (9).

Dextran and polyethylene glycol are not toxic chemicals, they are included in the pharmacopoeias of many countries and have applications in the food

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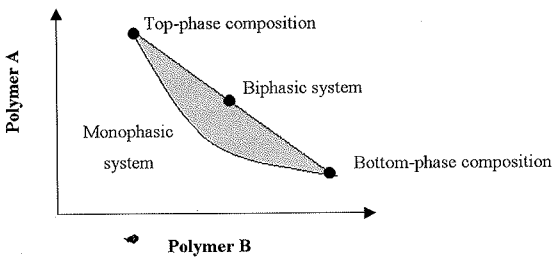
industry (17). Utilization of these polymers can be considered an advantage in the development of new technologies because a lot of applications of enzymes and biologically active proteins in the food and pharmaceutical industries (11).

In order to achieve a high recovery and the concentration of a target protein in one phase of the ATPS, it is necessary to increase the difference between the value of the partition coefficient (K = concentration of the protein in the top phase/bottom phase) of the target protein and the K value of the contaminant material (K_p). Fig. 1 shows a diagram of an ATPS.

Figure 1. Binodial curve of an aqueous two-phase system.



Parameters such as polymer molecular mass and concentration, type and concentration of salt, pH and temperature can affect K (4).

The process of purification in ATPS by bioaffinity combines the property of biological recognition and partitioning in a liquid environment. The ligand is coupled by covalent bonds to the polymer phase; therefore it will have a high partition coefficient towards the phase enriched with the polymer. If the target protein to be isolated has a specific affinity for the ligand, the formation of a ligand-biomolecule complex will induce an increase in the partition coefficient of the protein towards the phase enriched in the ligand (5, 15), while the presence of contaminant molecules will be predominant in the opposite phase (9).

The enzyme lactase or ß-galactosidase has many applications in dairy technology, such as for industrialized products containing lactose. ß-galactosidase can also be applied in crystal removal, production of sweeteners, solubilisers and toothpaste and research and analytic activities (12). The transglycosylation activity of ß-galactosidase, leading to the synthesis of oligosaccharides, has been reported by Prenosil *et al.* (23) and Brena *et al.* (6). Lactase-hydrolyzed products are currently being manufactured in Brazil, and the demand for such products would increase if this enzyme were produced at a lower cost and a higher quality.

This enzyme is intracellularly formed in yeasts and bacteria and secreted by fungi. The most important microorganisms which produce this enzyme are species of *Aspergillus niger, A. oryzae, Kluyveromyces fragilis* and *K. lactis* (16). Its production by *Neurospora* (19), *Escherichia coli* (29), *Saccharomyces lactis* (13), *Bacillus circulans* (14), *Scopulariopsis* sp (22) and *Erwinia aroidea* (10) has also been reported.

MATERIALS AND METHODS

PEG 4000 was purchased from Fluka (Switzerland) and tresyl chloride from Sigma (St. Louis, MO, USA). The *Kluyveromyces fragilis* strain was obtained from the American Type Culture Collection (ATCC 46537). A commercial β-galactosidase (Lactozym) was the kind gift of Novo Nordisk.

Enzyme production

For the production of β-galactosidase, *K. fragilis* was grown at 37°C for 24 h in the following culture medium: 30 ml of commercial milk, 0.15 g of $(NH_4)_2SO_4$ and 0.06 g of KH_2PO_4, with pH adjusted to 6.5. The fermented culture broth was centrifuged at 3,000 g for 20 min. The mass of cells was weighed and chloroform was added in the proportion of 1:1 (w/w). This suspension was kept under magnetic stirring for 1 hour, and then 20 ml of 0.05 M phosphate buffer, pH 7, was added and centrifuged. This procedure was repeated three times. The supernatant containing intracellular material of *K. fragilis* was used in the experiments.

Determination of β-galactosidase activity

The enzyme activity was determined by using o-nytrophenyl-β-galactopyranoside (ONPG) as the substrate and by spectrophotometric measurement at 420 nm. The enzyme was assayed at 37°C. One β-galactosidase unit corresponds to 1.0 µmol of orthophenol released per minute under the given condition.

Partitioning in the ATPS

One hundred ml of enzyme sample was mixed into the ATPS, using a Vortex for 30 s. Phase

separation was achieved by centrifugation for 5 min at 3,000 g and the interface of each system discharged. A known volume of each phase was collected and the activity was determined. The partition coefficient (K_E) of the enzyme was calculated from the ratio of β-galactosidase activity found in the top phase and activity found in the bottom phase.

Protein assay

The main contaminant protein concentration was determined by the dye-binding technique of Sedmak and Grossberg (25). Fifty to 100 µl of the top phase was transferred from each prepared system to a cuvette containing 2.4 ml of water and 1.0 ml of Coomassie blue solution and mixed well, and the OD_{595} was measured in a spectrophotometer, versus a blank which had 50 µl of a top phase of a system which had been equally prepared without any sample. The blanks were done to correct the interference of the phase components. The procedure was repeated for the bottom phase of each system. A BSA standard curve was used to calculate protein concentration. The partitioning of the main contaminant protein, K_P, was calculated as the ratio of protein in the top phase to that in the bottom phase at room temperature.

Synthesis of PEG-APGP

The PEG-APGP was synthesized according to Delgado *et al.* (8) and Nilsson and Mosbach (21).

Activation of PEG 4000 with tresyl chloride

(I) Solid PEG 4000 (20 g) was dried by azeotropic distillation in toluene and then dried in vacuum. The white solid was dissolved in 45 ml of dry dichloromethane at room temperature. The mixture was cooled to 0°C and stirred magnetically, and 1.125 ml of pyridine and 1 g of tresyl chloride at 0°C were added drop by drop. The reaction was continued at room temperature with constant stirring for 1.5 h, and the dichloromethane was removed by evaporation under reduced pressure. The white solid was dried in vacuum overnight at room temperature and formed the PEG-tresilated precursor (TPEG). TPEG was washed twice with HCl-ethanol (1:250, v:v), precipitated at 4°C and kept in a dissecator. This procedure was repeated sixtimes and the white solid was collected and dried in vacuum. In a second step, TPEG (I) reacted with Tris-HCl 0.2 M buffer, pH 8.0 for 12 hours at 4°C. It was dialyzed against water to remove the excess Tris-HCl and then concentrated by ultrafiltration (50 ml) **(II)**.

Attachment of APGP to TPEG

(III) - APGP (0.60 g) was added to the TPEG (II) and it reacted for 12 hours, ultrafiltered with water and then dried in vacuum.

Aqueous two-phase system preparation

Aqueous two-phase systems were prepared according to Franco *et al.* (11). They were prepared from stock solutions of PEG 4000 (50%, w/w), dextran T505,000 (30%, w/w) and potassium phosphate solution with K_2HPO_4 to KH_2PO_4 (40%, w/w) with a molar ratio of 0.6, pH 6.5. In the first step, the compounds were mixed to form 8.0 g of a system having a final concentration of 6% PEG 4000-APGP (compound III) and 8% dextran T505, pH 6.5. In the second step, the bottom phase of the PEG 4000-APGP/dextran system was discarded and replaced with a fresh phosphate phase, pH 6.5. The composition of the system (6.0 g total weight) was 13% PEG 4000-APGP and 9% K_2HPO_4/KH_2PO_4, pH 6.5 The system was mixed and centrifuged.

Electrophoresis

SDS electrophoresis (SDS-PAGE) was carried out in 12% homogeneous gel (18). The gels were stained with Bio-Rad silver. The molecular mass markers consisted of thyroglobulin (330 KDa), ferritin (220 KDa – half unit), albumin (67 KDa), catalase (60 KDa), lactate dehydrogenase (36 KDa) and ferritin (18.5 KDa), available as a standard kit (Pharmacia Biotech).

Specific β-galactosidase activity ($SA_{\beta\text{-gal}}$)

It is defined as the ratio of enzyme activity (U/ml) to the total protein concentration (mg/ml) and is expressed in U/mg of protein (equation 1).

$$SA_{\beta-gal} = \frac{\text{Enzime activity}}{\text{Protein concentration}} \qquad (1)$$

Purification factor (PF)

The PF concept has been used in this work as a measurement to follow the purification operations (24, 27) and is defined as the ratio of the specific β-galactosidase activity after a purification step to the initial specific β-galactosidase activity (from the aqueous enzyme extract or from a previous purification step) (equation 2).

$$PF = \frac{SA_{\beta\text{-gal}} \text{ in the collected phase}}{\text{Initial } SA_{\beta\text{-gal}}} \quad (2)$$

Recovery (R)

It is defined as the ratio of the enzyme activity collected from an aqueous phase after partitioning to the total enzyme activity added to the system (equation 3).

$$R(\%) = \frac{\text{enzyme activity of the phase}}{\text{total enzime activity added to the system}} \quad (3)$$

Selectivity (S)

It is defined as the ratio of the partition coefficient of the enzyme, K_E, to the partition coefficient of the protein, K_P.

$$S = \frac{K_E}{K_p} \quad (4)$$

RESULTS AND DISCUSSION

Our earlier experiments showed that β-galactosidase from *Kluyveromyces lactis* was not separated from the main contaminant proteins of the broth (27) in conventional aqueous two-phase systems. The pool of total proteins was mainly partitioned towards the bottom salt-rich phase of PEG/phosphate systems independently of PEG molecular mass. In order to achieve a good and efficient separation of the β-galactosidase and its main contaminants, it would be desirable to find an ATPS composition where they are mostly extracted in opposite phases. Therefore an affinity system was developed in which the APGP biospecific ligand chemically attached to PEG 4000, which was accomplished in two chemical reactions. In the first reaction PEG hydroxyls were activated with chloride to become more reactive. In the second reaction the APGP ligand was finally bound to PEG. In this present work, the amount of activated and reacted PEG was not measured because our earlier results had shown that approximately 74% of the total number of hydroxyl groups in PEG had been transformed into tresyl esters in the first activation reaction. Also, the amount of APGP bound to PEG in the second reaction had been indirectly observed to be higher than 80% of the activated hydroxyls after five hours of reaction (27). Therefore in order to assure at least the same amount of APGP bound to PEG in this work, the second reaction lasted 12 hours. Another modification of the synthesis of the PEG-APGP was the removal of the excess of free APGP molecules and of the excess of Tris-HCl buffer, which was done by extensively washing by ultrafiltration.

Partitioning of the *Kluyveromyces fragilis* β-galactosidase

In order to extract and separate β-galactosidase from the contaminant proteins, a strain of *K. fragilis* was fermented and the cells were disrupted with chloroform. The enzyme extract was partitioned in the aqueous two-phase systems described in Table 1. The observed results show a 3,280-fold increase in the K_E value, when PEG-APGP replaced the plain PEG in the 6% PEG 4000 and 8% dextran system, indicating the strong affinity of β-galactosidase for the phase containing the APGP ligand. The purification factor rose from 2.8 to 9.7 and the selectivity factor of the system rose from 1.6 to 1,650. The high selectivity value indicates the potentiality of the system for a selective extraction of β-galactosidase by liquid extraction. Enzyme yield in the first-step partitioning in the affinity system was 55%, and the 45% β-galactosidase loss at the interface would possibly be due to the strong interaction between the APGP and the enzyme, which was not totally disrupted by the condition of the β-galactosidase assay with the ONPG substrate. STEERS *et al.* (26) observed that in affinity chromatography the interaction between APGP and β-galactosidase was stronger than the interaction between β-galactosidase and other substrates such as lactose, o-nitrophenyl-β-D-galactopyranoside and isopropyl-β-D-galactopyranoside. They found that the APGP-enzyme binding could only be disrupted when a low degree of ligand substitution was used or when an alkaline buffer of borate, pH 10, was employed to elute the enzyme. A second-step purification was developed by separating the top PEG-APGP-rich phase where the enzyme was collected and mixing it with a new fresh phosphate phase.

Albertsson (2) reports on the binodial curve of the PEG 4000/dextran system, where the top phase contained 8% PEG 4000 and 3% dextran and the bottom phase contained 5% PEG 4000 and 9%

Table 1. Liquid-liquid extraction process of β-galactosidase from *Kluyveromyces fragilis*, pH 6.5.

System	K_E	K_P	R (%)	SA (U/mg)	PF	S
6% PEG 4000 8% Dextran	0.71	1.2	57 (bottom phase)	104	2.8	1.6
6% PEG 4000-APGP 8% Dextran	2,330	1.4	55 (top phase)	361	9.7	1,650
13% PEG 4000-APGP 9% phosphate	2.2 x 10-5	0.8	39 (bottom phase)	708	19	2.7 x 10-5

β-galactosidase activity = 295 U/ml
Protein concentration = 7.92 mg/ml
Specific activity = 37 U/mg

dextran. As a new PEG 4000/phosphate system, pH 6.5, had to be used in order to invert the partition coefficient of the β-galactosidase by disrupting the interaction between the APGP and the enzyme, the top PEG-APGP phase was mixed with a stock phosphate solution to give a final composition of 13% PEG-APGP and 9% phosphate system. It was assumed that the binodial curve of the PEG-APGP/dextran system was similar to the PEG 4000/dextran system.

The results from Table 1 show that the partition coefficient of β-galactosidase decreased from 2,330 to 2.2 x 10^{-5} in the second partitioning procedure in the PEG 4000/phosphate system. The purification factor was 19, the specific activity was 708 U/mg and the selectivity was 2.7 x 10^{-5}, respectively. Due to the extremely low enzyme concentration in the salt-rich phase from the PEG-APGP systems (first step) and in the PEG-APGP-rich phase (second step), the partition coefficients given are lower limit estimates based on the limit of detection of the spectrophotometer.

A commercial enzyme (Lactozym) produced by the same specie of microrganism was also extracted and purified by the procedure developed in this work (Table 2).

Table 2. Liquid-liquid extraction process of β-galactosidase from Lactozym.

Systems	K_E	R (%)
6% PEG 4000 8% Dextran	0.40	87 (bottom phase)
6% PEG 4000-APGP 8% Dextran	2,380	50 (top phase)
13% PEG 4000-APGP 9% phosphate	5.3 x 10^{-4}	26 (bottom phase)

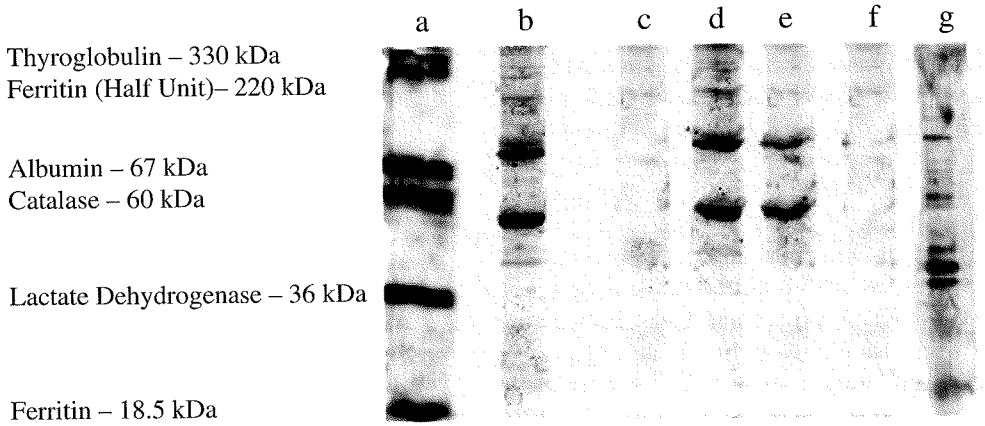
The K_E increased 5,950-fold and recovery was only 26% of the enzyme activity. The K_E observed for the Lactozym enzyme was 2,330, similar to the K_E observed for the β-galactosidase from fermentation, which was equal to 2,380 for *K. fragilis*. The system composed of 13% PEG 4000-APGP and 9% phosphate favoured the disruption of the complex enzyme ligand, and in the same way, the commercial β-galactosidase was concentrated in the bottom phosphate-rich phase.

In systems without a ligand, contaminant proteins were almost evenly distributed in both phases (K = 1.2). When the ligand was added, an insignificant increase in the partition coefficient of the contaminant proteins was found. Fig. 2 shows an electrophoretic gel of the β-galactosidase purification.

The gel shows two major bands of corresponding molecular mass, 117 KDa and 70 KDa, on the purified material. According to the literature, the molecular mass of β-galactosidase from *Kluyveromyces fragilis* is 201 KDa (20), calculated by size exclusion chromatography. They also found by SDS-PAGE that this enzyme was composed of two protein chains of MM of 120 KDa and 90 KDa, which in native conditions, would behave as a dimmer of approximately 200 KDa. If the enzyme purified in our work is assumed to be globular, a molecular mass of 187 KDa is calculated, which is only 5% below the β-galactosidase described by Mahoney and Whitaker (20).

The K/K_0 value determines the efficiency of an affinity partitioning procedure. It is described by the ratio of K, the partition coefficient in system with ligands and K_0, the partition coefficient of the enzyme in a system without ligands under otherwise identical conditions. When a ligand is coupled to the top

Figure 2. SDS-PAGE gel electrophoresis of β-galactosidase from Lactozym: a) marker proteins; b) Lactozym β-galactosidase; c) top phase of ATPS, without ligand; d) β-galactosidase extracted without ligand, bottom phase of ATPS; e) β-galactosidase extracted with ligand, top phase of ATPS; f) bottom phase of ATPS, with ligand, g) β-galactosidase (fermented).

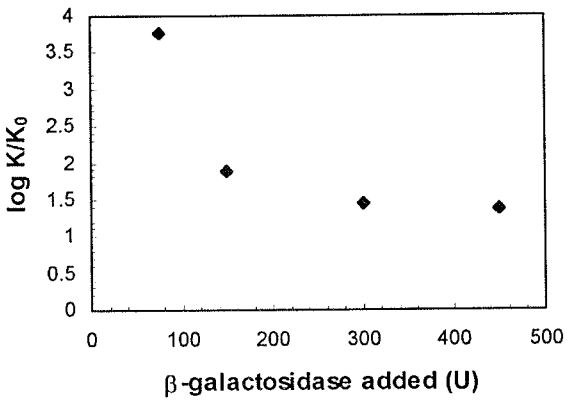


polymeric phase, K_E increases until some saturating value is reached. The value K/K_0 is proportional to the number of available ligands in the polymeric phase (1, 7).

Fig. 3 shows the effect of the enzyme concentration added to the system on the K/K_0 values. It is observed that the highest K/K_0 value 5,950, was obtained when 75 U of commercial β-galactosidase was added to the 8.0 g system.

Pastore and Park (22) purified β-galactosidase from *Scopulariopsis sp* by precipitation with ammonium sulphate and two chromatographic steps leading to a 4% yield of the pure enzyme desired. Veide *et al.* (28) developed a process of industrial isolation and purification by PEG 4000/potassium phosphate partitioning systems, for *E. coli* β-galactosidase which is followed by an ultrafiltration step to recycle the salt-rich phase. They were able to recover 95% of the β-galactosidase. Silva *et al.* (27) observed that the partition coefficient of β-galactosidase from *Kluyveromyces lactis* increased 3.7-fold in a 6% PEG 4000-APGP and 12% dextran system. The purification factor increased 1.6-fold and the recovery of the target enzyme was 83%.

Figure 3. Effect of the enzyme concentration added to the system on the K/K_0 values.



It seems clear that partitioning in ATPS can be an effective way of purifying and concentrating enzymes and other biomolecules. It can be greatly improved by designing efficient and specific bioligands which are not expensive and can be recycled (i.e., ultrafiltrated). It is desirable that the interaction between the ligand and the target enzyme can be easily disrupted by salts or by pH changes. Research on affinity techniques, binding chemistry and the designing of specific equipments for extraction in ATPS will be of great help in the improvement of downstream enzyme processing.

CONCLUSIONS

An extractive liquid-liquid system was developed with the objective of purifying the microbial enzyme β-galactosidase. As the enzyme from *Kluyveromyces fragilis* is very hydrophilic, being partitioned with the main contaminant proteins to the saline phase in conventional PEG/phosphate systems, a new two-step process was developed for the extraction and

purification of β-galactosidase. In the first step, the system involves a biospecific ligand composed of 6% PEG4000-APGP and 8% dextran. In this system the partition coefficient of β-galactosidase increased 3,280-fold compared to the initial value in a system without ligands. In the second step, a system formed of PEG 4000 and phosphate was used to revert the value of the partition coefficient of the β-galactosidase ($K = 2.2 \times 10^{-5}$), providing the purification and recovery of 39% of the enzyme. The main benefit of this work was the development of a feasible process of chemical activation of the polymer polyethylene glycol and the subsequent binding of a ligand to PEG (a hydrosoluble polymer), capable of completely altering the value of the partition coefficient of the enzyme. In the affinity step, a great number of contaminant materials were removed to the bottom phase, as the enzyme was partitioned to the top phase. In the second step, the enzyme-ligand bond was broken in the presence of a high concentration of phosphate, providing a 19-fold purification of the β-galactosidase in the saline phase in a procedure of just two steps.

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RESUMO

Purificação de β-galactosidase de *Kluyveromyces fragilis* por partição por bioafinidade

Foi desenvolvido um método novo de extração e purificação da β-galactosidase de *Kluyveromyces fragilis* em sistema de duas fases aquosas (SDFA). PEG 4000 foi ativado quimicamente com cloreto de tresila e o ligante bioespecífico p-aminofenil-β-D-tiogalactopiranosídeo (APGP) foi acoplado ao PEG 4000 ativado. Na primeira etapa foi usado um sistema composto de 6% PEG-APGP e 8% dextrana 505, onde a partição da β-galactosidase ocorreu na fase superior (K = 2.330). Na segunda etapa foi usado um sistema composto por 13% PEG-APGP e 9% fosfato para reverter o valor do coeficiente de partição da β-galactosidase ($K = 2,2 \times 10^{-5}$), obtendo-se recuperação de 39% da enzima na fase salina.

Palavras-chave: β-galactosidase, sistema de duas fases aquosas, purificação de proteína, afinidade.

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PRODUCTION OF BACTERIOCIN-LIKE INHIBITORY SUBSTANCES (BLIS) BY *STREPTOCOCCUS SALIVARIUS* STRAINS ISOLATED FROM THE TONGUE AND THROAT OF CHILDREN WITH AND WITHOUT SORE THROAT

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SHORT COMMUNICATION

ABSTRACT

Streptococcus salivarius strains, isolated from children with and without sore throat, were tested for bacteriocin production against *Streptococcus pyogenes*. *S. salivarius* strains producing bacteriocin-like inhibitory substances (BLIS) against *S. pyogenes* were more frequently found in children without sore throat. These results suggest that these children may be protected against sore throat by the presence of BLIS-positive *S. salivarius* strains.

Key-words: *Streptococcus salivarius, Streptococcus pyogenes*, bacteriocin-like-inhibitory-substances, BLIS

Bacterial interference has been recognized as a tool to prevent certain diseases. Several researchers believe that this is a natural protection against some pathogenic bacteria. *Streptococcus* are usually isolated from the mouth and are the dominant bacteria of this habitat. Among them, *S. salivarius,* a non pathogenic bacterium, is numerically the most significant of those on the tongue (5). Antagonism between *Streptococcus*, based on production of bacteriocins, has been investigated (2, 12).

The term "bacteriocin" was first coined by Jacob *et al.* (4). More recently, this term has been used to designate bacterial peptides and protein antibiotics which are ribossomally produced or derived from gene-encoded precursor peptides, and towards which the producing strain possesses a specific self-protection mechanism (8). Inhibitors that do not fulfill this criteria should be classified as bacteriocin-like inhibitory substances (BLIS) (12).

A wide range of interactions have been observed within oral bacteria including interactions with agents of throat infection. The potential protective role played by the resident microflora was explained by Sanders (11), who compared the inhibitory viridans streptococci in the throat of children infected with Group A streptococci with children who had not been infected. This inhibitory activity was present in children without *S. pyogenes* and was independent of the Group A test organism and inoculum size. Fantinato and Zelante (2) noted that among the oral bacteria which produce BLIS

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against *S. pyogenes, S. salivarius* was one of the most common in the mouth.

The aim of the present study was to verify if the presence of BLIS-positive *S. salivarius* strains in the mouth and throat of children with and without sore thoats, could be associated to protection against throat infection.

The samples were collected from 54 children who had frequently experienced sore throat (Group I) and from a control group of 50 children who had not experienced this disease in the last 3 years (Group II). The samples of Group I were collected in the Taubate Hospital (São Paulo - Brazil), from children with clinically diagnosed throat infections. The samples of Group II were collected from an Intermediate School in S. José dos Campos (São Paulo - Brazil), from children who did not experience sore throats. All children were 7 to 14 years old. A consent form and a questionaire about the frequency of the illness were full filled by the parents or guardians of all children.

Samples were collected by swabbing the tongue and throat. Mitis Salivarius Agar (Difco) was used to isolate *S. salivarius* strains. Ten colonies from each plate were submitted to biochemical identification, following the scheme outlined by Hardie and Bowden (3). Bacteriocin production was tested by performing the deferred antagonism test (1), using *S. pyogenes* ATCC 8133 (Type 23) as the indicator strain.

Any child was presenting BLIS positive *S.salivarius* strains either on the tongue or in the throat was considered a carrier.

Results in Table 1 show that 53.7% of children from Group I and 98% of children from Group II were BLIS-positive *S. salivarius* carriers. A statistically significant difference between results for Group I and II was found, at 5% level.

The results suggest that the presence of BLIS-positive *S. salivarius* strains in children who had not developed sore throats may be related to protection against throat infection.

As noted by Pichichero (6), sore throat is one of the most commom problems in children and Group A *Streptococcus* infections account for nearly all sore throats of bacterial etiology. The author also commented about the therapy failure in 20% of children under antibiotic treatment.

Sanders *et al.* (10) evaluated the effects of orally administered antibiotics on the composition of the normal throat flora and its ability to inhibit the growth of group A streptococci; the interfering activity of the flora was remarkedly diminished or absent during penicillin therapy.

Several studies were performed on the treatment of recurrent streptococcal tonsilitis by Roos *et al.* (7). Alpha-streptococci, inoculated into the throat of the patients, presented a successful protective effect against recurrence.

S. salivarius can be considered more appropriate than other streptococci for prevention of throat infections since this species is one of the most active antagonist against group A streptococci.

RESUMO

Produção de substâncias inibidoras semelhantes à bacteriocina por cepas de *Streptococcus salivarius*, isoladas da língua e garganta de crianças com e sem dor de garganta

Cepas de *Streptococcus salivarius,* isoladas de crianças com e sem dor de garganta, foram testadas quanto à produção de bacteriocina contra *Streptococcus pyogenes*. Os resultados mostraram que as crianças que não tinham dor de garganta

Table 1 - Isolation of BLIS[a] producing *Streptococcus salivarius* strains from children with and without sore throats

Group[b]	Number of Children	Total of strains[c]		Total of BLIS + strains		BLIS + strains Carriers
		Tongue	Throat	Tongue	Throat	
I	54	46/85.1	48/88.8	24/44.4	22/40.7	29/53.7
II	50	48/96.0	49/98.0	43/86.0	43/86.0	49/98.0
Total	104	94/90.3	97/93.2	67/64.4	65/62.5	78/75.0

[a]=Bacteriocin-like inhibitory substances
[b]=Group I, children with sore throat
Group II, children without sore throat
[c]=Number/%

possuiam, na boca, cepas de bactérias produtoras de substâncias inibidoras semelhantes à bacteriocina contra *S. pyogenes*.

Palavras-chave: *Streptococcus salivarius, Streptococcus pyogenes*, substâncias inibidoras semelhantes à bacteriocina, BLIS

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CANDIDA SP IN THE ORAL CAVITY WITH AND WITHOUT LESIONS: MAXIMAL INHIBITORY DILUTION OF PROPOLIS AND PERIOGARD

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ABSTRACT

Fifty individuals of both sexes aged on average 45.2 years were evaluated at the Semiology Clinic of FORP-USP in order to isolate and identify yeasts from the oral cavity, with and without lesions, and to determine the maximal inhibitory dilution (MID) of the commercial products Propolis (Apis-Flora) and Periogard (Colgate) against the strains isolated. Yeasts of the genus *Candida* were detected in the saliva of 9/19 (47.4%) individuals with a clinically healthy mouth, 18/22 (81.8%) of individuals with oral lesions, and in 4/9 (44.4%) of patients with deviation from normality, and were detected in 19/22 (86.4%) of the lesions. In the group with oral candidiasis, we isolated in tongue and lesion, respectively for each specie: *C.tropicalis* (8% and 10.7%), *C.glabrata* (4% and 3.6%) and *C.parapsilosis* (2% and 3.6%), in addition to *C.albicans* (71.4% and 67.8%) as the only species and the prevalent. The total cfu counts/ml saliva showed a higher mean value in the group with oral candidiasis (171.5% x 10^3) than in the control group (72.6 x 10^3) or the group with abnormalities (8.3 x 10^3). Most of the test strains 67/70 (95.71%) were sensitive to the antiseptics, with Propolis presenting a MID of 1:20 for 54/70/77.1%), and Periogard a MID of 1:160 for 42/70 (60%) strains from healthy sites, results similar to those obtained with strains from oral lesions. Different results were mainly observed among different species. The results indicate the possibility of using the antiseptics Propolis and Periogard (chlorhexidine) for the prevention and treatment of oral candidiasis.

Key words: Oral candidiasis, Propolis, chlorhexidine, yeasts

INTRODUCTION

Yeast-like fungi establish a biological link with the host which guarantees their saprophytic condition by establishing an ecological equilibrium denoted "amphibiosis" (17). When this equilibrium is broken due to different endogenous and/or exogenous factors these amphibionts behave as opportunistic microorganisms, eventually causing multiple oral infections which, if unresolved, may become generalized, leading to more severe mycoses (2).

The lesions of candidiasis are more frequent on

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the tongue, cheeks and palate (5), sites that may be more frequently and densely colonized in subjects arraying yeasts of the genus *Candida* (24). Although the most frequent etiologic agent of oral candidiasis is *Candida albicans* (9,24), other species of the genus, such as *C.tropicalis*, *C.glabrata*, *C.krusei* and *C.parapsilosis*, among others, may be responsible for this type of mycosis (24).

Antifungal prophylaxis may be indicated for the prevention of colonization or multiplication of *Candida* spp in patients susceptible to primary infection and also for the prevention of recurrence in patients previously submitted to antimycotic treatment (2).

Propolis is widely used in the preparation of medications in different countries in the world, not only in Europe when it has been used as a therapeutic substance for many years, but also in some South American countries such as Brazil and Uruguay (14,21). Its presence has been reported in compounds with antifungal activity against yeasts (6) and filamentous fungi (11). Rojas and Lugo (16) demonstrated the presence of antifungal activity of an alcohol extract of Propolis against 23 strains of yeasts of the genus *Candida* isolated from different biological materials, with a fungistatic action at low concentrations (0.85mg/ml for 2 hours of treatment and 0.55mg/ml for 24 hours of treatment).

Periogard contains chlorhexidine gluconate as the active principle, a substance that has proved to be effective in the chemical control of dental plaque and the consequent prevention of gingivitis, mainly in special patients (3,13), and also as an antifungal agent (4).

Although standard mechanical methods are generally preferred to the use of some chemical product for the routine control of dental plaque, the supervised use of chlorhexidine is indicated in certain situations in which it is difficult and painful to maintain the desirable level of oral hygiene (8), i.e., among older individuals and among patients with mental problems and with Down's Syndrome (13,20) or medicinally compromised (diabetes, anticancer treatment, bone marrow transplant, etc.).

The *in vitro* evaluation of the yeast sensitivity to antiseptics has been little studied even though the application of oral antiseptics deserves to be considered at least as a preventive measure or as an alternative or a complementary procedure in treatment (10,13).

The objectives of the present study were: 1) to isolate, identify and determine the prevalence of yeasts in the oral cavity of individuals with and without lesions, and 2) to test the minimum inhibitory dilution (MID) of Propolis and Periogard against the isolated yeasts.

MATERIALS AND METHODS

The study was conducted on 50 adults of both sexes aged on average 42.56 years, 19 of whom (38.0%) presented oral health (control group), 22 (44.0%) had some type of intraoral lesion with suspected oral candidiasis, and 9 (18.0%) showed deviation from normality (fissured and/or coated tongue).

Approximately 2.0 ml of nonstimulated saliva was collected from each patient into 20 x 150 mm sterilized tubes containing glass beads. The tubes were then shaken in a Mixtron-Toptronix apparatus until a uniform suspension was obtained, to be used for serial decimal dilutions in phosphate buffered saline (PBS).

Aliquots of 0.1 ml of pure saliva from each of three dilutions (10^{-1}, 10^{-2} e 10^{-3}) were added to the center of a 15 x 100 mm Petri dish containing agar Sabouraud plus chloramphenicol (Sba) and then seeded with a sterilized L-shaped glass rod. The plates were then incubated at 37°C for 24-48 h and stored at room temperature for the subsequent tests.

Material was collected aseptically from the dorsum of the tongue of each patient. After clinical examination of the individuals with lesions clinically suspected to be caused by yeasts, material was collected from the lesions with the aid of a flame-sterilized, and cooled platinum loop and directly seeded onto agar (Sba) distributed into different test tubes. After seeding, all tubes containing samples from the tongue, or from the lesions were incubated at 37°C for 3 to 5 days, reisolated in agar Sabouraud, and distributed into different plates by the depletion technique.

The yeasts were identified by classical methods (12,22) using the following tests: formation of germinative tubes, study of micromorphology, assimilation of carbon and nitrogen sources, fermentation, urea hydrolysis, and triphenyltetrazolium reduction.

The inocula for the identification tests were obtained from recent cultures (24 to 48 hours at 37°C) on agar Sabouraud after culture purity was confirmed.

For the detection of the MID of Propolis and Periogard, 70 yeast strains of different species of

the genus *Candida* isolated from the oral cavity of subjects without lesions were tested. The Propolis sample tested was supplied by Apis-Flora (Ribeirão Preto/SP) in the form of an alcohol extract containing quantities corresponding to 10 g% of the soluble solids present. Periogard, a pharmaceutical product launched on the Brazilian market by Colgate (Osasco/SP) which contains 0.12% chlorhexidine was purchased on the local market.

The antifungal activity of the products tested was determined by the technique of dilution on a solid medium (19). Serial dilutions of the antiseptics were prepared in duplicate in sterilized distilled water, corresponding to 1:20 to 1:300 for Periogard and to 1:20 to 1:320 for the alcohol extract of Propolis for which a tube containing only 60% ethyl alcohol was prepared in addition to the control tube. A sufficient amount of Mueller Hinton agar medium (MHA-Difco) to provide a final volume of 20 ml, cooled to approximately 50°C, was added to the serial dilution tubes and to the control tubes, followed by homogenization and distribution among sterilized 15 x 100 mm Petri dishes.

Suspensions in sterilized physiological saline containing approximately 1 x 10^6 cells/ml were applied with Steers replicator onto the series of agar plates. Incubation were to 24 h at 37°C and the results were read by observing the presence of absence of microorganism growth at the corresponding dilution of the antiseptic tested.

RESULTS

Thirty-two (64.0%) of the samples obtained from the oral cavities of 50 individuals were positive for yeast. Yeasts of the genus *Candida* were isolated from 9/19 (47%) saliva samples from clinically healthy individuals, 18/22 (81.8%) samples from patients suspected to have oral candidiasis and from 4/9 (44.4%) individuals with deviation from normality (fissured and/or coated tongue), and were detected in 19/22 (86.4%) samples from the oral lesions.

C.albicans was the most prevalent among the species isolated and was the only one detected in all types of samples analyzed, occurring at a frequency of 48% (24/50) in saliva, at frequencies of 28.0% (07/20), 14.3% (01/07) and 12.5% (01/08) on normal, fissured and coated tongues, respectively, and at a frequency of 67.8% (19/28) in samples from lesions. Other species were also isolated, mainly from saliva and lesions, at a higher frequency for *C.tropicalis*: 8.0% (04/50) of saliva samples and 10.7% (03/28) of lesion samples, and *C.glabrata*: 4.0% (02/50) of saliva samples and 3.6% (01/28) of lesion samples, with emphasis on the fact that the latter species was isolated from fissured tongues: 28.6% (02/07).

The study of the salivary levels of yeasts of the genus *Candida* by the methods of serial decimal dilution showed a mean number of total cfu/ml saliva of 171.5 x 10^3 in the group of patients with lesions (range: 0.42 x 10^3 to 2,760.0 x 10^3 cfu/ml). It can be seen that these levels were higher than in the groups of patients with no lesions (control) or with deviation normality, with respective values of 72.64 x 10^3 cfu/ml and 8.6 x 10^3 cfu/ml. Among the species isolated from the 3 study groups, *Candida albicans* was the most frequent, with a mean level of 227.52 x 10^3 cfu/ml in the groups with lesions, 72.28 x 10^3 cfu/ml in the group without lesions and 11.02 x 10^3 cfu/ml in the third group. Yeasts of the species *C.tropicalis* and *C.glabrata* were isolated only from patients with lesions, at respective levels of 14.7 x 10^3 cfu/ml and 48.25 x 10^3 cfu/ml saliva. *C.guilliermondii* (0.06 x 10^3 cfu/ml) and *C.parapsilosis* (4.70 x 10^3 cfu/ml)

Table 1. Number and frequency of species of the genus *Candida* isolated from oral cavity.

Specie	Saliva (50)		Normal (20)		Fissured (7)		Saburrough (8)		Lesion (28)	
	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%
C.albicans	24	48.0	07	28.0	01	14.3	01	12.5	19	67.8
C.tropicalis	04	8.0	-	-	-	-	-	-	03	10.7
C.glabrata	02	4.0	-	-	02	28.6		-	01	3.6
C.guilliermondii	01	2.0	-	-	-	-	-	-	-	-
C.parapsilosis	01	2.0	-	-	-	-	-	-	01	3.6
C.krusei	02	4.0	-	-	-	-	-	-	-	-

Legend: (-) non detected.

were only detected in control individuals, from whom *C.krusei* was not isolated from the oral cavity.

In the determination of the action of the antiseptics Periogard and Propolis against the 70 strains of yeasts of the genus *Candida* isolated from the oral cavity of individuals with a clinically healthy mouth or a mouth with lesions, we observed that most strains 67/70 (95.71%) were sensitive to the two antimicrobial agents.

With respect to the action of the different dilutions of the antiseptics tested, we noted that a large number of strains was inhibited by the 1:20 dilution of Propolis 54/70 (77.1%), a MID corresponding to the final concentration of 10.0 mg soluble solids per ml of the commercial product, and by the 1:160 dilution of Periogard 42/70 (60%), which contains a final concentration of chlorhexidine gluconate of 0.0075 mg/ml. We emphasize that all strains tested grew when submitted to Propolis dilutions higher than 1:40.

Of all strains tested, 29/70 (41.42%) were simultaneously sensitive to a MID of 1:160 of Periogard and to a MID of 1:20 of Propolis. Of these strains, 28/29 (96.55%) were of the species *C.albicans*.

Different results were obtained for different species, when they were tested with Periogard, with the MID ranging from 1:100 to 1:200 for this species and from 1:200 to 1:300 for the remaining ones. With respect to Propolis, *C.albicans* and *C.tropicalis* presented strains that were sensitive to the same concentrations (1:20 or 1:40), whereas the other species were sensitive to a MID of 1:20 (Table 2).

With respect to the *Candida* species, the MID of Periogard for *C.albicans*, the species most often detected 24/31(77.4%), was 1:100 to 1:160, whereas the MID against *C.tropicalis* 4/31 (12.9%) ranged from 1:200 to 1:300. Whit respect to Propolis, *C.albicans* was sensitive to a MID of 1:20 - 18/24 (75.0%) or 1:40 - 5/24 (20.8%), whereas the remaining species were sensitive to a MID of 1:20 (Table 3).

The 3 species that were resistant to all dilutions of Propolis tested were *C.albicans*, *C.tropicalis* and *C.glabrata* isolated from oral lesion.

DISCUSSION AND CONCLUSION

The density of yeasts in oral is usually high and more than one species is frequently isolated that it is difficult to determine the relative role of individual species in the disease process (1,2).

Total quantitation of yeasts of the genus *Candida* varied in the three groups studied here, with emphasis on the mean level detected in patients with oral candidiasis (171.5 x 10^3 cfu/ml saliva) compared to the control group (72.64 x 10^3 cfu/ml) and to the group with deviation from normality (8.26 x 10^3 cfu/ml).

With respect to the species isolated, *C.albicans* showed the highest levels (72.28 x 10^3, 227.52 x 10^3 and 11.02 x 10^3 cfu/ml) in the group with lesions and in the group with deviation from normality,

Table 2. Number and frequency of species of the genus *Candida* isolated from oral cavity. Maximal inhibitory dilution (MID) using Periogard and Propolis.

Specie / MID	*C. albicans* (55)		*C.tropicalis* (8)		*C.glabrata* (2)		*C.parapsilosis* (2)		*C.krusei* (2)		*C.guilliermondii* (1)		Total
	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%	
1:100	02	3.6	0		0		0		0		0		02
1:120	10	18.2	0		0		0		0		0		10
1:160	41	74.6	0		0		0		0		01	100.0	42
1:200	02	3.6	04	50.0	01	50.0	01	50.0	01	50.0	0		09
1:240	0		03	37.5	0	50.0	0		01	50.0	0		05
1:300	0		01	12.5	01		01	50.0	0		0		02
1:20	42	76.4	06	75.0	01	50.0	02	100.0	02	100.0	01	100.0	54
1:40	12	21.8	01	12.5	0		0		0		0		13
R	01	1.8	01	12.5	01	50.0	0		0		0		03

R = resistant to Propolis at the dilutions studied.

Table 3. Number and frequency of species of the genus *Candida* isolated from oral lesions. Maximal inhibitory dilution (MID) using Periogard and Propolis.

Specie / MID	*C.albicans* (24)		*C.tropicalis* (4)		*C.glabrata* (2)		*C.parapsilosis* (1)		Total
	Nº	%	Nº	%	Nº	%	Nº	%	
1:100	02	8.3	0		0		0		02
1:120	04	16.7	0		0		0		04
1:160	18	75.0	0		0		01	100.0	18
1:200	0		02	50.0	01	50.0	0		04
1:240	0		01	25.0	0		0		01
1:300	0		01	25.0	01	50.0	0		02
1:20	18	75.0	03	75.0	01	50.0	01	100.0	23
1:40	05	20.8	0		0		0		05
R	01	4.2	01	25.0	0	50.0	0		03

R = resistant to Propolis at the dilutions studied.

respectively, among individuals with oral candidiasis. *C.tropicalis* and *C.glabrata* presented a mean of 14.7 x 10^3 and 48.25 x 10^3 cfu/ml, respectively, in individuals with oral candidiasis.

The results obtained for the genus *Candida* in saliva samples from individuals with suspected oral candidiasis (18/81.8%) were lower than those obtained by Davenport (7) (98.0%) and higher than those obtained by Jorge Junior *et al.* (9) (45.5%). The results for lesion samples (86.4%) were lower than those obtained by Rindum *et al.* (15) (98.1%). *C.albicans* was detected in 59.1% of the saliva samples and in 67.8% of the lesion samples, a result lower than that obtained by Davenport (7) in saliva (70.0%) and by Rindum *et al.* (15) in oral mucosa lesions (94.3%).

The results of the present study are reported as MID because we tested a commercial preparation both for chlorhexidine (chlorhexidine gluconate/ Periogard) and Propolis (alcohol extract). Since these preparations might contain substances that inhibit the yeasts tested in addition to the active principle itself of the antiseptics studied, this would invalidate the calculation of minimum inhibitory concentration for the products employed.

Different results were obtained for different species tested with Periogard (0.12% chlorhexidine gluconate). The action of the product against *C.albicans* occurred at a MID of 1:200 to 1:300, indicating that the species isolated at highest frequency from individuals with and without oral lesions had the lowest sensitivity.

In contrast, the antifungal action of Propolis was more uniform against all species tested, with a MID of 1:20 for most of them, whether or not they were isolated from lesions.

Periogard, whose active principle is chlorhexidine, has been shown to be active against bacteria and fungi (4,13), a fact that was confirmed in the present study, in which strains of the yeasts tested were inhibited by a concentration ranging from 4 to 12ug with an intermediate concentration of 7.5ug/ml for a MID of 1:160 inhibiting 60% of the strains. This result is similar to that obtained by Candido *et al.* (4) who obtained 90% inhibition of the strains at a concentration of 6.4ug/ml, indicating that the commercial product can be used diluted.

Most of the strains tested (77.1%) were sensitive to Propolis at a dilution of 1:20 corresponding to 10 mg/ml of the product (soluble solids) present in the product (soluble solids) present in the alcohol extract. Of these, 18.6% were sensitive to a higher dilution of 1:40, results that are difficult to compare since other authors have testes the action of Propolis using a pure Propolis extract (PPE) and/ or yeasts obtained from different sites.

Ota *et al.* (14), in a study of *C.albicans* sensitivity to PPE, observed that the minimum fungicidal concentrations against the 15 strains tested ranged from 3 to 7 mg/ml, indicating their possible use in the prevention of oral diseases.

Rojas and Lugo (16) demonstrated the antifungal activity of the alcohol extract of Propolis against 23 yeast strains isolated from different sites in the human body and observed that the product was fungistatic at a concentration of 0.55 mg/ml.

The diversity of the results obtained with Propolis may be related to the methods employed, the strains

tested and mainly the fact that the antimicrobial effect is directly proportional to its concentration and also depends on its origin since its chemical composition and consequently its antimicrobial effect vary according to such origin (18).

Even considering the fact that *in vivo* cannot be directly extrapolated to *in vivo* effects (20), our results indicate that yeasts of different species of the genus *Candida* could be inhibited by the application of the commercial products Periogard and Propolis to the oral cavity for therapeutic and/or preventive purposes against oral candidiasis.

RESUMO

Candida sp na cavidade bucal com e sem lesão: diluição inibitória máxima de Própolis e Periogard

Foram avaliados 50 indivíduos, de ambos os sexos e faixa etária média de 42,5 anos, da clínica de Semiologia da FORP-USP, objetivando-se isolar e identificar leveduras na cavidade bucal, com e sem lesão, e determinar a DIM dos produtos comerciais Própolis (Apis-Flora) e Periogard (Colgate) frente às cepas isoladas. Leveduras do gênero *Candida* foram detectadas na saliva de 9(47,4%) indivíduos com boca clinicamente sadia, 18/81,8% portadores de lesões bucais e de 4/44,4% pacientes com desvio de normalidade; sendo detectadas em 19/86,4% lesões. No grupo com candidose bucal, respectivamente de língua e lesão, isolou-se *C.tropicalis* (8% e 10,7%), *C.glabrata* 4% e 3,6%) e *C.parapsilosis* (2% e 3,6%), além de *C.albicans* (71,4% e 67,8%) como espécie única e prevalente. A contagem de ufc total/ml de saliva demonstrou um valor médio no grupo com candidose bucal (171,5% x 10^3) maior do que no controle (72,6 x 10^3) e portadores de anormalidade (8,3 x 10^3). A maioria das cepas testes 67/70 (95,7%) foi sensível aos anti-sépticos, sendo que a Própolis apresentou uma DIM igual a 1:20 para 54/70 (77,1%) e, o Periogard uma DIM de 1:160 para 42/70 (60%) cepas de nichos sadios; semelhante ao obtido com cepas de lesões bucais. Resultados diferentes ocorreram, principalmente, entre espécies diferentes. Os resultados indicam a possibilidade de se empregar os anti-sépticos Própolis e Periogard (clorexidina), na prevenção e na terapêutica da candidose bucal.

Palavras-chave: Candidose bucal, Própolis, Clorexidina, leveduras.

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BACTERIOCIN-LIKE ACTIVITY OF ORAL *FUSOBACTERIUM NUCLEATUM* ISOLATED FROM HUMAN AND NON-HUMAN PRIMATES

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ABSTRACT

Fusobacterium nucleatum is indigenous of the human oral cavity and has been involved in different infectious processes. The production of bacteriocin-like substances may be important in regulation of bacterial microbiota in oral cavity. The ability to produce bacteriocin-like substances by 80 oral *F. nucleatum* isolates obtained from periodontal patients, healthy individuals and *Cebus apella* monkeys, was examinated. 17.5% of all tested isolates showed auto-antagonism and 78.8% iso- or hetero-antagonism. No isolate from monkey was capable to produce auto-inhibition. In this study, the antagonistic substances production was variable in all tested isolates. Most of the *F. nucleatum* showed antagonistic activity against tested reference strains. These data suggest a possible participation of these substances on the oral microbial ecology in humans and animals. However, the role of bacteriocins in regulating dental plaque microbiota *in vivo* is discussed.

Key words: *Fusobacterium nucleatum*, bacteriocin-like substance, oral bacteria.

INTRODUCTION

Anaerobic bacteria comprise a large percentage of the oral and gut indigenous microbiota (9). Some anaerobic bacteria possess several potentially pathogenic factors, particularly Gram-negative rods which appear to be present in several anaerobic infections, such as periodontal diseases (23).

Fusobaterium nucleatum is indigenous of the human oral cavity and has been involved in several infectious processes such as, sinusitis, osteomyelitis, brain or liver abscesses (3, 9, 14,17). Also, this organism constitutes a considerable part of the subgingival microbiota of gingivitis in children and adults and of periodontitis in juveniles and adults (18).

The microbial composition of the established dental plaque may be controlled by nutrient requirements or production of antagonistic substances (6). However, the nature of the inhibitory substances is still a matter of discussion. Microbial antagonistic substances were first studied by Gratia and Fredericq (11), who showed the iso-inhibitory activity produced by *Enterobacteriaceae.* These colicin-like substances were called bacteriocins (13). The knowledge of bacteriocin production has been extended to Gram-positive and Gram-negative aerobes, facultative and strict anaerobes.

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From an ecological viewpoint, there are few studies concerning production of antagonistic substances, such as bacteriocins, in Gram-negative anaerobes indigenous to the human microbiota, particularly, in oral *F. nucleatum* from human and animal origin. However, the production of metabolic products such as bacteriocin-like substances may be important in ecological processes to the colonization of periodontal tissues by *F. nucleatum* or other members from human indigenous microbiota (5).

In this study, the production of bacteriocin-like substances by *F. nucleatum* isolated from humans and *Cebus apella* monkeys is reported. The monkeys were used because of anatomic similarities with the human oral cavity.

MATERIALS AND METHODS

Microorganisms

A total of 80 oral *F. nucleatum* isolates were tested for their bacteriocinogenic activity. Fourty-nine isolates were obtained from 30 patients with adult periodontitis (age range 18 - 40 years), with clinical and radiographic evidence of periodontal disease, including pockets of depth equal or exceeding 5 mm, 21 isolates from 20 healthy individuals (age range 20 - 30 years) from the Clinic of Periodontology, University of São Paulo, SP, Brazil, and 10 isolates were from 10 *Cebus apella* monkeys without evidence of periodontal disease (Núcleo de Procriação do Macaco Prego, São Paulo, SP, Brazil). Subgingival bacterial samples were taken using sterilized absorbent paper points (Dentsply Ind. & Co. Ltda., Rio de Janeiro, RJ, Brazil), which were introduced into the periodontal pocket or gingival sulcus for 60 s and then transferred to tubes containing 2.0 ml of Ringer-PRAS solution, pH 7.2, under CO_2 flux (22). The bacterial isolation and identification to the species level was performed by using Omata & Disraely selective agar as described previously (1, 10) and by using conventional biochemical tests (12, 24).

Bacteriocinogenic activity

The isolates were tested for their inhibitory activity against themselves and against *F. nucleatum* ATCC 10953, *F. nucleatum* ATCC 25586, *Bacteroides fragilis* ATCC 23745, *Eubacterium lentum* ATCC 25559 and *Peptostreptococcus anaerobius* ATCC 27337. The bacteriocinogenicity was determined by using a double layer method (4). The bacterial inoculum was prepared in brain-heart infusion broth (BHI, Difco) supplemented with 0.5% extract yeast (12). 10 ml of 24 h cultures were inoculated in 5 equidistant spots onto BHI agar (BHI-A), using a standard platinum loop. Plates were incubated at 37°C for 72 h under anaerobic conditions (10% CO_2/ 90% N_2). After incubation, the cells were killed by exposure to chloroform for 30 min (8). Residual chloroform was allowed to evaporate and BHI-A plates were overlayed with 3.5 ml of BHI-soft agar (0.7%) inoculated with 0.1 ml of a 48 h culture of each indicator. After 48 h of incubation under anaerobic conditions, the plates were evaluated for the presence of bacteriocin-like substances. The inhibitory halo was measured. Experiments were done in duplicate.

Test for the presence of bacteriophages

A piece (3.0 mm in diameter) of agar at the inhibitory halo was removed aseptically (26). This block of agar was homogeneized, placed onto BHI-A and overlayed with 3.0 ml of BHI-soft agar inoculated with 0.1 ml of the indicator culture. After 48 h of incubation under anaerobic conditions, the plates were evaluated for the presence or absence of lytic zones. Tests were done in duplicate.

RESULTS

Table 1 shows the inhibitory activity of 80 oral *F. nucleatum* isolates recovered from periodontal patients, healthy individuals and monkeys, tested against themselves. Auto-antagonism was observed in nine isolates from periodontal patients, and in five isolates from healthy individuals. However, no auto-antagonism was observed among the monkey isolates. Iso-antagonism was not observed only in three isolates from periodontal patients, seven isolates from healthy individuals and six monkey isolates. The non-producing hetero-antagonism isolates were six from patients, nine from healthy individuals, and one from monkey.

All isolates showed antagonistic activity against all tested reference strains (Table 2). None of the *F. nucelatum* isolates from periodontal patients or monkeys produced antagonistic substances against *F. nucleatum* ATCC 10953. On the other hand, none of the isolates obtained from healthy individuals was capable of inhibiting *B. fragilis* ATCC 23745. None of the *F. nucleatum* isolates obtained from healthy individuals or monkeys produced antagonistic

substances against *P. anaerobius* ATCC 27337. However, only 3 (6.1 %) of periodontal isolates inhibited this microorganism (Table 2). All the diameters of the inhibitory zones were between 10 and 13 mm (data not shown). In tests for the presence of phages, no lytic zone with the same indicator organisms was observed, indicating that the inhibition halos were not due to the presence of bacteriophages.

DISCUSSION

The production of antagonistic substances may play an important role in the microbial colonization of the human or animal oral cavity, leading to ecological alterations in the indigenous microbiota. Because bacteriocin-like substances are produced by a high number of oral microorganisms, a constant and complex intra- or inter-specific regulation can be expected (8).

Among *F. nucleatum* isolated from humans (periodontal patients and healthy individuals) and from *Cebus apella* monkeys, in terms of auto-, iso- and hetero-antagonism, 14 (17.5%) presented auto-antagonism and 63 (78.8%) iso- or hetero-antagonism. Interestingly, the inhibitory activity produced by isolates from periodontal patients was those intense than that produced by isolates from healthy individuals or monkeys (data not shown). Significant differences among hetero-antagonism levels from periodontal and from healthy isolates were observed (Table 1), suggesting an association with the predominance of this organism in periodontal sites.

Auto-antagonism is more prevalent in Gram-positive than in Gram-negative organisms (21, 28). In some Gram-negative bacteria, such as *Pseudomonas solanacearum* and *B. fragilis*, this phenomenon has not been observed (7, 8, 16). Our results show auto-inhibition in 18.4 % and 25 % of the periodontal and healthy isolates, respectively. No auto-antagonism was observed in animal isolates. Azevedo *et al.* (2) showed that the frequency of bacteriocin-producing strains among oral streptococci, including auto-, iso- and hetero-antagonism, is variable.

Antimicrobial proteins are produced by several pathogenic and non-pathogenic bacteria. However, little is known about antagonistic substances in *F. nucleatum*. Auto-antagonism among the tested *F. nucleatum* was observed in a small number of isolates, where as, iso- and hetero-antagonism were observed in a large number of them, suggesting that the bacteriocin-like activity in *F. nucleatum* is variable. This can have a great ecological significance, particularly in colonization or

Table 1. Production of bacteriocin-like substances by 80 oral *F. nucleatum* isolates

Sources of isolates	Antagonism type					
	Auto-		Iso-		Hetero-	
(N)	N	(%)	N	(%)	N	(%)
P* (49)	9	(18.4)	46	(93.9)	43	(87.8)
H** (21)	5	(25.0)	13	(65.0)	11	(55.0)
M*** (10)	0	(0.0)	4	(20.0)	9	(90.0)
Total (80)	14	(17.5)	63	(78.8)	63	(78.8)

*Periodontal patients
**Healthy individuals
***_Cebus apella_ monkeys

Table 2. Antagonistic activity of 80 oral *F. nucleatum* isolates against themselves and against reference strains.

Source of isolates	Percentage of positive isolates							
	P*	H**	M***	*F. nucleatum* ATCC 10953	*F. nucleatum* ATCC 25586	*B. fragilis* ATCC 23745	*E. lentum* ATCC 25559	*P. anaerobius* ATCC 27337
P*	93.8	69.4	67.3	0.0	12.2	8.1	14.3	6.1
H**	90.5	66.6	57.1	9.5	33.3	0.0	4.4	0.0
M***	90.0	80.0	40.0	0.0	20.0	10.0	10.0	0.0

*49 Periodontal patient isolates
**21 Healthy individual isolates
***10 Monkey isolates

regulation of the autochtonous microbiota of the host (15).

Tagg *et al.* (25) showed that bacterial immunity to their own auto-antagonistic products is due to the synthesis of a specific protein which acts as an immunity factor, and the formation of a complex between bacteriocin and this factor would protect the bacteriocin-producing strain.

The diameters of the inhibition zones varied from 10 to 13 mm with a higher frequency of 13 mm and with sharp delimitation (data not shown). These data are similar to those reported by Farias *et al.* (8). Bacteriophages were not observed since the inhibitory action was not transmissible as shown by the tested method (26), excluding the possibility of phages causing the bacteriocin-like effect have been suggested by several authors (14, 20).

Bacteriocin synthesis appear to be an unstable characteristic since some microorganisms lose and recover the capacity to produce it. However, Oliveira and Drozdowicz (19) suggested that the capability to produce bacteriocin is expressed by a small proportion of the bacterial population. On the other hand, it also is possible that one isolate may produce more than one antagonistic substance with different physico-chemical and biological properties. However, some components of the growth medium have been implicated in the induction of bacteriocins (25). However, Weerkamp *et al.* (27) suggested that some components of the nutrient media protect some organisms against bacteriocin action. The chemical nature, genetic determinants and environmental factors that affect the phenotypic expression of the bacteriocin-like production are not totally determined. Since the production can be masked by presence of similar or different substances, studies about characterization and purification of these products should help to a better knowledgement of its action on oral microbiota of human and animal origin.

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RESUMO

Atividade semelhante a bacteriocina de *Fusobacterium nucleatum* orais isolados de primatas humanos e não-humanos

Fusobacterium nucleatum é indígena da cavidade oral humana e tem sido envolvido em diferentes processos infecciosos. A produção de substâncias semelhantes a bacteriocinas pode ser importante na regulação da microbiota bacteriana da cavidade oral. A capacidade de produzir substâncias tipo bacteriocina de 80 isolados de *F. nucleatum* orais, obtidos de pacientes com doença periodontal, indivíduos sadios e macaco *Cebus apella*, foi avaliada. 17,5% de todos os isolados mostrou auto-antagonismo e 78,8% iso- ou hetero-antagonismo. Nenhum isolado de macaco foi capaz de produzir auto-inibição. Neste estudo, a produção de substâncias antagonístas foi variável em todos os isolados testados. A maioria dos *F. nucleatum* mostrou atividade antagonísta para as cepas de referência testadas. Esses dados sugerem a possível participação dessas substâncias sobre a ecologia microbiana em humanos e animais. Entretanto, o papel das bacteriocinas na regulação da microbiota da placa dental *in vivo* é discutida.

Palavras chave: *Fusobacterium nucleatum*, substância tipo bacteriocina, placa dental.

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CRITICAL CONTROL POINTS FOR MEAT BALLS AND KIBBE PREPARATIONS IN A HOSPITAL KITCHEN

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ABSTRACT

Hazards and critical control points (CCP) associated with meat balls and kibbe preparations in a hospital kitchen were determined using flow diagrams and microbiological testing of samples collected along the production line. Microbiological testing included counts of mesophilic and psicrothrophic microorganisms, yeasts and molds, total and fecal coliforms, *C. perfringens*, coagulase positive staphylococci, bacteria of the *B. cereus* group and detection of *Salmonella*. Time/temperature binomial was measured in all steps of preparation. A decision tree was used to help in the determination of CCPs. The detected hazards were: contamination of raw meat and vegetables, multiplication of the microorganisms during meat manipulation, poor hygiene of utensils and equipment, and survival of microorganisms to the cooking process. Cooking and hot-holding were considered CCPs. The results stress the importance of the implementation of a training program for nutricionists and foodhandlers and the monitoring of CCPs and other measures to prevent foodborne diseases.

Key words: HACCP, microbiological quality of foods, meat, hospital kitchen

INTRODUCTION

Mishandling of foods in food service operations is frequently associated to outbreaks of foodborne diseases (7). In these establishments, the traditional method to control the quality of the food includes the inspection and microbiological evaluation of the end product. However, this method is not ideal because microbiological analysis results become available only after the food has been already eaten, leaving no opportunity for corrective measures.

In recent years, the hazard analysis and critical control point (HACCP) concept has been proposed as the best approach to assure food safety (6,10,13,20). HACCP includes identification, evaluation and control of potential hazards before they occur (14). HACCP is based on several principles, which are described in different ways. In March 1992, The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) (12) published a document which defined hazard as "a biological, chemical or physical property that may cause a food to be unsafe for consumption". This document delineates the seven HACCP principles,

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gives the definitions of HACCP terms, and describes the initial steps to develop a HACCP plan. It also describes the use of a decision tree to aid the identification of critical control points.

The seven HACCP principles were summarized by Savage (14): (1) conduct a hazard analysis by preparing a list of steps in the process where significant hazards occur and describe the preventive measures, (2) identify the critical control points (CCP) in the process, (3) establish critical limits for preventive measures associated with each identified CCP, (4) establish CCP monitoring requirements, (5) establish corrective action to be taken when monitoring indicates that there is a deviation from an established critical limit, (6) establish effective recordkeeping procedures that document the HACCP system, and (7) establish procedures for verification that HACCP system is working properly.

In the application of HACCP, physical and chemical tests and even visual observations are used to monitor critical control points since microbiological testing is time consuming.

The objectives of this study were to evaluate the hazards associated with foods prepared with ground meat, and to determine the critical control points for preparation of these foods in a Brazilian hospital kitchen, using time/temperature measurements and microbiological testing. Foods prepared with ground meat were selected for this study because this type of foods has been implicated repeatedly as vehicle in reports of foodborne disease outbreaks.

MATERIALS AND METHODS

Food preparation place:

The study was done in the kitchen of a hospital that belongs to São Paulo University, São Paulo, Brazil. This hospital serves daily nearly 700 meals at lunch and 250 at dinner. The kitchen has an area for reception of goods, butchery and distinct areas for manipulation of vegetables and cooking.

Development of flow diagrams:

Flow diagrams of preparation of two ground meat based products (meat balls and kibbe) were constructed, in order to provide a clear, simple description of the steps involved in the process.

Determination of critical control points:

To determinate CCPs, the decision tree described by NACMCF was used (12).

Sampling procedures:

Portions of 100 g of meat were asseptically collected at each step of preparations of meat balls and kibbe. A pool of the seasonings and all other ingredients but eggs were also sampled.

Samples of water were collected in screw cap sterile flasks. Equipment and utensils like basins, dishes, polipropilene chopping boards, wooden spoons, and blenders were also sampled before use by rubbing a known area with a sterile swab, previously moistened in sterile buffered water (3).

All samples were analyzed on the same day of collection. Samples collected along the preparation were examined three times. Equipments and utensils were tested five times.

Laboratory procedures:

For testing of solid products (raw meat, meat balls, kibbe), samples were homogenized with sterile buffered water, pH 7.2, in a Stomacher Lab Blender 400 for 2 minutes and then, decimal dilutions were made in the same diluent. The dilutions were used for microbiological analysis as described below.

For equipments and utensils testing, the tubes containing the swabs and Buffered Water were vortexed. This Buffered Water was used in the microbiological analysis.

Water samples were tested for total and fecal coliforms using the presence-absence test (1).

The microbiological analyses carried out in the samples are summarized in Table 1.

Aerobic mesophilic bacteria (AMB) were enumerated using pour plate in Plate Count Agar (Difco). Plates were incubated at 35°C for 48 h (3).

Psychrotrophic microorganisms (APB) were enumerated by spreading 0.1 ml of the homogenates and their dilutions on Plate Count Agar (Difco) plates, which were incubated for 10 days at 7°C (3).

Yeasts and molds were enumerated by spreading 0.1 ml of homogenates on Potato Dextrose Agar (Difco) (pH 3.5) plates, which were incubated for five days at room temperature (3).

The number of total and fecal coliforms was estimated by the most probable number method. Lauryl Sulfate Broth (Difco) was used in the presuntive test. After incubation at 35°C for 48 h, total coliforms were confirmed in Brilliant Green Broth (Difco) incubated at 35°C for 48 h, and fecal coliforms were confirmed in EC Broth (Difco), incubated at 44.5°C for 24 h, as described by APHA (3).

Table 1 – Microbiological analysis of samples collected in a hospital kitchen

Samples	Microbiological analysis
Frozen meat Refrigerated meat Frozen ground meat Refrigerated ground meat Manipulated meat	Aerobic mesophilic colony count Aerobic psychrotrophic colony count Yeasts and molds colony count Enumeration of *B.cereus* group Enumeration of *Clostridium perfringens* Enumeration of *Staphylococcus aureus* Enumeration of total coliforms Enumeration of fecal coliforms Detection of *Salmonella*
Cooked meat Last meal to be served	Aerobic mesophilic colony count Yeasts and molds colony count Enumeration of *B.cereus* group Enumeration of *Clostridium perfringens* Enumeration of *Staphylococcus aureus* Enumeration of total coliforms Enumeration of fecal coliforms Detection of *Salmonella*
Pool of ingredients	Enumeration of *B.cereus* group Enumeration of *Clostridium perfringens* Enumeration of *Staphylococcus aureus* Enumeration of total coliforms Enumeration of fecal coliforms Detection of *Salmonella*
Eggs	Detection of *Salmonella*
Wheat	Enumeration of *B .cereus* group
Equipments and utensils	Aerobic mesophilic colony count

Bacteria of *B. cereus* group were enumerated according to the methodology described by APHA (3) with some modifications. Phenol-red-egg-yolk-polymixin agar plates were used for isolation of colonies, which were submitted to Gram staining and tests for catalase, motility, haemolysis in sheep blood agar and tyrosine decomposition.

Coagulase positive staphylococci were enumerated on Baird-Parker agar (Difco) plates. Gram staining, tests for coagulase, thermonuclease and catalase production tests were used to confirm suspected colonies (3).

Clostridium perfringens was enumerated by spreading 0.1 ml of the homogenates and their dilutions onto SPS agar (Difco) plates, which received an overlay of the same media. These plates were incubated at 43°C for 48 h under anaerobic conditions. Gelatin-lactose and motility-nitrate tests were used for confirmation of suspected colonies (modification of APHA (3)).

For isolation and identification of *Salmonella*, 25 g of food were homogenized in 225 ml of Buffered Peptone Water. Selenite Cystine broth (Difco) with novobiocin (100 µl/10ml) (Sigma) (35°C/24h) and Tetrathionate Brilliant Green Broth (Difco) (43°C/24h) were used as selective enrichment media. Broths were plated on Brilliant Green Agar and on SS Agar, and incubated at 35°C for 24 h. Typical colonies were transferred to Triple Sugar Iron Agar (Difco) and to Phenylalanine Agar (Difco). The complete identification was done using the API20E system (BioMérieux) . Serological testing with somatic and flagella antibodies was also performed (modification of APHA (3)), using polivalent antisera supplied by Probac do Brasil Produtos Bacteriológicos Ltda.

Measurements of temperature:

The internal temperature of the food was measured in the geometric center using a thermocouple (Instrutherm TH200C). The temperature was taken at all steps from meat reception up to serving the last meal. The time of each preparation step was also registered, and a time-temperature binomial was determined. Thermometers for maximum and minimum temperatures were used to evaluate storage conditions of meat. The temperature of hot-holding devices was also measured.

RESULTS AND DISCUSSION

Flow charts and critical control points for the preparations of meat balls and kibbe are presented in Figs 1 and 2, respectively. Laboratory results are listed in Tables 2, 3 and 4.

Description of meat balls and kibbe preparations:

Frozen vacuum packaged meat was delivered to the hospital 10 days before use. After checking the temperature, the meat was introduced in a cold-chamber for thawing. Meat temperature ranged from – 9.0°C to – 3.0° C at delivery. After thawing, temperature reached a maximum of 7.8°C.

One day before planned use, thawed meat was transferred from the cold-chamber to the butchery, where it was ground by butchers wearing disposable gloves. This process was done in approximately 68 minutes. Before use, chopping board, blanches, knifes and mincer were disinfected with a solution of 2000 ppm sodium hipoclorite for 15 minutes and washed with hot water. After grounding, the meat was kept in the cold-chamber until the next day.

To prepare meatballs and kibbe, ground meat were mixed with vegetables and condiments. All vegetables were washed with tap water and chopped using the same knife and the same cutting board. Wheat used to prepare kibbe was soaked in water overnight at 7°C. In the meat balls preparations, eggs and milk were also used.

All ingredients were mixed with a wooden spoon. While part of the mixture was transformed in balls by manipulators wearing disposable gloves, the remaining part was kept in the cold-chamber. Rolled meatballs and kibbe were kept in the cold-chamber until fried.

After frying, part of the meatballs and kibbe were served in a steam table and part was held in a hot pass-through until served.

Microbiological analysis and time/temperature measure:

Samples were collected 3 times in each step of the process and submitted to microbiological analysis. Results of pathogen enumeration are listed on Table 2.

It must be pointed out that in two occasions, no pathogens were detected in the samples.

All raw meat samples delivered to the kitchen were in accordance to specifications of the Ministry of Health of Brazil (Portaria nº 451/98) (11) since *Salmonella* was not detected in 25 g of any sample. However, considering the recommendation of Solberg *et al.* (17), 33.3% of the samples could not be considered acceptable due to the number of *S. aureus* (1.0×10^2 CFU/g) (Table 2). As shown in Table 2, these bacteria remained viable during the cold storage since they were also found in samples of thawed meat.

During cold storage, an increase of psychrotrophic microorganisms was detected (Table 3) which is not surprising since meat remained 10 days under refrigeration for thawing.

Table 3 shows that the number of some indicator microorganisms increased during grinding of meat. The process of grinding was done at room temperature and took about 68 minutes, leading to bacterial multiplication. Besides, the contact of the meat with the chopping board could introduce new contamination. Although desinfected, the chopping board contained a significant numbers of mesophilic microorganism (Table 4). Tebutt (19) described that it is not easy to clean and disinfect utensils made of polypropylene due to the irregular surface.

In general, a high number of *Enterobacteriaceae* or coliforms ($>10^4$/g) is found in raw vegetables (18). In the present study, total (TC) and fecal coliforms (FC) were detected in the pool of ingredients (Table 3). This reveals an inadequate hygiene since samples were collected after their disinfection. The flora coming from the ingredients could be responsible for the increase in the MPN of total coliforms, determined in meat samples collected after mixing (Table 3).

During mixing, no increase in the counts of fecal coliforms, mesophilic microorganisms and yeasts and molds was detected (Table 3). Correct manipulation procedures, in which foodhandlers weared disposable gloves, and exposure of meat at room temperature for less than 30 minutes may explain the absence of microbial multiplication.

S. aureus are normally found in warm blooded

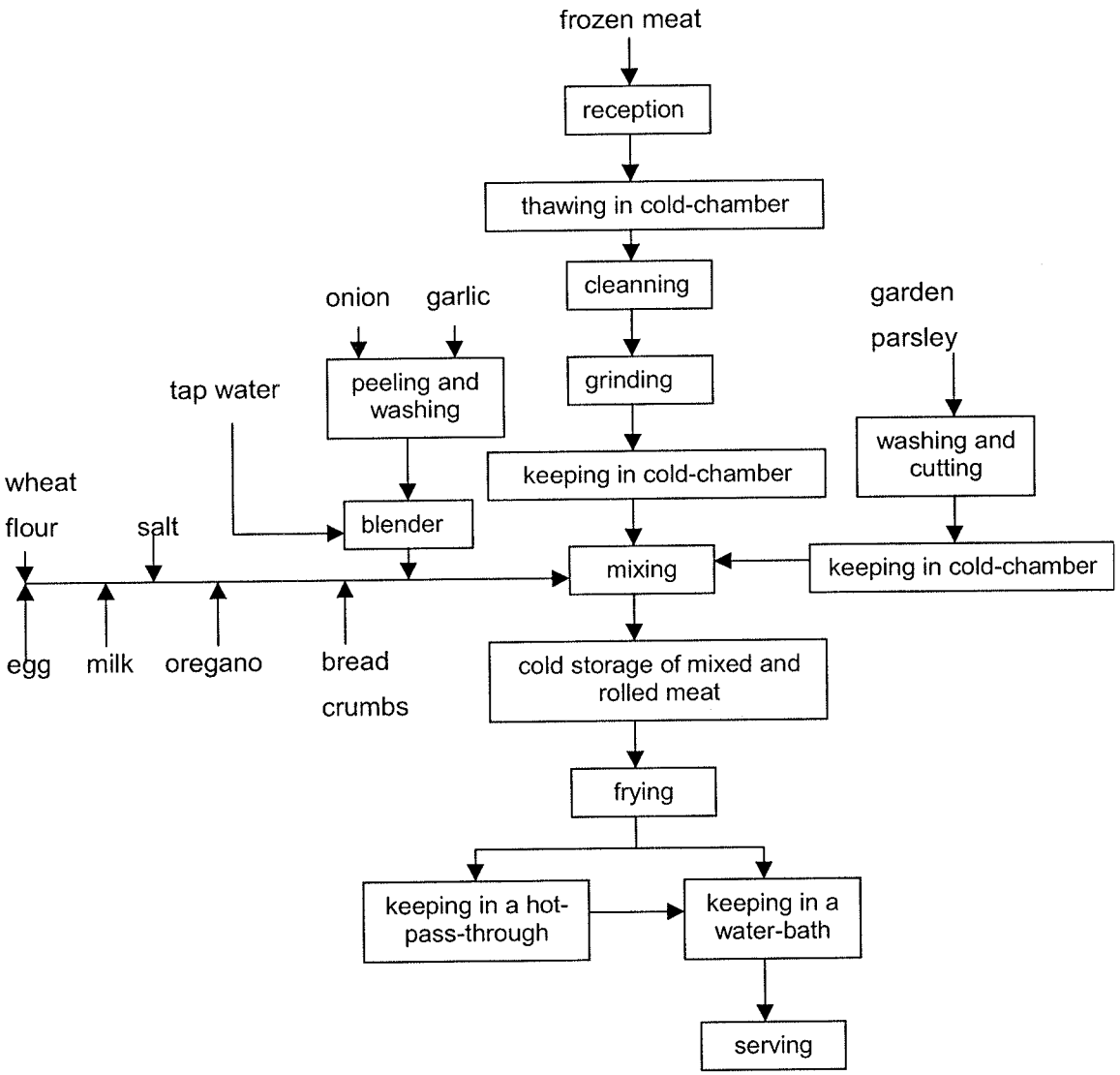

Figure1 – Flow diagram of meat balls preparation

animals and can be used as indicators of inadequate manipulation (9). These microorganisms were detected in two samples of meat delivered to the hospital. In one case (Table 2), coagulase positive staphylococci survived during refrigeration and mixing. These results agree with the literature which reports that these bacteria can survive for long periods in temperatures lower than the necessary for multiplication (21). However, the number of coagulase positive staphylococci found in the samples was not enough to produce toxin and cause a foodborne intoxication. According to Schmitt *et al.* (15), foods would represent a risk only if maintained at 14-15°C for several days or at room temperature for many hours, which did not happen in the present study.

The average times for frying meat balls and kibbe were 4 and 6 minutes, respectively. The maximum temperature during frying was 82.3°C and 95.0°C, respectively. Meat balls remained in a temperature above 63°C for 3.3 minutes and kibbe for 5,0 minutes. In both cases, the time/temperature binomial was enough to eliminate total and fecal coliforms and to reduce mesophilic bacteria and yeasts and

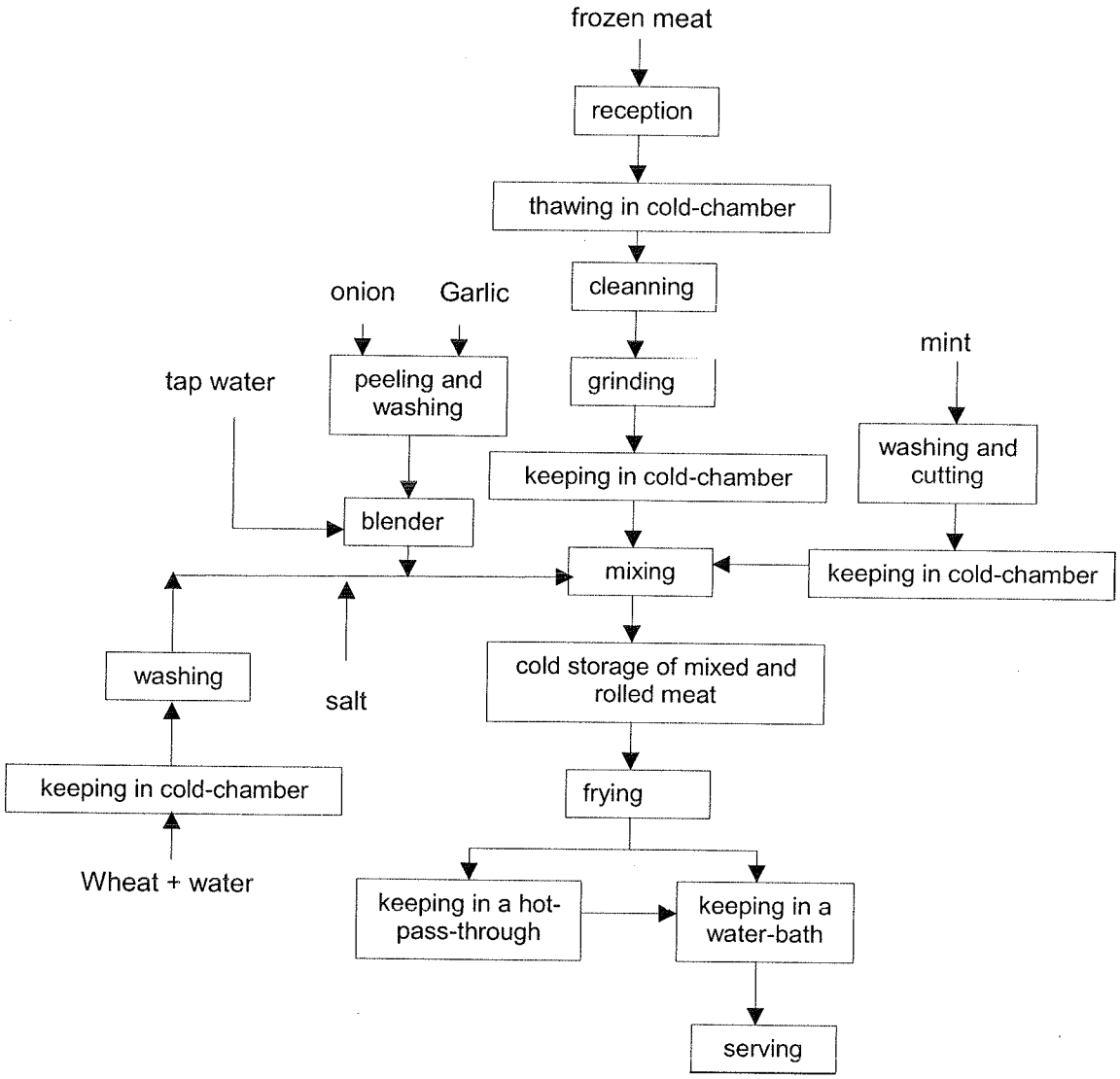

Figure 2 – Flow diagram of kibbe preparation

molds, as it can be observed in Table 3. It seems that the attained temperature was sufficiently high to kill the vegetative cells, but not enough for spores of pathogenic bacteria. Bacteria of the *B.cereus* group were not eliminated by frying (Table 2).

Between frying and serving, meat balls and kibbe were maintained hot in the steam table or in a hot pass-through for an average period of time of 135 and 108 minutes, respectively. The average temperature of the steam-table water was 92.5°C , which is enough to maintain the food temperature above 60°C (16). However, 33.3% of samples showed lower temperatures for a period of one hour. During this period, indicator microorganisms and pathogen levels did not increase (Tables 2 and 3).

C. perfringens was not detected in any analyzed sample.

Table 4 shows the average numbers of mesophilic bacteria detected in equipments and utensils. It seems that only dishes were efficiently cleaned. Wooden spoons presented the highest level of contamination, followed by blender and basins. These equipments and utensils could contaminate the food during preparation.

Hazard analysis:

According to NACMCF (12), to conduct a hazard analysis it is necessary to prepare a list of steps in the process where significant hazards occur, to list all identified hazards associated with each step, and to establish the preventive measures.

The steps with identified hazards and the preventive measures for both preparations were the following:

Reception: raw meat may present spores of *C. perfringens, Salmonella, S.aureus* and other pathogens (5). In the present study, coagulase positive staphylococci were detected in 33.3% of the incoming meat. To prevent these hazards it would be necessary to require periodical microbiological analysis of raw meat supplied by the butchery, and to change the meat supplier when the analysis shows an inadequate count of microorganisms. Raw vegetables and condiments may also have large numbers of bacteria, including bacterial spores (7).

Thawing: no important hazard was detected in the thawing step. The growth of *C.perfringens, Salmonella* and *S.aureus* during thawing in a refrigerator (5) under an adequate temperature is not expected. However, temperature higher than 4°C was found once in this study which allowed an increase

Table 2 – Pathogens found in different steps of preparation of meat balls and kibbe.

Preparation	Date	Sample	Pathogen	CFU/g
Meat balls	11/12/95	Thawed meat	*B. cereus*	$1.0x10^2$
	1/22/95	Received meat	*S. aureus*	$1.0x10^2$
		Thawed meat	*S. aureus*	$1.6x10^2$
		Mixed meat	*S. aureus*	$1.0x10^2$
Kibbe	8/24/95	Received meat	*S. aureus*	$1.0x10^2$
		Mixed meat	*B. cereus*	$2.0x10^2$
		Cooked meat	*B. cereus*	$1.0x10^2$
	11/20/95	Mixed meat	*B. cereus*	$1.0x10^2$

Table 3 – Enumeration of total (TC) and fecal (FC) coliforms, aerobic mesophilic bacteria (AMB), aerobic psichrotropic bacteria (APB), and yeasts and molds at each step of the preparation of meat balls and kibbe.

Preparation	Food sample	TC	FC	AMB	APB	Yeasts and molds
		(MPN/g)*	(MPN/g)*	(CFU/g)*	(CFU/g)*	(CFU/g)*
	Received meat	4.7x10	1.6x10	$4.5x10^4$	$1.2x10^4$	$7.5x10^3$
	Thawed meat	$4.4x10^2$	$3.7x10^2$	$7.9x10^6$	$4.7x10^7$	$1.5x10^6$
	Ground meat	$2.1x10^3$	$3.8x10^2$	$3.6x10^6$	$6.1x10^7$	$1.8x10^6$
Meat balls	Mixed meat	$3.8x10^4$	$2.2x10^2$	$5.7x10^6$	$3.3x10^7$	$1.6x10^6$
	Cooked meat	< 3.0	< 3.0	$3.2x10^3$	NT	$2.7x10^2$
	Served meat	< 3.0	< 3.0	$2.3x10^3$	NT	$2.3x10^3$
	Pool of ingredients	$4.2x10^3$	6.0	NT	NT	NT
	Received meat	$6.8x10^2$	4.7x10	$9.1x10^4$	$1.9x10^5$	$8.0x10^2$
	Thawed meat	$1.9x10^2$	8.0x10	$5.8x10^4$	$2.2x10^7$	$1.6x10^4$
	Ground meat	$1.9x10^2$	1.1x10	$1.7x10^6$	$1.3x10^7$	$1.5x10^6$
Kibbe	Manipulated meat	$1.5x10^3$	6.0x10	$7.5x10^6$	$4.0x10^6$	$3.5x10^5$
	Cooked meat	< 3.0	< 3.0	$2.2x10^4$	NT	$4.6x10^3$
	Served meat	< 3.0	< 3.0	$7.2x10^2$	NT	$2.0x10^2$
	Pool of ingredients	$3.8x10^2$	2.6	NT	NT	NT

NT – not tested.
* average of three determinations;

Table 4 – Enumeration of acrobic mesophilic microorganisms (AMB) in utensils.

Utensils	AMB (CFU/cm²)*
Basin	$2.0x10^4$
Wooden spoon	$4.9x10^6$
Flat cake tin	$4.8x10^2$
Blender	$5.5x10^4$
Dish	$< 1,0 \times 10^2$
Chopping board	$2.3x10^3$

* average of five determinations

of the meat temperature to 7.8°C. So, it would be necessary to monitor the cold-chamber temperature.

Manipulation: during manipulation, thawed meat was ground, mixed with vegetables and condiments, and transformed in meat balls and kibbe. As humans may carry foodborne pathogens on the skin, they can transfer these pathogens to the food during handling (7). However, all foodhandlers worn gloves. So this source of contamination was not considered significant. On the other hand, poorly-cleaned surfaces of utensils and equipments that harbor and promote microorganisms spread like wooden spoons and chopping board were used (Table 4), being a new source of contamination.

To prevent these hazards it would be necessary to implement Good Manufacturing Practices (GMP), like better hygiene of utensils and equipments and reduction of exposure time to room temperature.

Frying: Two hazard were detected in this step. One is the microbial survival, mainly of sporeforming bacteria due to insufficient heating and survival of resistent microorganisms (7). The other is the presence of preformed toxins, like staphylococcal toxins, which are not destroyed by heat. These hazards indicate that it is important to control the time/temperature binomial during storage because meat balls and kibbe are fried for few minutes only. To prevent theses hazards it should be necessary to promote adequate cooking through reduction of the size of meat balls and kibbe, and setting correctly the time of frying and the oil temperature.

Hot-holding: microorganisms which were not destroyed during frying multiply during hot-holding if temperature is not high enough. Kibbe remained for up to 50 minutes in a temperature below 60°C (data not shown).

Service: there was no apparent hazard during service because ready foods were distributed by cookers wearing disposable gloves and the dishes were adequately hygienized (Table 4).

Determination of Critical Control Points:

Using the NACMCF decision tree (12), two steps in the preparations of meat balls and kibbe were considered CCPs: cooking (frying) and hot-holding, where the time/temperature binomial should be controlled.

During cooking, temperature should reach 80°C for 5 minutes in the geometric center of food.

During hot-holding food temperature should be at least 60°C. To maintain this temperature, the water temperature in the water bath should be higher than 85°C and pass-through temperature should be at least 60°C. Besides, the bottom of the pans should be in contact with the steam table water.

Hazards analysis was also applied in a hospital kitchen by Bryan and Lyon (8) in the cook/freeze, cook/chill, assemble/serve and cook/hot-holding operations. CCPs were detected in each operation. In the cook/freeze and cook/chill operations, the identified CCPs were cooking, cooling and handling after cooking. In the assemble/serve operation, the CCP was the incoming food, and in the cook/hot-hold operation the CCPs were cooking and hot-holding. No hazard was observed during thawing.

Working in hospital kitchens with cook/hot-hold, cook/chill and cook/freeze systems, Bobeng and David (4) considered ingredients, foodhandlers and equipments hygiene and time/temperature control as CCPs.

Due to several hazards determined during meat balls and kibbe preparations it seems that training programs for nutritionists and foodhandlers are necessary. This training program shoud contain principles of food microbiology, food safety, microbiological hazards, food processing, determination of critical control points, practical control measures and monitoring procedures which are important to prevent foodborne diseases.

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RESUMO

Pontos críticos de controle na preparação de almôndegas e kibes em uma cozinha hospitalar

Foram determinados os perigos e os pontos críticos de controle (PCC) associados a preparações de almôndegas e kibes em uma cozinha hospitalar, baseados em fluxogramas de preparo e análises microbiológicas de amostras coletadas ao longo da preparação. As análises microbiológicas incluíram a contagem de microrganismos mesófilos, psicrotróficos, bolores e leveduras, *C. perfringens,* estafilococos coagulase positiva, bactérias do grupo *B. cereus*, número mais provável de coliformes totais e fecais e pesquisa de *Salmonella*. Foram medidos o tempo e a temperatura em todas as etapas da preparação. Para a determinação dos PCCs foi utilizada uma árvore decisória. Foram detectados os seguintes perigos: a contaminação da carne e vegetais crus, a multiplicação dos microrganismos durante a etapa de manipulação da carne, a falta de higiene dos utensílios equipamentos, a sobrevivência de microrganismos ao processo de cocção. A cocção e a manutenção à quente foram considerados PCCs. Os resultados indicam a importância da implantação de um programa de treinamento de nutricionistas e cozinheiros e de um sistema de monitoramento dos PCCs, assim como de medidas para a prevenção de doenças de origem alimentar.

Palavras-chave: HACCP, qualidade microbiológica de alimentos, carne, cozinhas hospitalares.

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MOLECULAR CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM FOODS

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ABSTRACT

A total of 30 strains of *Listeria monocytogenes* isolated from different foods (16 of differents kinds of sausage, 14 cheese,) purchased at groceries of São Paulo City were ribotyped and analysed for the presence and expression of hemolysin gene and production of phosphatidylinositol-specific phosphalipase C - PI-PLC enzyme. The *L. monocytogenes* strains were differentiated into six ribotype classes. A total of 13 (43.3%) from these strains belong to the same ribotype (ribotype I), and was coincident to the ribotype of the standard *L. monocytogenes* prototype strain (ATCC-15313). The hemolytic activity was observed in 29 (96.7%) strains when incubated at 37°C, but not at 4°C. The direct colony hybridization method for hemolysin gene detection showed a positive reaction whit all the 30 *L. monocytogenes* strains, while showed negative reaction with other *Listeria* spp. The PI-PLC was produced by 27 (90%) of the strains analysed. There was no correlation between the six identified ribotypes and the virulence factors (hemolysin and PI-PLC) studied.

Key words: *Listeria monocytogenes,* ribotyping, virulence factors, food

INTRODUCTION

Several food-borne outbreaks have highlighted the importance of the *L. monocytogenes* to the public health (8, 9, 11, 18, 25). Establishing new methods to detect this pathogen in food (21) is very important because different food can be contaminated with this bacteria (11). Characterization using biochemical tests relies on expression of phenotypes that may not discriminate between species or strains within a same species (2). Alternative methods ideally should be based on the detection of *L.monocytogenes* virulence genes or gene products. One marker should be the production of a hemolysin, listeriolysin O (LLO), which is required for intracellular survival of invading bacteria in mammalian host (10). The gene encoding LLO had been named *hly*A. *L.monocytogenes* produces other hemolysins besides LLO like phosphatidylinositol-specific phospholipase C - PI-PLC (17) and phosphatidylcholine-specific phospholipase C - PC-PLC (5). Unlike LLO, which lyses host cells by forming pores in the membrane, the phospholipases disrupt host membranes by hydrolyzing membrane lipids (24).

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Methods for the characterization of *Listeria* spp. have been developed, and isoenzyme analysis shows some promise as a typing method (3). Nucleic acid hybridization has contributed significantly to the progress of bacterial classification and identification. One possible disadvantage of DNA probes is that each probe will detect only one taxonomic entity or clones harboring a given gene (14). Species specific probes for *L monocytogenes* have been developed, and used in colony hybridization assay (6, 7, 22, 23). The DNA fingerprint reveals DNA restriction fragment length polymorphisms, and has already been used in epidemiological investigations (2), but, with no success. Ribotyping, based on restriction fragment length polymorphism in the chromosomal DNA containing rRNA genes has also been used as important tool.

The purpose of this study was to type *L.monocytogenes* strains, isolated from foods in São Paulo city, by using a probe complementary to ribosomal RNA (plasmid pKK3535). Haemolytic activity was analysed in blood agar plates and the presence of the hemolysin gene detected by using the plasmid pIP5 as a probe. We also examined the enzymatic activity of phosphotidylinositol-specific phospholipase C, in order to examine the eventually relationship between the ribotypes and virulence associated genes

MATERIALS AND METHODS

Bacterial strains

A total of 30 strains of *L.monocytogenes*, serovars 1/2b and 4b, isolated from different kinds of food (16 different kinds of sausagesand 14 cheeses) purchased at groceries of São Paulo city were studied. The non-*monocytogenes Listeria* and *L. monocytogenes* type strains were from the American Type Culture Collection. The bacteria were maintained at room temperature in a solid medium (trypitic soy agar supplemented with 0.6% of yeast extract). Host strain *E.coli* (ED 8654-pKK3535), and *E.coli* DH5a -pIP5-[F$^-$, *end*A1 *hsd* R17 (r^-_k m^+_k) *sup*E44 thi-1l$^-$ l$^-$*rec*A1 *gyr*A96 *rel*A1 (*arg* F-*lac* ZYA) U169 f80d *lac* DAM15] were maintained under selective pressure with ampicilin (100μg/ml).

Preparation and labeling of DNA probe

Plasmid pKK3535 (4) was isolated from a culture of *E.coli* (ED 8654 pKK3535), and the plasmid pIP5 (23) was isolated from a culture of *E.coli* DH5a (pIP5), grown overnight at 30°C in 2xYT broth contaning ampicilin (100μg/ml) as described by Ish-Horowicz and Burke (15). The plasmid pKK3535 was labeled using digoxigenin-dUTP (1) by nick-translation (Genius Kit, Boehring Manheim Biochemicals, Indianapolis, IN) following the manufacturer's instructions. The plasmid pIP5 was used as a probe after linearization with restriction endonuclease *Kpn*I (Promega) (23), and labeled as described above.

Ribotyping

Total DNA of *Listeria monocytogenes* strains was extracted from one liter 48h culture in 2xYT using the procedure of Sambrook *et al.* (25). DNA samples (0.5-2ug) were cleaved by restriction endonucleases *Hin*d III (Promega) according to the manufacturer's instructions, electrophoresed on 0.7% agarose gel, in Tris-acetate buffer (0.04M Tris acetate, 0.001M EDTA, pH 6.7), using 1 Kb DNA ladder (Gibco) as a marker, and stained in ethidium bromide (1.0mg/ml) for 20 min. Gels were then photographed. The DNA was transfered to nylon membrane by the capillary transfer method (27), and the fixed DNA (14) was prehybridized at 37°C in hybridization solution [25ml 20xSSC; 30ml formamide; 1ml NaCl a 10%; 0.2ml SDS a 10%; 5g blocking reagent (Genius Kit-Boehringer Mannheim); 43.8ml dd water]. The solution was replaced by 2.5ml of hybridization solution containing the labeled probe pKK3535. After 24 h incubation, the membrane was washed and positive reactions were colorimetrically visualized according to Genius Kit manufacturer's recommendations.

Hemolytic activity

The strains were stabbed on 5% horse blood agar plates (19), incubated at 37°C for 48h, and at 4°C for 30 days. The production of clear zones around the colonies indicated beta-hemolytic activity.

Colony hybridization

Bacterial colonies, from blood agar plates, were transferred to the nylon membrane, which were prepared for hibridization according to Peterkin *et al.* (23). The treated membrane, carrying DNA, was prehybridized at 68°C for 1 h in a bag containing hybridization solution. This solution was replaced by fresh hybridization solution containing the labeled pIP5 and after 24h incubation, the membrane was washed, and positive reactions were colorimetrically

visualized according to Genius Kit manufacturer's recommendations.

Detection of PI-PLC activity

PI-PLC activity were determinated as described by Notermans *et al.* (21). The plates were observed up to 5 days of incubation at 37°C, for turbid halos around colonies.

RESULTS

The 30 *L. monocytogenes* strains were differentiated in six ribotypes. The schematic representation of the ribotype patterns can be observed in Fig. 1. The type I (rI) presented 5 fragments: 2.3; 3.3; 3.6; 7.3 and 7.4Kb, the type II (rII) with 4 fragments: 2.3; 3.3; 6.9 and 7.4Kb, the type III (rIII) with 6 fragments: 2.3; 3.3; 4.0; 4.7; 7.3 and 7.4Kb, the type IV (rIV) with 4 fragments: 2.0; 2.3; 2.7 and 7.4Kb, the type V(rV) with 5 fragments: 2.3; 4.0; 4.7; 7.3 and 7.4Kb and the type VI(rVI) with 3 fragments: 2.3; 3.3 and 6.9Kb. All ribotypes presented a 2.3Kb common band, and another band of 7.4Kb was observed in five ribotypes (I, II, III, IV and V). Interestingly, a total of 13 (43.4%) of the 30 strains typed as a ribotype I were identical to the profile of the standard *L.monocytogenes* strain (ATCC-15313). The probe used was successful to differentiate *L. monocytogenes* strains from other *Listeria* spp, which lacked the 2.3Kb band. The hemolytic activity was observed in 29 (96.7%) strains when they were incubated at 37°C in blood agar, but at 4°C the listeriolysin was not produced (Table 1). The hemolytic activity using the direct colony hybridization method showed a positive reaction with all the *L.monocytogenes* strains. The phosphatidylinositol-specific phospholipase C activity was detected in 27 strains (Table 1).

DISCUSSION AND CONCLUSION

Epidemiological tracking of food-borne pathogens requires methods of analysis that allow discrimination between phenotypically indistinguishable strains within a species (2).

Grimont and Grimont (14) analyzed the DNA from 41 different bacterial species, and demonstrated that patterns of hybridized fragments could be used to identify biochemically indistinguishable strains. Baloga and Harlandar (2) studied 28 strains of *L.monocytogenes* isolated from food implicated in food-borne illness and from patients with listeriosis. The authors determinated the fingerprints, ribotypes, and the resulting subtypes were compared with multiloccus enzyme electrophoresis classification schemes. They observed that the serotypes were distributed among several distinct fingerprints and ET categories.

The mechanisms of pathogenicity of *L. monocytogenes* have been studied, and usually is associated with hemolysin production (12). There have been a number of reports suggesting the possibility of the temperature regulation of virulence genes expression in *L. monocytogenes*, but the results have been conflicting (13, 17). The hemolytic activity of *Listeria* spp., and the level of listeriolysin produced may be dependent on enrichment procedures, selective media, temperature and virulence of the bacteria (8, 21).

In our study, hemolytic activity was detected in 29 *L. monocytogenes* strains when incubated at 37°C, but, this activity was not observed when incubated at 4°C. Ours data agree with the Girard *et al.* (13) and Leimeister-Wachter *et al.* (17) results, which

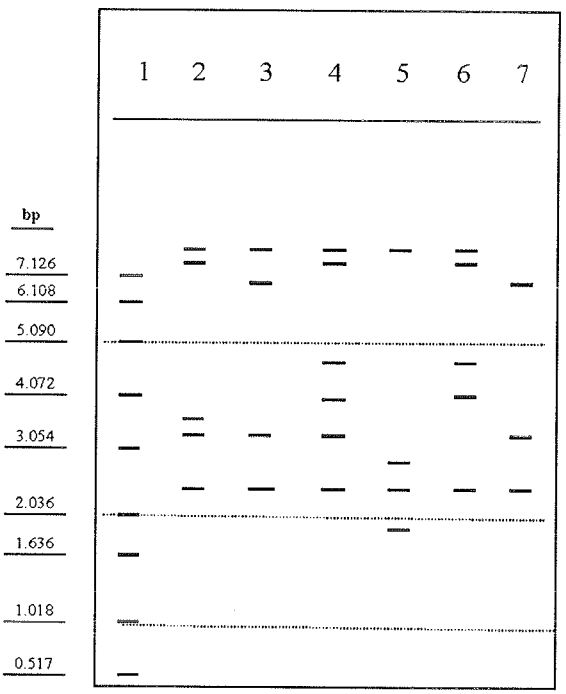

Figure 1: Schematic representation of six *L. monocytogenes* ribotype patterns.
Lane 1: 1Kb DNA ladder (Gibco-BRL); Lane 2: ribotype I; Lane 3: ribotype II; Lane 4: ribotype III; Lane 5: ribotype IV; Lane 6: ribotype V; Lane 7: ribotype VI.

observed higher levels of the hemolysin production in different temperatures (between 20 to 37°C), when compared to 4°C.

Leimeister-Wachter *et al.* (17) found that low temperature have no significant effect on the *L. monocytogenes* virulence factors production. A likely explanation for the absence of hemolytic activity in one strain could be an alteration in the environment or in the regulatory gene (17, 20).

The most recent developments in methodologies for the detection of *L. monocytogenes* have involved gene probes. A commercial hybridization assay based on 16S rRNA sequences (Gene Trak) was developed for the detection of *Listeria* spp. in dairy products and environmental samples (16). Some researchers developed probes specifically for *L. monocytogenes* (7, 23). Those probes are used to detect the listeriolysin gene (*hly*A), and are used in colony

Table 1. Ribotype (*Hin*dIII), hemolytic activity, presence of *hly*A gene and phosphatidylinositol-specific phosfolipase C (PI-PLC) production of *Listeria* spp, and *L. monocytogenes* isolated from food in São Paulo-BR.

Strains	Ribotype	Hemolytic activity 4°C	Hemolytic activity 37°C	(Gene *hly*A)	PI-PLC
L. gray		-	-	-	-
L. innocua		-	-	-	-
L. ivanovii		+	+	-	-
L. monocytogenes	I	-	+	+	+
L. murrayi		-	-	-	-
L. seeligeri		-	+	-	-
L. welshimeri		-	-	-	-
1	I	-	+	+	+
2	II	-	+	+	+
3	II	-	+	+	+
4	VI	-	+	+	+
5	VI	-	+	+	+
6	I	-	+	+	+
7	I	-	+	+	+
8	I	-	+	+	-
9	V	-	+	+	+
10	II	-	+	+	+
11	V	-	+	+	+
12	I	-	+	+	+
13	I	-	+	+	-
14	I	-	+	+	+
15	I	-	+	+	+
16	II	-	+	+	+
17	I	-	+	+	+
18	V	-	+	+	+
19	II	-	+	+	+
20	II	-	+	+	-
21	III	-	+	+	+
22	III	-	+	+	+
23	IV	-	+	+	+
24	I	-	+	+	+
25	I	-	+	+	+
26	I	-	-	+	+
27	III	-	+	+	+
28	I	-	+	+	+
29	IV	-	+	+	+
30	IV	-	+	+	+

(+) positive reaction (-) negative reaction

hybridization assays. In our study, the direct colony hybridization method gave positive reaction with all the 30 *L. monocytogenes* strains analysed, while showed a negative reaction with other *Listeria* spp, as expected.

The *pfr*A gene is a positively acting factor that transcriptionally activates the expression of the *pic, hly*A and other genes (17). In this study, the PI-PLC activity was expressed only by the majority of the strains. Probably, the negative PI-PLC activity in three tested strains should be explained by the absence of or mutation on the *pic* or *prf*A genes as suggested by Notermans *et al.* (21)

Our data permit us to conclude that there were no correlation between the six identified ribotypes and some *L. monocytogenes* virulence factors (hemolysin and PI-PLC).

RESUMO

Caracterização molecular de *Listeria monocytogenes* isolada de alimentos

Foram estudadas 30 cepas de *Listeria monocytogenes* isoladas a partir de diferentes alimentos (16 diferentes tipos de linguiça, 14 de queijo), adquiridos em supermercados da cidade de São Paulo. As cepas foram classificadas através da ribotipagem e analisadas quanto à presença e expressão do gene da hemolisina e à produção da enzima fosfolipase C fosfatidilinositol-específica PI-PLC. As cepas de *L. monocytogenes* foram diferenciadas em 6 ribotipos. As cepas do tipo I possuiam o mesmo perfil da amostra padrão de *L.monocytogenes* (ATCC 15313), sendo 13 (43,3%) das cepas estudadas correspondentes a ele. A atividade hemolítica foi observada em 29 (96,7%) das cepas, quando incubadas a 37°C em agar sangue, mas não a 4°C. O método da hibridização direta de colônias, utilizando sonda para hemolisina, revelou resultado positivo para todas as cepas. A PI-PLC foi produzida por 90% das amostras analisadas. Deste modo, foi possível concluir que não há relação entre os seis ribotipos de *L. monocytogenes* identificados nesse estudo e os fatores de virulência estudados (hemolisina e PI-PLC).

Palavras-chave: *Listeria monocytogenes*, ribotipagem, fatores de virulência, alimentos.

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OCCURRENCE OF *LISTERIA MONOCYTOGENES* IN SALAMI

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SHORT COMMUNICATION

ABSTRACT

Eighty-one samples of four different types of salami (Friolan, Hamburguese, Italian and Milanese), belonging to five brands, and purchased at Rio de Janeiro market, were evaluated for the occurrence of *Listeria monocytogenes*. The pathogen was detected in 13.3% of Italian type samples of salami, while *L. innocua* occurred in 6.5% of the Italian type and in 16.6% of the Milanese type. The remaining samples were negative for *Listeria* spp.

Key words: *Listeria*, meat products, salami

Listeriosis occurs mainly in immunocompromised individuals such as patients who depend on hemodialysis and prolonged therapies, AIDS patients, alcoholics, drug addicts and elderly, newborn and pregnant women (12, 18). The involvement of *L. monocytogenes* as an infecting agent, in these cases, has been related to the consumption of "in nature" and / or processed foods of vegetal and animal origins, such as milk (14, 17), cheese (5, 20), poultry (8, 17, 24), red meats (6, 13), meat products (5, 9, 23), vegetables and fruits (6, 11).

The incidence of *Listeria* in fresh meat may vary from 0 to 68%, while in processed meat products, including ready-to-eat food, the contamination ranges from 8 to 92% (13). However, a higher incidence of *L. innocua* in meat products, compared to *L. monocytogenes* was reported (17). In salami, the occurrence of *L. monocytogenes* varies from 5 to 23% (6, 7). Salami do not undergo heat treatment and are fermented under variable temperatures (mostly between 25-30°C according to the processing method adopted by the manufacturer), having a final pH between 4.8 and 5.2, and water activity around 0.85-0.90 (3). The fermentation process is also variable and can be conducted by the meat natural flora or by the addition of lactic acid starter cultures. The characteristics of these products make them susceptible to the survival of *L. monocytogenes* and, therefore, there is a potential risk to the consumer's health. Despite the importance of *L. monocytogenes* in meat products, only few studies on the occurrence of this microorganism in fermented sausage

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manufactured in Brazil are available (5, 19). So far neither clinical cases nor outbreaks of listeriosis associated with the consumption of any food have been described (1, 16, 21). Therefore, the aim of this study was to verify the occurrence of *L. monocytogenes* and other species of *Listeria* in different types of salami sold at Rio de Janeiro retail market.

Three units of the same production batch of four types of salami (Friolan, Hamburguese, Italian and Milanese) from different commercial brands were collected at the retail market in the city of Rio de Janeiro. Commercial brands included five of the Italian type, two of Milanese type and one of Hamburguese and Friolan types. Each experiment was repeated three times, using samples from different production batches, in an overall total of eighty-one samples. Each sample was constituted by three sub-samples of 50 g, finely ground in a blender for two minutes. 25g of each sample were analyzed according to a modification of the method suggested by Mc Clain and Lee (15) and Van Netten *et al* (25), using PALCAM agar (Merck) instead of Lithium chloride-phenylethanol-moxalactam medium (LPM). The procedure included the following steps: primary enrichment (UVM I *Listeria* enrichment broth – Merck), secondary enrichment (UVM II *Listeria* enrichment broth – Merck), plating (PALCAM agar) and biochemical identification. In all steps, standard cultures of *L. monocytogenes* Scott A, *L. monocytogenes* ATCC 7644, *L. monocytogenes* ATCC 19111 and *L. innocua* L6A were used as controls.

One hundred and ten colonies with characteristics of the genus *Listeria* were recovered from PALCAM agar. After initial selection by the motility test, eighty isolates with umbrella-shaped motility were submitted to morphological and biochemical characterization. In the carbohydrate fermentation tests, all cultures were able to use α-methyl-D-mannoside and rhamnose, with the production of acid, but they could not ferment xylose and mannitol. All eighty isolates presented positive and negative reactions to catalase and urea tests, respectively. Among these, sixty-seven were unable to reduce nitrate, as occurs in the *Listeria* genus. The sixty-seven isolates were further submitted to β-hemolysis, CAMP-*Sa* and CAMP-*Re* tests. According to the results, thirty isolates belonged to *Listeria* genus and twenty-three of them were identified as *L. innocua* and seven as *L. monocytogenes* (Table 1) .

The seven strains of *L. monocytogenes* were isolated from six samples of a single brand of Italian type salami, whereas the twenty-three strains of *L. innocua* were detected in six samples of two types of salami (Italian and Milanese) belonging to two different brands.

Therefore, 14.8% (12 out of 81) of the samples of salami were positive for *Listeria* spp. 50% of the positive sample (13.3% of Italian type samples) harbored *L. monocytogenes*, and 50% of them (6.5% of the Italian type and in 16.6% of the Milanese type) were positive for *L. innocua*.

The incidence of these microorganisms in similar meat products is quite diversified. Simón Serra *et al.* (22) observed an incidence of *L. innocua* of 14.3% of samples of raw-cured sausage while *L. monocytogenes* was not detected. Other authors, testing same kind of products, reported the occurrence of *L. monocytogenes* in 12.0% (2) to 17.5% of samples (4, 10).

L. monocytogenes was detected in a significant number of salami samples, indicating that it is important to monitor the presence of this pathogen in this type of meat product, specially because it is consumed without previous heat treatment.

Table 1. Biochemical profile of thirty isolates of *Listeria* spp.

Isolates	Biochemical profile								
	Mannitol	Rhamnose	Xylose	α-methyl-D mannoside	urea	nitrate	β-Hemolysis	CAMP-Sa[1]	CAMP-Re[2]
1 to 5	-	+	-	+	-	-	+	+	-
6 to 7	-	+	-	+	-	-	+	+	+
8 to 30	-	+	-	+	-	-	-	-	-

[1]CAMP test with *Staphylococcus aureus*
[2]CAMP test with *Rhodococcus equi*.

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RESUMO

Ocorrência de *Listeria monocytogenes* em salame

A ocorrência de *Listeria monocytogenes* foi avaliada em oitenta e uma amostras de quatro diferentes tipos de salame fermentados (Friolano, Hamburguês, Italiano e Milano), pertencentes a cinco marcas comerciais, adquiridas no mercado varejista da cidade do Rio de Janeiro. *L. monocytogenes* foi detectada em 13,3% das amostras de salame do tipo Italiano, enquanto que *L. innocua* ocorreu em 6,7% das mostras do tipo Italiano e 16,6% das amostras do tipo Milano. As demais amostras foram negativas para *Listeria* spp.

Palavras-chaves: *Listeria*, produtos cárneos, salame

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CHARACTERIZATION OF TYPICAL AND ATYPICAL ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC) STRAINS OF THE CLASSICAL O55 SEROGROUP BY RAPD ANALYSIS

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ABSTRACT

The genetic diversity of 41 typical and atypical enteropathogenic *Escherichia coli* (EPEC) strains of the serogroup O55 was analyzed by using the random amplified polymorphic DNA (RAPD) method. All typical EPEC O55 strains were grouped in two clusters (A and C) and belonged to the serotype O55:H6, while cluster B included all atypical strains, which were of the serotype O55:H7. The three groups also included non-motile strains. RAPD may be a useful method for epidemiological studies on *E. coli* O55 infection.

Key words: Enteropathogenic *Escherichia coli*, EPEC, genetic diversity, RAPD analysis

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of diarrhea in many countries. Typically, EPEC strains possess a pathogenicity island, named LEE (locus of enterocyte effacement) and a 50-70 MDa plasmid, designated EAF (EPEC adherence factor). The LEE pathogenicity island contains the *eae* gene encoding an outer membrane adhesin (intimin) and all the other genes involved in formation of the attaching and effacing (A/E) lesion, characterized by bacterial adherence and destruction of intestinal microvili. The EAF plasmid encodes the BFP fimbriae (bundle-forming pilus) and some regulatory proteins. Routinely, LEE is detected by the *eae* gene probe and the EAF plasmid by the EAF probe (reviewed in 7).

It has been reported that the EAF plasmid can be lost during storage or even during infection (3, 6), but the absence of this plasmid in certain serotypes (i.e, O111:H9 and O26:H11) seems to be a natural occurrence. Natural EAF-negative strains belong to distinct electrophoretic types or clones (2, 9). Kaper, in 1996, proposed the designation of atypical EPEC for these strains (4).

E. coli O55 is one of the most important of the classical EPEC O serogroups not only because of its

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isolation frequency, but also because this serogroup includes strains with variable virulence properties (8, 9). O55 strains may be motile or non-motile and when motile they usually belong to serotypes O55:H6 and O55:H7. Most strains of the serotype O55:H6 possess the EAF plasmid while the plasmid has not yet been found in O55:H7 strains (9, 10). When studied by multilocus enzyme electrophoresis (MLEE), O55:H6 and O55:H7 strains are separated in two clonal groups, both including non-motile strains. The non-motile strains in O55:H7 clonal group may be EAF-negative or EAF-positive or express other virulence characteristics (9). In this study we investigated whether EAF-positive and EAF-negative O55 strains, isolated in different places and periods of time, could be distinguished by RAPD analysis. RAPD is a reproducible and inexpensive method that has been used to characterize both bacterial groups and strains (1).

MATERIALS AND METHODS

Bacterial strains. A total of 41 *E. coli* strains of the serogroup O55 were studied. Of these 26 were *eae*+ EAF+ and 15 were *eae*+ EAF–, as determined by the corresponding gene probes (9). None of the strains produce Shiga toxins as verified by tissue culture assay in Vero cells (5). Six strains were of serotype O55:H7, 5 of the serotype O55:H6 and 27 were non-motile. In 3 strains the H antigen could not be determined. With the exception of some H7 strains that were isolated in other countries, the remaining strains were isolated in São Paulo and in Rio de Janeiro from children with diarrhea between 1965 and 1994 (Fig. 2). From the time of isolation to the beginning of the studies, the strains were kept at room temperature or in glycerinated broth at -70°C.

RAPD reaction. Genomic DNA was extracted and purified from bacterial cultures in Luria-Bertani broth as described previously (1). PCR for the RAPD reaction was performed in 20 µl reaction volumes containing: 10 ng of DNA, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of dNTP (dATP, dCTP, dGTP, dTTP, Gibco BRL), 0.3 µM of random primer (OPE-16: 5'-GGTGACTGTG-3'; OPK-01: 5'-CATTCGAGCC-3'; OPK-04: 5'-CCGCCCAAAC-3'; OPP-03: 5'-CTGATACGCC-3'; Operon Technologies), 1.5 unit of Taq DNA polymerase (Gibco BRL) and overlaid with mineral oil. Amplification reactions were performed in a thermalcycler (Perkin-Elmer model 480, Cetus) and included one previous step at 94°C for 5 min and 40 cycles with the following steps: denaturation at 95°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 2 min. An additional extension step of 72°C for 7 min was included at the end of the PCR cycles. Amplified products were electrophoresed in 1.4% agarose gels, stained with ethidium bromide and visualized by using UV light. The 1kb and 123 bp DNA ladders (Gibco BRL) were used as molecular size markers in all gels.

Analysis of data. The statistical analysis of the data was performed by using the NTSYS-pc program (Numerical Taxonomy and Multivariate Analysis System) 1.7 version (Exeter Software, Setaked, N.Y.).

RESULTS AND DISCUSSION

Each primer yielded distinct polymorphism for the 41 strains and discriminated relatively well between EAF-positive and EAF-negative strains. This is exemplified in Fig. 1, which shows the

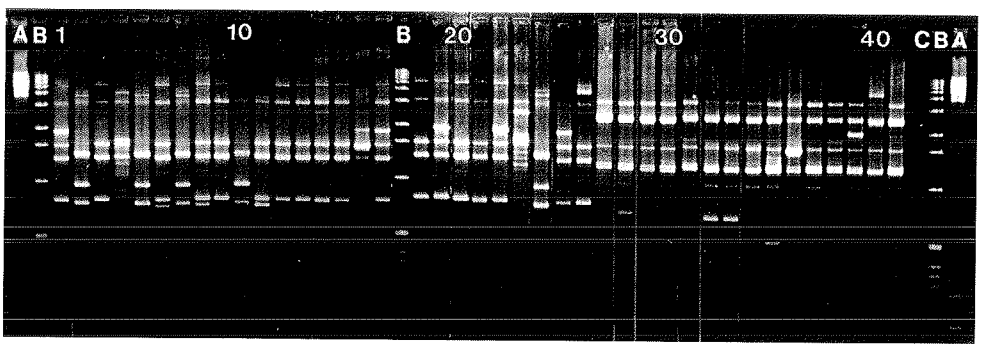

Fig. 1. RAPD fingerprints patterns of 41 *E. coli* O55 strains with primer OPE16. Lanes 1-26: EAF+ strains and lanes 27-41: EAF- strains. Molecular weight markers: 123bp (lane A) and 1kb (lane B) DNA ladders. In lane C no template DNA was added.

polymorphism and discrimination obtained with primer OPE16. Preliminary analysis of the data showed that better discrimination could be obtained by using the polymorphism's yielded by the four primers. Analyzing all 61 polymorphism, using the UPGMA method, we constructed a matrix and dendogram (Fig. 2), which showed that the 41 strains could be divided into three clusters designated A, B, and C. Clusters A and C included all EAF-positive strains and cluster B, all the EAF-negative ones. The two São Paulo O55:H6 strains in cluster A were isolated in 1966 and 1968 and the three ones in cluster C were isolated in 1981-1985.

The results of this study show that the O55 EAF-positive and the O55 EAF-negative strains are genetically distinct since they belonged to different RAPD clusters (Fig. 2). These results confirm previous findings obtained by MLEE and are consistent with the concept that typical and atypical EPEC are distinct bacterial lineages. The occurrence of the São Paulo O55:H6 strains in clusters A and C may represent the existence of two clones in this serotype predominating in different periods of time. The results of this study provide further evidence that O55:H6 and O55:H7 strains correspond to typical and atypical EPEC, respectively and show that RAPD provides an informative and simple tool that can be applied for epidemiological studies.

It has been shown that the occurrence of EAF-negative strains is rather frequent in several EPEC O serogroups and that these strains may be more important than the EAF-positive ones in some countries (i.e. England) (10). Further characterization of these strains is required to gain better

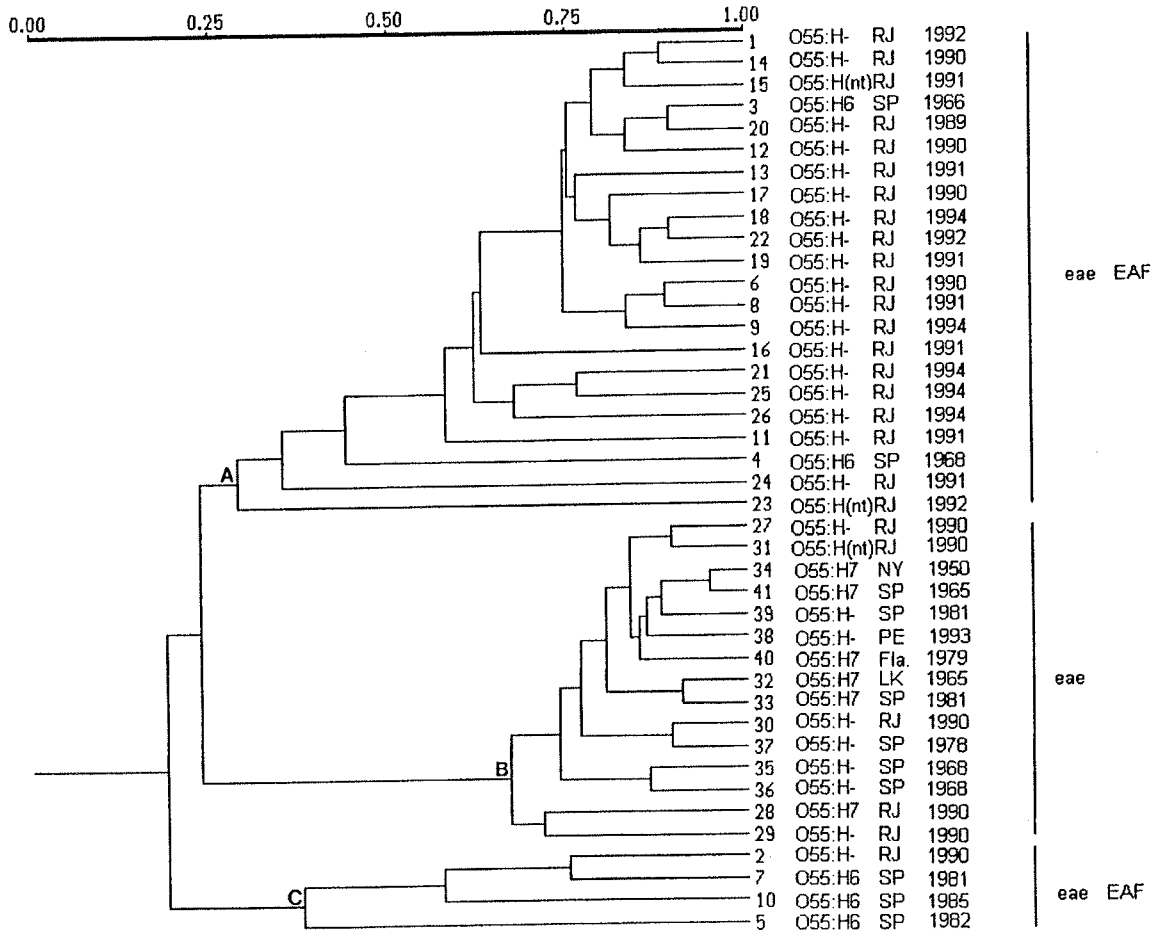

Figure 2. Dendogram based on UPGMA cluster analysis of Jaccard coefficients. Column 1 shows the numbers of the strains, column 2 shows their serotypes, columns 3 and 4 indicate the location and the year of isolation, respectively. Symbols: SP, São Paulo (Brazil); RJ, Rio de Janeiro (Brazil); Fla, Flórida (USA); NY, New York (USA); PE, Pernambuco (Brazil); LK, Srilanka (India).

understanding of their evolution and pathogenic properties, since there are other evidences suggesting that atypical EPEC strains may be ancestral to the typical EPEC and EHEC bacteria (11).

RESUMO

Caracterização de amostras de *Escherichia coli* enteropatogênica (EPEC) típicas e atípicas do sorogrupo O55 através da análise por RAPD

Quarenta e uma amostras de *Escherichia coli* enteropatogênica (EPEC) típicas e atípicas do sorogrupo O55 foram analisadas com relação à diversidade genética através da técnica de RAPD (random amplified polymorphic DNA). Todas amostras de EPEC O55 típicas ficaram localizadas em 2 grupos (A e C) e pertenciam ao sorotipo O55:H6, enquanto que o grupo B compreendia todas as amostras atípicas, as quais eram do sorotipo O55:H7. Os três grupos apresentavam também amostras imóveis. A técnica de RAPD pode ser uma ferramenta de grande utilidade em estudos epidemiológicos da infecção pela *E. coli* O55.

Palavras-chave: *Escherichia coli* enteropatogênica, EPEC, diversidade genética, análise por RAPD

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AN EXPERIMENTAL STUDY OF NANOFLAGELLATE BACTERIVORY

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SHORT COMMUNICATION

ABSTRACT

Heterotrophic nanoflagellate *Pseudobodo tremulans* (4.8 to 7.0 μm) and heterotrophic bacteria, isolated from coastal waters in Ubatuba, SP, Brazil, were used in experiments to analyze quantitatively the relationships between bacteria and nanoflagellates. The meaning of these results for the role of heterotrophic nanoflagellates in the Ubatuba coastal ecosystem is discussed.

Keys words: nanoflagellate, heterotrophic bacteria, coastal ecosystem, bacterivory

It has been widely accepted that a highly dynamic microbial loop consisting of pelagic bacteria, autotrophic pico- and nanoplankton, heterotrophic nanoflagelattes and microciliates is an integral part of planktonic food webs (3). Protozoa in the size range between 2 - 20μm (nanoplankton) are the major consumers of free-living pelagic bacteria in the sea (15). Several investigations on the dynamics of microbial loop have used a combination of laboratory and field methods (2, 12). The present investigation aims to quantify the bacterivory by a heterotrophic nanoplanktonic flagellate in "in vitro" conditions in order to get some indication of the importance of bacteria and nanoflagelattes in the Ubatuba coastal water ecosystem.

Surface seawater samples were collected in Ubatuba coastal region (southeast of Brazilian coast - 23°S 25°W). The system is considered to be meso-oligotrophic and is characterized by temperatures ranging from 14 to 24°C and salinities between 35 and 36‰. The primary production is low and nitrogen has been considered to be its main limiting factor (1).

Water samples were enriched with sterile rice grains and incubated at 20°C during 10 days before use. A heterotrophic nanoplanktonic flagellate (4.8 to 7.0 μm) identified as *Pseudobodo tremulans* was isolated from these enrichment cultures by micropipeting and a heterotrophic bacteria was isolated from the same enrichment by direct plating onto rice agar (9). The isolated microorganisms were maintained in stock cultures using a broth culture media (9) prepared with natural seawater and sterile rice grains, in the dark at 20°C (ambient water temperature at sampling time).

The experiments were carried out in duplicate and represented two different situations. In the first experimental procedure, the two microorganisms were grown without Cycloheximide, whereas in the second, microorganisms were grown in the presence

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of Cycloheximide used to inhibit the growth of nanoflagellates and reduce or eliminate predators in the system. Aliquots (5 ml) of BACTERIA + NANOFLAGELLATE stock cultures in the exponential growth phase (bacteria $10^8 ml^{-1}$ and nanoflagellate $10^5 ml^{-1}$) were transferred to filtered rice media and filtered rice media plus Cicloheximide (200mg l^{-1}). The cultures were maintained in the dark at 20°C. During 13 days at different time intervals 5ml aliquots were taken from each culture flask and fixed with formaldehyde (5% final concentration).

Bacterial abundance was estimated in preserved samples by epifluorescence microscopy using the Acridine Orange direct count method (AODC) (11). Nanoflagellates were quantified by epifluorecence microscopy according to the procedures described by Fenchel (7). Bacteria and nanoflagellate growth curves were done using the average values of the microorganism densities obtained from the duplicates of the experiments. Nanoflagellate biovolume was estimated by measuring the diameters of approximately 50 individual cells in different samples taken during the bacterivory experiments and assuming a spherical morphology (the equation used was $4/3\pi R^3$). The nanoflagellate carbon conversion factor used was 0.08 pg C mm^{-3} (16). An estimated biovolume of 0.10 μm^3 was used for bacteria and a carbon conversion factor of 0.22 pg C μm^{-3}, according to Bratbak and Dundas (4).

Microbial dynamics in the experiments were described according to a modified Lotka-Volterra type predator-prey model (2). The data used were obtained from growth curves of flagellate and bacteria in both situations (with and without Cycloheximide) at the first bacterial peak.

Fig. 1A represents the growth curves of bacteria and nanoflagellate populations during the experiment without inhibitor (BACTERIA + NANOFLAGELLATE) showing the predator-prey nature of the populations involved. In these experiment, bacterial density increased during the first 71h (maximum 1.75 x 10^8 ml^{-1}) and then decreased, reaching constant numbers after a time lapse of 167h (Fig. 1A). Approximately 96h after, the bacterial maximum nanoflagellates reached a peak of 5.00 x 10^5 ml^{-1} and then declined.

Fig. 1B shows the growth curves of both bacteria and nanoflagellate populations in presence of Cycloheximide. Cycloheximide inhibited both nanoflagellate growth and bacterivory for a period of about 168h. After a time interval of 239h, the nanoflagellates reappeared probably because the

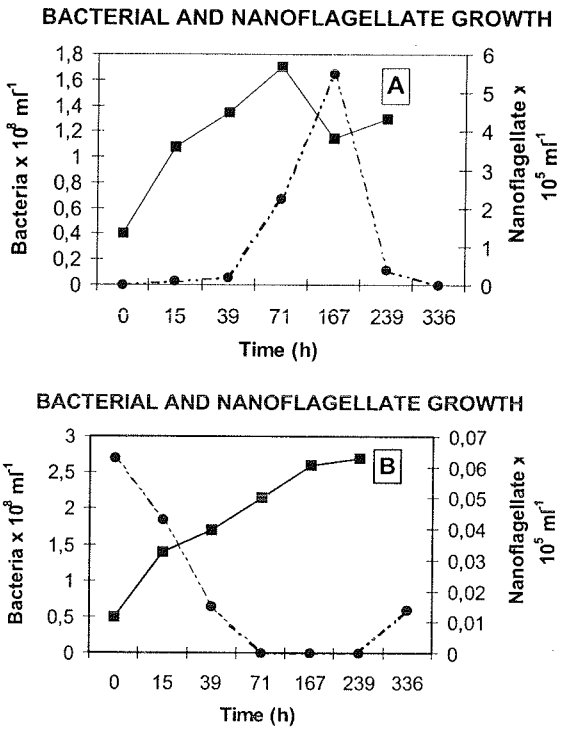

Figure 1. Growth of bacterial (■—■) and nanoflagellate (●—●) populations in the experiments without Cycloheximide (A) and with Cycloheximide (B).

Cycloheximide was metabolized by these microorganisms (6). At this time, protozoan grazing was repressed and bacterial abundance increased during the first 167h, reaching a value of 2.75 x $10^8 ml^{-1}$.

Table 1 shows the arithmetical means of the grazing data for experiments without and with Cycloheximide, prey and predator densities, growth rates, grazing rates, clearance rates and gross growth efficiency. The average bacterial growth rate in the grazing system was 0.102 divisions h^{-1} and the nanoflagellate one was 0.079 divisions h^{-1}. In the experiment without Cycloheximide each nanoflagellate consumed 82 bacteria per hour. The estimated average clearance rate obtained for one nanoflagellate was 4.6 x 10^{-7} ml^{-1} h^{-1}. The nanoflagellate gross growth efficiency was 39%. The extrapolation of the clearance rate obtained without Cycloheximide to the nanoheterotrophic population densities (mainly nanoflagellates) found in the Ubatuba coastal waters (8, 13) revealed that between 1 and 16% of the water column is cleared of bacteria per day by nanoflagellate populations.

In the experiment with inhibitor, Cycloheximide

Table I. Avarege values of grazing parameters obtained in the first bacterial peak. Experiment 1 was done without inhibitors and experiment 2 in the presence of Cycloheximide.

Cycloheximide	x	y	u(x)	u(y)	f(x)	f(x)/x	Y	Y
	Bacteria x 10^{-8} ml^{-1}	*Flagellates* x 10^{-5} ml^{-1}	*(d h^{-1})*	*(d h^{-1})*	*Bacteria* flagellate^{-1} h^{-1}	*ml flagellate^{-1} h^{-1}*	*Flagellate* bacteria^{-1}	*%*
absent	1.75	2.2	0.102	0.079	82	4.6 x 10^{-7}	1.0 x 10^{-3}	39
present	2.75	0	0.104	0	0	0	0	0

u(x) = bacterial growth rate
u(y) = nanoflagellate growth rate
f(x) = grazing rate
f(x)/x = clearance rate
Y = Yield (gross growth efficiency)

inhibited nanoflagellate growth and bacterivory and therefore the clearance rate. Comparatively to the experiment without Cycloheximide, the average value found for bacterial densities in this experiment was higher, in spite of the bacterial growth rate being similar (0.104 divisions h^{-1}) (Table 1).

The nanoflagellate growth rate obtained in this study is within the range of values of 0.01 to 0.25 d h^{-1} reported by Sherr and Sherr (15) and is very similar to that reported by Parslow *et al.* (14) for *Pseudobodo* sp (0.083 d h^{-1}) growing on *Micromonas pusilla*. In the absence of grazing, when nanoflagellate growth was inhibited by Cycloheximide, the bacterial population reached higher densities showing that the functional and numerical responses of the protozoa are probably adequate to control the size of bacterial populations (7).

The nanoflagellate grazing rate obtained in the abscence of Cycloheximide is close to those observed by several other authors (2) specially by Fenchel (7) for *Pseudobodo tremulans* (84 bacteria flagellate^{-1} h^{-1}). However, the clearance rate obtained was lower than those reported in literature. Since clearance is inversely proportional to prey densities and cellular volume (6), the high bacterial densities and bacterial volume observed in this work may be responsible for the low clearance rate obtained. Although low, this rate was equivalent to that obtained by Fenchel (8.0 x 10^{-7} ml flagellate^{-1} h^{-1}) (7) using hydrodynamic mathematical models.

The extrapolation of the nanoflagellate clearance rate to "in situ" bacterial and nanoheterotrophic densities in Ubatuba coastal waters showed that the nanoflagellate populations cleared a significant portion of bacteria in water column. This value, although significant, is not as high as those obtained by other authors (2, 12). According to Fenchel (7) and Lucas *et al.* (12), the nanoflagellate grazing rates and the clearance rates depend upon predator and prey species and their densities, relative sizes and the feeding mode of the predator. These factors explain the wide range noted in the literature of grazing and clearance rate values obtained in "in vitro" conditions.

The gross growth efficiency found in the present work is in the range of values reported by Fenchel (7) and Caron *et al.* (5) and is very close to that obtained by Lucas *et al.* (12) for *Pseudobodo* sp. A gross growth efficiency of 39% may indicate either a highly efficient transference of organic matter to higher trophic levels or a high nutrient regeneration rate. The amount of organic matter that could be transferred to higher trophic levels should depend upon the nanoflellate respiration and excretion rates (10). If the nanoflagellate gross growth efficiency is high and respiration and excretion rates are low, the protozoa will have an important role in transference of organic matter to higher trophic levels. On the other hand, if the nanoflagellate gross growth efficiency is high but respiration and excretion rates are also higher, nanoflagellates will be more important in the nutrient regeneration processes.

The results obtained in this work suggest that heterotrophic nanoflagellates and bacteria occupy an important role in planktonic food web of Ubatuba coastal ecosystem. Therefore, studies on nanoflagellate metabolism and nutrient regeneration by these organisms are needed to provide a better understanding of the role of nanoflagellates in this ecosystem either as a source of biomass for higher trophic levels or as organic matter mineralizers.

RESUMO

Um estudo experimental da bacterivoria por nanoflagelados

O nanoflagelado heterotrófico de dimensões entre 4,8 e 7,0 µm (*Pseudobodo tremulans*) e uma bactéria heterotrófica, isolados das águas costeiras de Ubatuba, SP, Brasil, foram utilizados em experimentos com o objetivo de analisar quantitativamente as relações entre bactérias e nanoflagelados. O significado dos resultados obtidos em relação ao papel dos nanoflagelados heterotróficos no ecossistema costeiro de Ubatuba é discutido.

Palavras-chave: nanoflagelados, bactérias heterotróficas, ecossistema costeiro, bacterivoria

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SUSCEPTIBILITY OF CELL LINES TO AVIAN VIRUSES

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ABSTRACT

The susceptibility of the five cell lines – IB-RS-2, RK-13, Vero, BHK-21, CER - to reovirus S1133 and infectious bursal disease virus (IBDV vaccine GBV-8 strain) was studied to better define satisfactory and sensitive cell culture systems. Cultures were compared for presence of CPE, virus titers and detection of viral RNA. CPE and viral RNA were detected in CER and BHK-21 cells after reovirus inoculation and in RK-13 cell line after IBDV inoculation and with high virus titers. Virus replication by production of low virus titers occurred in IB-RS-2 and Vero cells with reovirus and in BHK-21 cell line with IBDV.

Key words: reovirus, infectious bursal disease virus, cell lines, susceptibility

INTRODUCTION

Avian reoviruses and infectious bursal disease virus (IBDV) are usually isolated and grown in embryonated eggs and/or in primary avian cell cultures (5, 13, 17, 18). However the use of a continuous cell line has several advantages over the use of primary cell cultures (1, 2, 9). Various authors have evaluated numerous cell lines for the isolation of these viruses. Of many mammalian established cell lines tested, reovirus has been grown in Vero, BHK-21, GBK, PK, RK and CRFK (1, 17, 18). Cells susceptible to the IBDV include mammalian cell lines such as RK-13, Vero, MA-104 and BGM-70 (9, 13) and the avian cell line-QT35 (2).

This report describes a comparative study of the susceptibility of one avian and four mammalian cell lines to avian reovirus S1133 and IBDV vaccine GBV-8 strain (Biovet Laboratory) through of presence of cytopathic effect (CPE), virus titration and detection of viral RNA by polyacrylamide gel electrophoresis (PAGE) to better define satisfactory and sensitive cell culture systems for their isolation.

MATERIALS AND METHODS

Cell cultures. Chicken embryo fibroblast (CEF) cultures were prepared from 9-to-11 day old embryos of specific-pathogen-free (SPF) chicken eggs (Biovet Laboratory) by standard procedures. Baby hamster kidney (BHK-21), african green monkey kidney (Vero), rabbit kidney (RK-13) and porcine kidney (IBRS-2 clone D-10) mammalian cell lines obtained from our own laboratory and a chicken fibroblast (CER) avian cell line obtained from the Microbiology Department of the State University of Campinas were

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used in the study. Cells were grown in Eagle's minimum essential medium (MEM) containing 8% fetal calf serum (FCS).

Viruses. IBDV vaccine GBV-8 strain (Biovet Laboratory) and avian reovirus S1133.

Virus propagation. 25cm^2 cultures flasks were inoculated with 0.1 ml of each virus and observed for 3-4 days for production of cytopathic effect (CPE) and for three blind passages. The monolayers were frozen and thawed on time and the supernatants fluids were collected for virus titer assay and electrophoresis.

Assay for virus yield. Virus yields from CEF and cell lines infection with both viruses was determined in the CEF culture. The serial 10-fold dilutions of each cell cultures-virus supernatants were prepared in growth medium and 50 µl of each dilution were transferred to each of the four wells of a 96-well microtiter plate that contained the same volume of fresh CEF suspensions (3.0 x 10^5 cells/ml). Plates were incubated at 37°C in 5% CO_2 atmosphere for 7 days and the virus titer was determined by the Reed and Muench method (16).

Polyacrylamide gel electrophoresis (PAGE) of viral RNA. The viral RNAs from infected cultures were isolated by phenol: chloroform extraction followed by ethanol precipitation (14). RNA was analyzed on 3.5% stacking gel and 7.5% separating gel using the discontinuous SDS-gel system of Laemmli (11). Electrophoresis was carried out for 18 h at 4°C at a constant current of 10 mA. The RNA bands were visualized by the silver staining method described by Herring *et al.* (6).

RESULTS

Primary culture-CEF was susceptible to both viruses. The RK-13 cell line was the only one that presented CPE after IBDV inoculation, while the CER and BHK-21 cells were sensible to the reovirus (Table 1).

CPE of reovirus infection was characterized by formation of syncytia followed by degeneration leaving holes in the monolayer and giant cells floating in the medium. The IBDV infection produced a CPE characterized by marked cell rounding and detachment from the substrate.

Results of virus titers obtained in CEF culture from infected supernatants of different cell lines with both virus are shown in Table 2. The RK-13 cell line and the CEF culture presented high virus titers (6.9 and 6.7 $\log_{10}$ $TCDI_{50}$ respectively) to IBDV. The reovirus caused similar virus titers in the BHK-21 and CER cells (6.4 and 6.15 $\log_{10}$ $TCDI_{50}$) however these were lower than the virus titers obtained from CEF culture (7.5 $\log_{10}$ $TCDI_{50}$). Low virus titers were detected only in IBRS-2 and Vero cells for reovirus infection and BHK-21 cells to IBDV infection without visible CPE manifestation (Table 1).

The detection of viral RNA can be seen in Table 3. The viral RNA bands from infected CEF cultures were always visible, while in infected cell lines it was only possible to observe them in cells which presented CPE. The electrophoretic profile of ds-

Table 1. Susceptibility of cell cultures to reovirus and IBDV.

Cell Culture	IBDV (Strain GBV–8)	Reovirus S1133
CEF	+	+
IB-RS-2	-	-
RK_{13}	+	-
Vero	-	-
BHK – 21	-	+
CER	-	+

+ ECP positive
- ECP negative

Table 2. Infectivity titers of cell cultures-adapted Reovirus and IBDV in CEF cells.

	Virus titre[a]	
Propagation System	IBDV	Reovirus
CEF	6.70	7.50
IB-RS-2	0	2.50
RK_{13}	6.90	0
Vero	0	1.12
BHK – 21	2.00	6.40
CER	0	6.15

a: values expressed as $\log_{10}$ $TCDI_{50}$/ml

Table 3. Detection of viral RNA from cultures infected with IBDV and reovirus by PAGE

	IBDV (GBV-8)	Reovirus S1133
CEF	+	+
IB-RS-2	-	-
RK-13	+	-
Vero	-	-
BHK-21	-	+
CER	-	+

RNA of both cell culture-passaged viruses strains was indistinguishable from the ds-RNA of original strain.

DISCUSSION

This study was carried out with the aim of better defining satisfactory and sensitive cell culture systems for isolation the reovirus and IBDV. In order to find the most susceptible cells, the results obtained were evaluated and correlated: observation of CPE, high virus titers and presence of viral RNA.

IBDV replicated and caused CPE in RK-13 cells as reported by Petek *et al.* (15) and presented virus titers similar to that in CEF cells. It did not cause CPE in BHK-21 cells, however there was some virus replication determined by the production of low virus titer. This is opposite of reported by Petek *et al.* (15). The Vero, CER and IBRS-2 cells presented neither CPE nor virus titer. Vero cells have been used to propagate this virus including for virus-neutralization test (4) as described by others authors (8, 9, 12) however initial passages can not produce visible CPE (7). On the other hand, viral RNAs were not detected in any of these cells, except RK-13. Since RK-13 cell line presented CPE, high virus titer besides the presence of viral RNA, it is the best to IBDV propagation.

Avian reovirus propagated in IBRS-2, Vero, CER and BHK-21 cell lines, where it caused a visible CPE, however it produced high virus titers, similar to that in CEF cultures, only in the two last cell lines. In other cells the only alteration observed was production of low virus titers. The RK-13 cell line was not susceptible (15). The presence of viral RNA was detectable only in those cells with CPE (CER and BHK-21 cells), which were therefore selected as the most sensitive to reovirus replication.

Passage of both viruses strains in cell lines did not result in detectable change in the electrophoretic profile of the ds RNA genome segments in PAGE.

The difference of ours results and those of others authors may be due, among others reasons, to cell-culture passage levels of virus strains used or variation in sensitivity of different strains of cell lines (3, 7,10, 19).

Further studies are needed to better determine whether CPE will occur and virus titers will increase after additional passages in all cases with negative CPEs and low virus titer. Moreover, the amount of the virus in the cells in these cases was also not high enough to detect the viral RNA by PAGE, which would also succeed after more passage and viral adaptation. However, it was a priority in this study, to obtain a cell line producing CPE as soon as possible, that would provide the greatest chance of recovery of viruses.

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RESUMO

Suscetibilidade de linhagens celulares a vírus aviários

Estudou-se a suscetibilidade de cinco linhagens celulares (IB-RS-2, RK-13, Vero, BHK-21, CER) ao reovírus S1133 e ao virus vacinal (GBV-8) da doença infecciosa bursal de galinha (IBDV). As culturas foram comparadas quanto a presença de efeito citopático (ECP), título viral e detecção de RNA viral. Nas linhagens CER e BHK-21 detectou-se ECP e RNA viral após inoculação com reovírus e na linhagem RK-13 após inoculação com IBDV, com produção de altos títulos virais. Replicação viral com produção de baixos títulos ocorreu nas linhagens IB-RS-2 e Vero inoculadas com o reovírus e na linhagem BHK-21 com IBDV.

Palavras-chave: reovírus, vírus da doença infecciosa bursal de galinha, linhagens celulares, suscetibilidade.

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PATHOGENICITY CHARACTERISTICS OF FILAMENTOUS FUNGI STRAINS ISOLATED FROM PROCESSED OAT

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SHORT COMMUNICATION

ABSTRACT

Nineteen strains of filamentous fungi isolated from processed oat were tested for pathogenicity factors, based on three parameters: growth at 37°C, production of phospholipase and urease. *Aspergillus niveus*, *Oidiodendron gryseum* and *Sporothrix cyanescens* were positive for the three parameters. The other species were positive only for one or two of them.

Key words: Filamentous fungi, processed oat, pathogenicity.

Fungi, like heterotrophic organisms, inhabit the most varied substrates, acting as saprophytes, parasites and symbionts. Saprophytes, provided the appropriate conditions, may become pathogenic, and are called opportunist fungi. Immunodepressed patients are susceptible to infections caused by such fungi, which may be located in the human body or come from the air or foods (6).

The purpose of this work was to characterize strains of filamentous fungi, isolated from processed oat (8) and preserved under mineral oil. These strains belong to the Collection of Fungi Cultures - Mycotheca-URM, Department of Mycology, Center of Biological Sciences (CCB), Federal University of Pernambuco (UFPE). This Collection is registered at the Comnonwealth Mycological Institute (CMI) under the abbreviature URM (University of Recife Mycology).

Seventeen strains of Hyphomycetes and two of Zygomycetes (Table 1) were used in this study. Strains were transfered to medium containing 40g/L glucose, 3g/L meat extract, 5g/L sodium chloride, 10g/L meat peptone and kept at room temperature (28°C±1°C) for five days. The strains were then transferred to test tubes containing specific media: Czapek Agar (7) for *Aspergillus, Paecilomyces* and *Penicillium* and Potato Dextrose Agar (7) for *Acremonium, Cladosporium, Nigrospora, Oidiodendron, Rhinocladiella, Rhizopus, Sporothrix, Syncephalastrum* and *Tritirachium*. The tubes were left at room temperature (28°C ± 1°C) for 48h.

After growth, a taxonomic review was carried out based on macroscopic (colony aspect, diameter and color pattern) and microscopic (microstructures) caracteristics (2, 3, 5, 10, 12).

Three pathogenic parameters were tested: growth

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at 37°C and production of phospholipase and urease. For the growth at 37°C, the strains were incubated on specific media for seven days and germination was monitored on a daily basis. For phospholipase activity, aliquots of five-day-growth cultures were transferred to the center of Petri dishes containing Medium I or Medium II, according to Price et al. (11). The basic medium for both Medium I and II was Sabouraud agar added of NaCl and $CaCl_2$. Medium I is basic medium plus 2% egg lecithin (LO) and Medium II is basic medium plus 2% soy lecithin (LS). After seven days at room temperature, the diameters of transparent halos were measured. For urease activity, strains were streaked on Christensen Agar medium (urea agar) (7) in test tubes and left at room temperature. Urease reaction was observed up to three days. A red-fucsin coloring pattern of the medium indicated a positive result.

Table 2 shows that seven out of the nineteen strains, incubated at 37°C, resulted excellent growth. Phospholipase activity was positive mainly when Sabouraud Agar medium containing soy lecithin was used. The positive species presented a variable halo diameter. On the medium with egg lecithin, only two species, *Oidiodendron gryseum* and *Paecilomyces lilacinus*, presented halos, which were small, indicating a poorly positive result. Seventeen species were positive for urease activity. Among the positive species, *A. fusidioides*, *A. griseoviride*, *O. gryseum*, *R. microsporus* and *S. cyanescens* were not referred until now to be opportunist fungi that cause mycosis(1).

In this work, the results of tests for pathogenicity indicated that *R. microsporus* was positive only for the growth at 37°C; *A. griseoviride* was positive for urease and phospholipase activity; but *A. niveus*, *O. gryseum* and *S. cyanescens* were positive for the three pathogenicity tests (Table 2). The other species, which were positive for one or two of the parameters tested, are regarded as opportunist agents by De Hoog and Guarro (1). However, *A. niveus, C. oxysporum* and *P. lilacinus* are referred to not only as opportunists but also as pathogenic agents as well, causing human otitis (13), keratitis (4) and ocular infections (9), respectively. *A. niveus* turned out to be positive for the three parameters tested and *P. lilacinus* and *C. oxysporum* were positive for urease and phospholipase production, but negative for growth at 37°C. The latter feature may not be indicative of pathogenicity.

Fungi in processed foods may cause mycosis, for most fungi isolated from processed oat turned out to

Table 1: Genus/species of filamentous fungi isolated from processed oat (Micoteca-URM, UFPE)

Genus/Species	Number of Register at Micoteca-URM
Acremonium fusidioides (Nicot) W. Gams	3563
Acremonium griseoviride (Onions & Barron) W. Gams	3530
Aspergillus janus Rapper & Thom	3555
Aspergillus niveus Blochwitz	3385
Aspergillus sydowi Bain. & Sarf.	3547
Aspergillus terreus Thom	3420
Cladosporium oxysporum Berk. & Curt.	3389
Cladosporium sphaerospermum Pens.	3546
Nigrospora sphaerica (Sacc.) Mason	3529
Oidiodendron gryseum Robac	3564
Paecilomyces lilacinus (Thom) Samson	3566
Penicillium citrinum Thom	3424
Penicillium decumbens Thom	3380
Penicillium expansum Link	3396
Rhinocladiella atrovirens Nannf.	3545
Rhizopus microsporus V, Thiegem	3388
Sporothrix cyanescens (de Hoog) de Vries	3428
Syncephalastrum racemosum Cohn ex. Schrot.	3384
Tritirachium oryzae Vicens (de Hoog)	3561

Table 2: Pathogenicity tests accomplished by strains of filamentous fungi from Mycotheca-URM, isolated from processed oat.

Genus/Species	Growth at 37°C	Phospholipase production*		Urease production*
		LO**	LS***	
Acremonium fusidioides	-	-	-	++
Acremonium griseoviride	-	-	++	++
Aspergillus janus	-	-	-	++
Aspergillus niveus	+	-	++	++
Aspergillus sydowi	-	-	+	++
Aspergillus terreus	+	-	-	++
Cladosporium oxysporum	-	-	+	++
Cladosporium sphaerospermum	-	-	++	++
Nigrospora sphaerica	-	-	-	++
Oidiodendron gryseum	+	+	+	++
Paecilomyces lilacinus	-	+	+	++
Penicillium citrinum	+	-	-	++
Penicillium decumbens	+	-	-	++
Penicillium expansum	-	-	-	++
Rhinocladiella atrovirens	-	-	+	-
Rhizopus microsporus	+	-	-	-
Sporothrix cyanescens	+	-	+	++
Syncephalastrum racemosum	-	-	-	++
Tritirachium oryzae	-	-	++	++

*: + = halo diameter between 0.2 and 0.6cm
++ = halo diameter between 0.7 and 1.8cm
- = absence of halo
** LO = egg lecithin
*** LS = soy lecithin

be positive for at least one of the pathogenicity tests. Nevertheless, the onset of the disease will depend on the immune system of the individual, since most fungi are regarded as opportunists.

RESUMO

Características de patogenicidade em amostras de fungos filamentosos isolados de aveia processada

Dezenove amostras de fungos filamentosos isoladas de aveia processada foram testadas quanto a fatores de patogenicidade, utilizando-se três parâmetros: crescimento a 37°C, atividades fosfolipásica e ureásica. *Sporothrix cianescens, Aspergillus niveus* e *Oidiodendron gryseum* apresentaram características de patogenicidade nos três testes realizados. As demais espécies apresentaram características de patogenicidade somente em um ou dois destes parâmetros.

Palavras-chave: Fungos filamentosos, aveia processada, patogenicidade

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