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Morphological and Cultural Characterization of some Strains of Unicellular Algae of the Genus *Prototheca* Sampled from Mastitic Cow Milk

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Abstract

Prototheca is a unicellular, achlorophillous, ubiquitary and saprophytic alga spread in the surrounding environment mostly in vastly damp locations in the presence of the organic matters. This is deemed to be pathogenic to a low degree, taking immediate advantage of the environment and triggering diseases at a time when the immunological capability of defense of the organisms is low or, when favorable factors come about. *Prototheca* is capable to induce disease in man as well as in several other animal species. Twenty strains of *Prototheca* sp, were sampled from cows with mastitis. The strains are characterized morphologically and developmentally on various culture media: glucose media (broth and agar), blood agar, potato medium, glucose medium of various pH values, medium containing antibiotics, Mac Conkey agar, Smith Baskerville medium (without antibiotics), and engine-oil medium. Growth took place within conditions of aerobic at 37° C and the culture became visible within 36-48 hours. On liquid media there has been noticed that the medium keeps settled and the tube bottom shelters granular drags, that lend to slight homogenization. On solid media the colonies become visible within 36-48 hours. By continuing to maintain them at room temperature, they increase in size. First, they are small, irregular in shape resembling ice crystals reflecting shiny irisations. With lapse of time, they increase in size reaching 3-5 mm, displaying pale-white colour. They also increase in height and the surface takes a mulberry or cauliflower shape. Morphological assessment was carried out on wet smear in Lügol solution or in thinned staining solutions (methylene blue, malachite green, Congo red or fuchsine), allowing for good differentiation in the inner structures. The prevailig shape of *Prototheca* is that of an egg, seldom round or, that of a kidney with variation in size between 9.5/10.5µm up to 27.5/30.4µm. With some cells 6 to 8 endospores were noticed. Based on morphological and cultural characters there was set down that the strains under surveillance belonged to the species of *Prototheca zopfii*.

Keywords: algae, *Prototheca*, morphology, cultivation, mastitic milk, cow

Introduction

The taxonomic position of *Prototheca* has for long been disputed. Currently, they display the following integration: Domain *Eukaryota*, Kingdom *Viridiplante*, Phylum *Chlorophyta*, Tribe *Chlorophyceae*, Order *Chlorellales*, Family *Chlorellaceae*, Genus *Prototheca*. *Prototheca* is considered as one achlorophyllous alga mutant in the genus *Chlorella*. It has lost its ability to synthesize chlorophyll by using a heterotrophic way (Lagneau, 1996). Within genus *Prototheca* there are recognized as valid species the following: P.*zopfii*, P.*wickerhamii*, P. *stagnora*, P. *ulmea*, P. *moriformis* and, more recently P. *blaschkeae* (Lass-Flürl and Astrd Mayr, 2007; Marques *et al*., 2008). The species in the genus *Prototheca* are to be found free in nature and were sampled with large spans of surrounding environments mainly in spots of water contents attended by organic matters (Huerre *et al*., 1993; Glusac, 2000). Numerous authors reported samples taken from slime flux of trees, damp soil around the foot of the tree; also, plants, flooded terrains, acid waterways, waste water, sewage, clogged ponds, sludge, mud (Casals and Solis, 1981; Anderson and Walker, 1988; Taniyama *et al*., 1994; Lagneau, 1996; Costa *et al*., 1997; Gonzales, 1996). Prototheca was also isolated in samples taken from green forage, shelters, waste dumps; also, in milk, milking devices, bulk-milk tanks; on shelter floors, store rooms, gutters etc. (Pore *et al*., 1987; Anderson and Walker, 1988). Several authors managed to secure sampling of *Prototheca* from faeces of various species of animals, such as: bovine (Enders and Weber, 1993; Costa *et al*., 1997): equine (Enders and Weber, 1993); swine (Pore and Shahan, 1988); wild boar (Weber and Enders, 1993). There are opinions that rats can go for contamination vectors for feeds (Pore and Shahan, 1988). There have been reports of samples taken from banana and potato peels, too (Pore, 1985).

Based on data provided by various authors one can assert that *Prototheca* is ubiquitously spread; however, its presence with various sources of environment correlates with humidity and organic matters. This genus is made up of heterotrophic microorganisms in need of outer organic carbon, nitrogen, and thyamine sources, secured from detritus, or waste, dropped by other organisms.

Asserting the pathogenic ability of *Prototheca* has been a study theme for many research workers. Cases of disease have been described with animals and man in numerous countries, on all continents. With man, infections are more often triggered by *Prototheca wickerhamii* whereas with animals, *Prototheca zopfii*; however, there is no possibility of drawing one secure line regarding this matter.

With man, disease is sporadically encountered and obvious through skin lesions, olecranon bursitis; seldom, systemic infection of fatal evolution (Kaminski *et al*., 1992; Huerre *et al*., 1993; Lass-Flőrl Cornelia and Astrid Mayr, 2007). Disease cases are encountered mostly with subjects having varying forms of immunosupression (Wolfe *et al*.,1976; Heney C., *et al*., 1991); systemic lupus erytematosus (Tsuji *et al*.,1993); AIDS syndrome (Kaminski *et al*.,1992; Woolrich *et al*.,1994; Wirth *et al*.,1999). Such an aspect has also been demonstrated via induced infections in experimental animals (Jensen and Aalbaek 1994; Răpuntean, 2002). The ability of *Prototheca* to be pathogenic is also favoured by the evolution of other diseases, such as *myastenia* gravis (Mohabeer *et al*.,1997); tuberculosis (Otto *et al*., 1981) in subjects submitted to peritoneal dialysis (Gibb *et al*., 1991), organ transplants, chemo- and radiotherapy or prolonged treatment with corticosteroids (Glussac, 1999). Most of the patients suffering from protothecosis are well over 30 of age; however, there were described cases with children too and, even in the newborn. There was described a rare case in an individual suffering from immunodeficiency as aftermath of a bite from a tick (Wirth *et al*.,1999).

Cases of animals becoming sick have been reported in domestic- and wild animals, in homoeothermic- and poikilothermic animals where granulomatous lesions were displayed, evolving as localized-, systemic- or generalized infections.

In the *cow* the most often encountered infection is mammitis displaying clinic- or subclinic evolution and many reports talk about the presence of the disease. On some diary farms the disease has endemic evolution, triggering substantial economic losses (Pore *et al*., 1987; Jensen *et al*., 1998; Lagneau, 1996; Costa *et al*., 1997; Moubamba, 1997; Taniyama *et al*.,1994; Janosi *et al*., 2001; Malinowski *et al*., 2002). A case of generalized protothecosis following repeated and prolonged treatments with antibiotics and corticosteroids has been reported (Taniyama *et al*., 1994).

In the *dog*, the disease may progress in the company of digestive disturbances shown as hemorrhagic enterocolitis (Kruiningen, 1970), confinement in the eye with abrupt blindness and deafness (Buyukmihci el al.,1975; Font and Hook, 1984; Gaunt *et al*.,1984; Blogg and Sykes, 1995; Cook *et al*.,1984; Hollingsworth, 2000); also, skin lesions, oedema and ulcer on the scrotum (Ginel P., *et al*., 1997), as well as cases affecting the nervous system (Tyler *et al*.,1980).

In the *cat*, the disease is produced by P. *wickerhamii* and is manifest in display of skin nodular lesions mostly on the head, limbs or in the nasal cavity (Dillberger *et al*., 1988; Finnie and Coloe, 1981).

In *wild animals* systemic or located diseases have been reported in several species: deer (Lagneau, 1996); fruit bat (Mettler, 1975); hamster (*Phadopus sungorus*) (Nichols, 1999); rabbit, mice, rats, beaver, weasel (Spalton, 1985; Gonzales, 1996), as well as in lower animals: snake (*Elaphe gutata gutata*) (Crispens and Marion, 1975); tree frog (*Litoria adelensis*) (Nichols, 1999); salmon (*Salmon parr*) (Gentles and Bond, 1977), and carp (*Ciprinus carpio*) (Loupal *et al*.,1992).

In animals with generalized forms, granulomatous lesions in kidneys, heart, liver, osseous muscles, thyroid, colon, bronchial lymphnodes, spinal cord, brains as well as in other tissues are building up. Identification of *Prototheca* in lesions is feasible through various laboratory techniques: cytologic and histopathologic examination; indirect immunofluorescence; electron microscopy; isolation on culture media; biochemical examinations: ELISA techniques and of molecular biology, infections induced in laboratory animals.

Prototheca is not phytopathogenic but it can be sampled on green fodder, tree barks, mostly elm tree (*Ulmus* sp.) and linden (*Tillia* sp.,). *Prototheca stagnora* is one common inhabitant of older banana crops (*Musa sapientum*) and roots of plantain tree (*Musa paradisiaca*), whereas P. *wickerhamii* colonize the fresh (young) roots of *Musa* sp., and flower bract water (*Heliconia* sp.) (Pore, 1985).

Materials and methods

There were examined twenty strains of *Prototheca zopfii* sampled from cows with subclinic mastitis; the strains were identified based of morphologic, cultural and biochemical characters (auxanogram and zymogram).

From the milk sample as such, or from sediment after centrifugation, dispersions were done on glucose agar plates medium incubated at 37°C and examined within 24, 48 and 72 hours. Examination of the plates was performed under magnifying glass as well as from colonies that could be classed as *Prototheca*; also, wet smears were made to observe the morphological characters; there were also made subcultures on separate plates on glucose media in order to obtain the strain in pure culture.

Culture was carried out on following media: blood agar, potato agar, MacConkey agar, glucose media with different pH-values (3 to 9), media containing antibiotics (lincospectin, spectinomycin), Smith-Baskerville medium (without antimicrobial supplement) and glucose agar medium with thin pellicle/film of engine oil.

Incubation was carried out within conditions of aerobic at 37°C during the first 48 hours; later, the culture containers were kept for 5-7 days at room/lab temperature

for inspection and for describing cultural aspects in due course.

For morphological characterization wet smears were done in one drop of Lügol solution; the examination was carried out under x20, x40 objective and even with immersion under slide. The inner structure of the cells, that of various phases of multiplication as well as the presence of endospores were better seen on wet smears using various staining methods (methylene blue, Congo red, fuchsine and malachite green). Assigning of sizes was performed by means of micrometric measurements.

Results obtained

Cultural characters on liquid medium.

One can catch sight of the culture within 24-36 hours in glucose broth and it becomes quite obvious the presence of a granular sediment and, on the surface a thin, fragile film displaying the tendency of climbing the tube wall. The remaining liquid in the tube withholds its clarity. On stirring, the drag disperses easily and uniformly but tends to rapidly deposit due to the weight of the cells. There were not noticed any alterations in viscosity, colour or peculiar odour.

Cultural aspects on solid media.

Within proper dispersion conditions *Prototheca* develop as individualized colonies. In order to catch characteristic aspects the examination of the colonies has to be done magnifying glass for several days.

Glucose agar and Sabouraud dextrose agar.

One can hardly discern the slightly gray colonies after 24-36 hours, as they are so puny. After 48, they are well shaped and show silvery- and shiny irisations resembling ice crystals. Left at room temperature colonies will rapidly increase in size reaching 3-5 mm in diameter or even larger, of pale white-grayish colour. Colonies grow in height and in the shape of mulberry displaying vague crenels on the edges (Fig. 1, 2). On thick dispersion, colonies are confluent resembling a white mass and the surface of cauliflower aspect (Fig. 3).

Characters noticeable under the magnifying glass, i.e., shiny ice crystals, growth in height, granular surface (in the shape of mulberry or cauliflower) are all characteristic elements for distinguishing them from other bacterial colonies (*Staphylococcus, Micrococcus, Neissera*) and yeasts (*Candida*). Following colonies in dynamics and catching the aspects just mentioned make up for defining elements meant to surely distinguish *Prototheca* from all other microorganisms. Even in case of heavy contamination of the sample with other germs, including yeasts, *Prototheca* colonies are easily discernable on solid culture media.

Fig.1. – Prototheca colonies on glucose agar (x25)

Fig.2. –Prototheca colonies on Sabouraud agar (x16)

Fig. 3. –Prototheca colonies on glucose agar (cauliflower shaped), (x10)

Blood agar.

Prototheca grows easily on blood agar very similarly with the description of Sabouraud medium having shiny aspect resembling that ice crystals aspect is more marked and does not produce haemolysis. After 48-72 hours, colonies are well developed, of 1.5-3 mm in diameter, pale-white in colour, easily discernable from other germs' colonies (Fig. 4).

Potato medium.

Prototheca strive on such medium. The microscopic aspect of the colonies resembles that described with glucose nutritive agar. Examined using the magnifying glass, the colonies are very well contoured, having their surface in the shape of bramble and grow in height, often displaying a "terraced" growth. (Fig. 5).

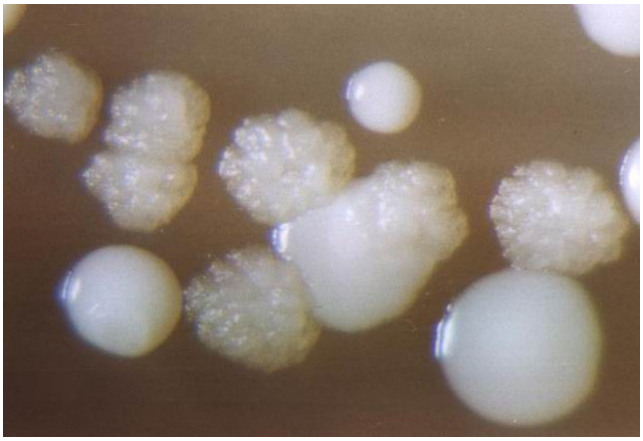

Fig. 4. – Prototheca (a) and Candida (b) colonies on blood agar (x16)

Medium containing antibiotics.

The antibiograma reveals that *Prototheca* is resistant to lincomycin and spectinomycin. Addition of antibiotics was done in order to inhibit bacterial development. In order to check on this supposition a mixture of *Prototheca* + E. coli + *Staphylococcus* was made: of this, there were carried out culturing on glucose agar including lincospectin (50 μg lincomycin and 100 μg spectinomycin). The plates were incubated for 48 hours then maintained at room

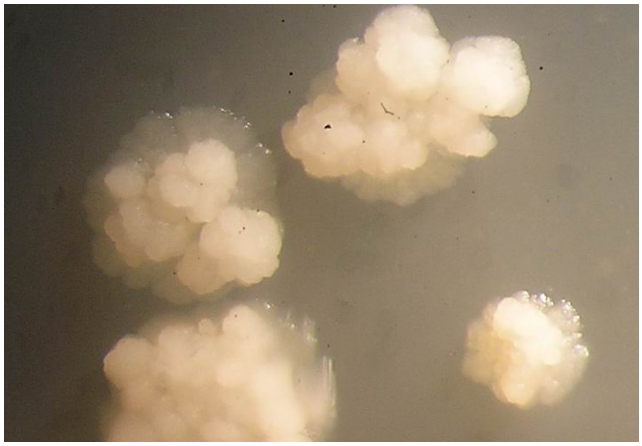

Fig. 5. Prototheca colonies on potato medium (magnifying glass) (x25)

temperature for 5 more days. The examination revealed that only *Prototheca* colonies developed while E. coli or *Staphyloccus* colonies were not present. Based on these observations we strongly recommend the utilization of media containing lincospectin (broad-spectrum antibiotic) for *Prototheca* sampling, mostly when samples examined are heavily contaminated by other germs.

Glucose agar of varying pH-values.

Each strain was cultured on a Petri dish with different pH value. The dishes were incubated at 37°C for the first 48 hours, and then were kept at lab temperature for 5-7 days. *Prototheca* develops within a very large register of pH values, starting from an intensely acid pH (pH-2) up to an intensely alkaline one (pH-9). In order to check the selective effect of acid pH, a strain of *Prototheca* was mixed in separate tubes with the following bacterial strains: *Prototheca* + E. *coli*; *Prototheca* + *Proteus*; *Prototheca* + *Bacillus*; *Prototheca* + *Staphylococcus*; *Prototheca* + *Candida*. It was found out that pH-3 acid medium possessed selective effect on both Gram negative and Gram positive germs by ousting them thus allowing only for the growth of *Prototheca*.

Smith Baskerville medium.

It possesses the ability to isolate *Bordetella bronchiseptica*. The primary medium without the antimicrobial supplement was used. Prototheca grows well in such a medium and the colonies become visible at 36 to 48 hours. Initially, the colonies are gray in colour having red berry-like surface. Such a trait allows the quick identification of *Prototheca* colonies within the frames of presence on cultivation plate and of other bacterial colonies, yeast or moulds (Fig. 6).

Cultivation medium containing engine oil.

Cultivation plates with glucose agar after having solidified were covered with an engine-oil (raw and burnt) pellicle. The plates were incubated at 37°C for the first 24 hours and then kept at lab temperature for 3-5 days. The colonies developed at 48 hours, smaller initially but gradually increasing in size up to 1-2 mm. They were white in colour, glossy having granular surface. Kept at lab tem-

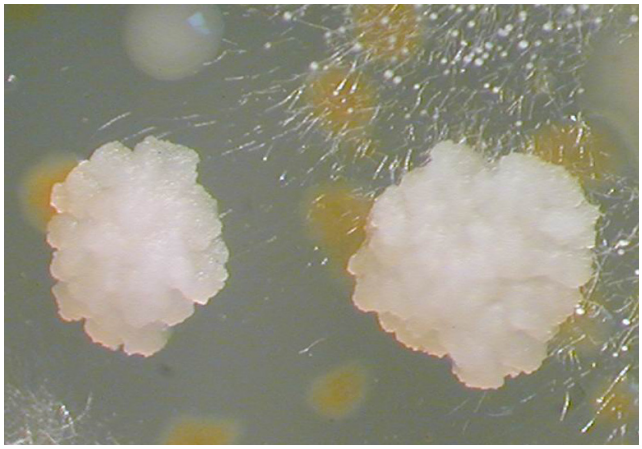

Fig. 6. – Prototheca colonies from a contaminated sample on Smith-Baskerville medium(x16)

perature the colonies increased in size displaying an irregular contour (Fig. 7). The microscopic examination of wet smears revealed that *Prototheca* withhold the characteristic morphology. Passage of colonies from the oily medium to fresh media with no oil led to obtaining typical *Prototheca* colonies.

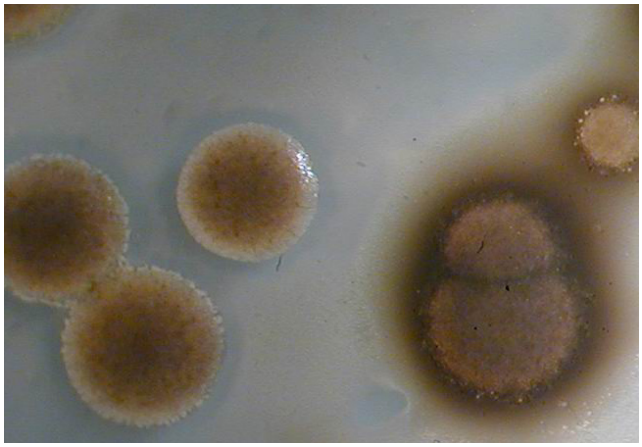

Fig. 7. – Prototheca colonies on glucose medium with engine-oil pelicle (x16).

Morphological characters.

In order to characterize morphologically the examined strains, wet smears in Lügol or diluted staining solutions (methylene blue, malachite green, Congo red or fuchsine) were realized. This examination method is highly feasible for *Prototheca* morphological examination using x20, x40 objectives and immersion. The outline of the cells is well observable; also, cell walls, inner structures (dense bodies, starch granules), sporangia in the making, revealing of endospores. Budding character, distinctive to yeasts was not observed. Regarding the sizes, established through micrometric measurements are concerned, there has been found out that *Prototheca* has variable sizes, and correlated with culture age and progressive cycle. The prevailing cell shape is that of an egg, of 9.5/10.5μm to 27.5/30.4μm in size (Fig. 8), seldom round, or even kidney-shaped. Mobility is not shown with *Prototheca*.

The inner structures were of pronounced variability. Some cells possess a uniform content of fine granular cytoplasm; others, still, display an irregular look, with inner structures well built and delimited, probably dense bodies, starch granules and reserve substances (Fig. 9).

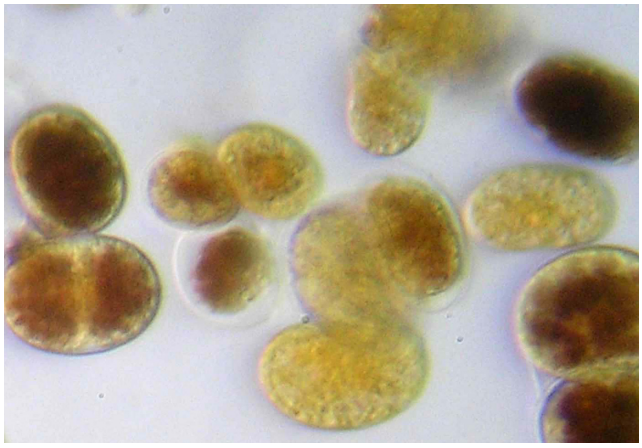

Fig. 9. Prototheca cells in several stages of development stained (Lügol stain, x1000)

Variability was also noticed in the thickness of the cell wall: some cells possessed thin walls made up of two distinct membranes; others, had a very thick irregular wall.

One rather important trait is called sporangia–mature cells in multiplication stage–that serves to easily identify *Prototheca* and differentiate it from yeasts. Initially, one can encounter the formation of a visible dividing septum, leading to the cell contents, in two symmetrical/equal structures; other times, these are asymmetrical/unequal (Fig. 10, 11).

There follows an irregular cleavage in the cytoplasm and a build-up of some thin septa outlining some formations, polygonal in aspect, less contoured initially. Such a process is called asexual multiple fission; as well as the set-up of dewberry-shaped structure (Fig. 12).

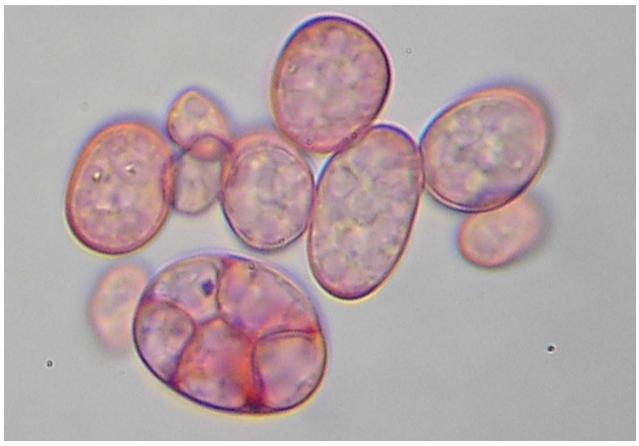

Fig. 8. – Prototheca cells in different phases of development, (Fuchsin stain, x1000)

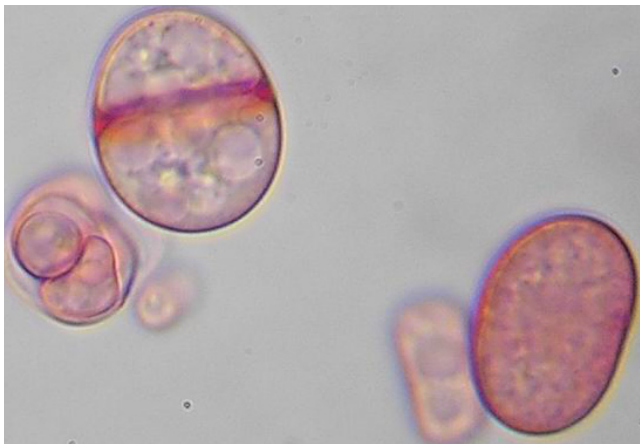

Fig. 10. Prototheca cell in the stage of dividing-septum (Fuchsin stain, x1000)

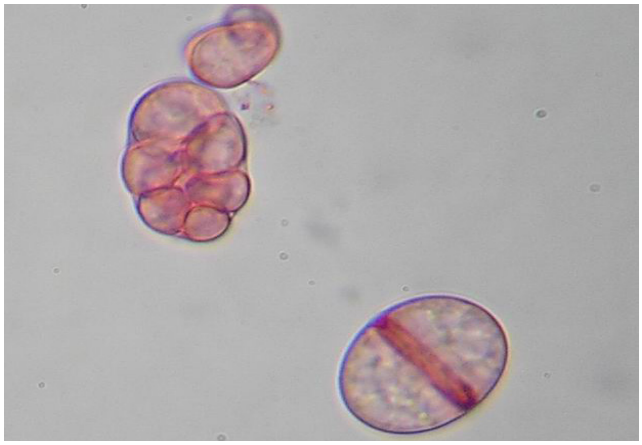

Fig. 11. – Prototheca cells during endospore forming (Fuxin stain, x1000)

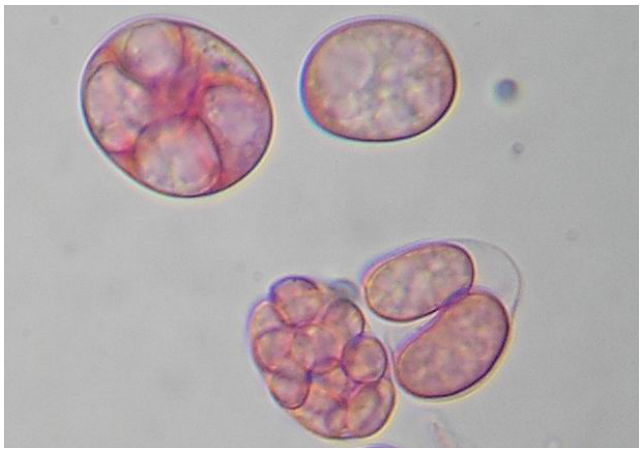

Fig. 12. – Prototheca cells with endospores in several stages of development (Fuxin stain, x1000)

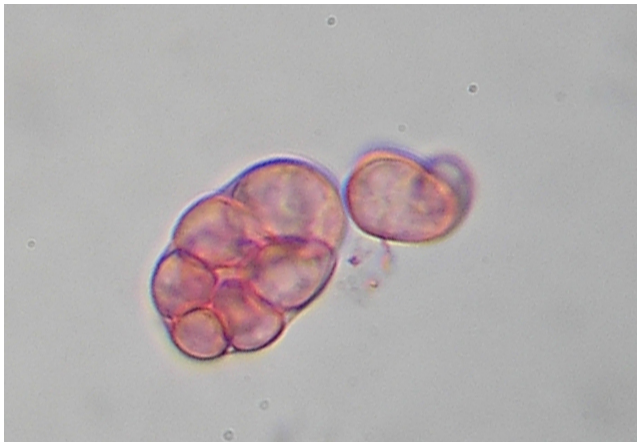

Fig. 13. – Prototheca cell with endospores,(Fuxin stain, x1000)

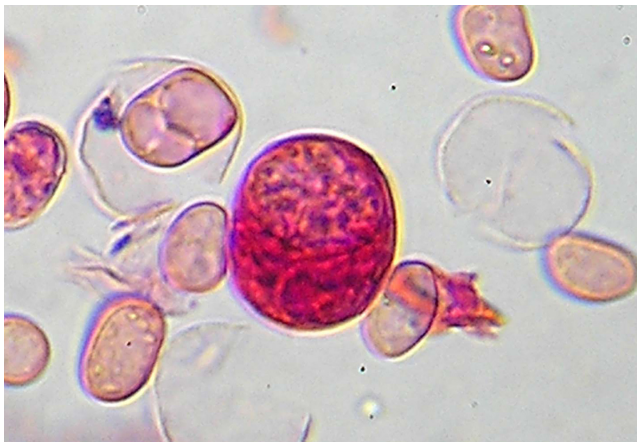

Fig. 14. Prototheca cells with granular content and empty cells (Fuxin stain, x1000)

Further on, the dividing walls among spores thicken and decide over a more clear delimitation of the endospores, these being better rendered and counTab. , too (Fig. 13). Once at maturity, sporangia undergo a process of lysis in one pole and elimination of the endospores take place. Then, one can notice the empty cells (Fig. 14).

Discussion of the results

Prototheca species are heterotrophic microorganisms needing outer sources of carbon, nitrogen and thyamine obtained by the species from organic detrition products or from wastes produced by other organisms. Species of *Prototheca* develop in aerobic conditions on various culture media. Literature data describe their growth on following types of media: Sabouraud, dextrose agar, corn-meal agar (Gonzales, 1996), blood agar, heart-brain infusion with sheep blood (Glusac, 1999), chocolate agar, eosin methylene-blue agar, Edward medium, MacConkey agar (Berkhoff *et al*., 1982), potato medium, malt medium, casitone medium, Chrome agar (Casal *et al*., 1997). They are also prosperous on special media containing various stimulating- or restricting substances, such as cycloheximide, chloramphenicol, 5- fluorocytosine, 2.6-dimethylnaphtalene (Pore, 1973). *Prototheca* species display tolerance to acidity (pH 2 to 2.5), and to sodium chloride 4-5% (Kessler, 1977). They can also grow on media containing hydrocarbonates (Walker *et al*., 1975; Walker and Pore, 1978; Cuc *et al*., 2008). By means of our research work we vouchsafe growth on some of the above mentioned media, as well as the tolerance to acidity, this acting as selective factor. This explains their resistance in acid-pH waters.

Regarding the incubation temperature the opinions differ. Some mention that the ideal temperature for growth would be 25-30° C, others 35-37°C (Tyler *et al*., 1980; Janosi *et al*., 2000). Several authors are of the opinion that growth at the temperature of 25°C and 35-37°C respectively represents a differentiating criterion among the species. The cultures, both on liquid and solid media (colonies development) become visible after 48 hours. Maintaining the test tubes/plates for several days, the culture aspects acquire some peculiarities and, on their basis, the colonies of *Prototheca* sp., can be identified. Tyler *et al*., 1980, recommend keeping the cultures for 14 days before being taken out for inspection. Preservation can be carried out at lab temperature.

Strains examined within the research in this work developed in aerobic conditions at 37°C; however, they continue to grow maintained at room temperature. For a better survey of the colonies characters', we recommend inspecting them under stereo magnifying glass for several days, in order to grasp the characteristic aspects of *Prototheca* (colony outline; the aspect of the edges; area; colour range; adherence to medium) (Răpuntean, 2002).

In accordance with Casals *et al*., 1997, in order to differentiate unicellular algae of genus *Prototheca* from *Candida* yeasts (both develop on media habitually utilized in mycology; both having almost identical colonies), they recommend the medium called "Chrome agar". On such a medium a chromatic differentiation between *Prototheca* and *Candida* is obtained. Differentiation is performed testing the susceptibility to Ribostamycin (60 mcg disc) an antibiotic to which *Candida* strains are resistant and inhibitory to *Prototheca* (Casals and Gutierrez, 1986).

The study of morphology in *Prototheca*, both in culture and in various pathologic products can be carried out by means of wet microscopic smears or following staining in differing techniques (Gonzales, 1996). In order to make inner structures better visible the procedure is to be performed in wet smears, in one drop of Lügol solution (iodinated solution), lactophenol, toluidine blue. Staining of *Prototheca* is also possible by following the Gram method; this method does not differentiate the inner formations/ structures (Lagneau, 1996; Rapuntean, 2002). *Prototheca* can also be revealed histological by Wright and Giemsa staining or, Gőmory (Taniyama *et al*., 1994; Glusac, 1999). By Phol staining (aqueous formaldehyde solution, glycerol and methilene blue) a proper staining of fungi and *Prototheca* and discrimination of young cells from the mature ones is revealed (Pal *et al*., 1990). However, performing the smears in thinned staining solutions (methylene blue, malachite green, Congo red or fuchsine) a proper discrimination of inner structures, of the cells in various stages of multiplication, (sporangia and endospores) is obtained (Răpuntean, 2002; Cuc *et al*., 2008).

Prototheca displays either a round- or an ovoid shape, rarely kidney-shaped and of varying magnitudes, according to species and stages of development. Most of the authors opine that dimensions are comprised between 3-30 μm. *Prototheca zopfii* is the largest of all species of the genus, frequently reaching proportions of 25-30μm. By means of computerized analysis the existence of a large span of width and shape in *Prototheca*, both at the level of lesion and of culture was found (Pierard *et al*., 1990). Under the influence of environmental factors protoplasts can be obtained; from this point of view there is a similarity with the Gram positive bacteria.

Outside *Prototheca* shows a thick wall (0.5 μm), hyaline-cellulose like, refractive, made up of two membranes with the outer one thicker. As a rule, there is a clear space between cytoplasm and wall cell. *Prototheca* is without cilia and uncapsulate, with the exception of P. *stagnora* which is capsulate. Cytoplasm is granular and in the inner side shows structures of various dimensions; some round in shape, some intensely colored (dense bodies); still, some reveal irregular and pale aspect (starch granules) (Tyler *et al*.,1980). However, cytoplasm has the tendency of basophilic staining in young cells and eosinophilic in the older ones.

Prototheca reproduces asexually, with a cycle which does not identify with that in *Chlorella* with endospore formation. The singular nucleus undergoes an irregular number of mitotic divisions immediately followed by fragmentations of the protoplasm. At the beginning, there starts a process of separation, revealing a separating septum, followed by an irregular cleavage in the cytoplasm and building of thin septa separating certain formations of polygonal aspect (multiple asexual fission) (Rapuntean, 2002; Marques *et al*., 2008). The separation walls between endospores frequently display a characteristic design, instantly recalling the well-known Mercedes logo (In "Wednesday Slide Conference 28 Preliminary Diagnoses" 1998-1999).

The content is gradually organizing itself, in that of a more obvious definition of some formations of ovoid expression and a variable number of endospores are constituted (Taniyama *et al*., 1994; Lagneau, 1996). Small spores thus created will synthetize their own wall cell called "theca" wherefrom the name of *Prototheca*. Greater part of the data reports the formation of 2 to 10 endospores. These are 6-9μm in diameter. In the company of proper nutrients spores will grow and subdivide forming 12 endospores within a large spore case that may break releasing spores even within 5 to 6 hours. These proliferating spores contain more endoplasmic reticules and mitochondria than the older spores which contain more lipids and quite often starch granules in the wall and a smaller number of membrane organelles.

Mature spores containing spores make up mulberry-like sporangia (Lagneau, 1996). Once having reached maturity and following pression exercised by the endospores, the breaking of cell wall at one end of the cell, the passive exit place for endospores is observed (Moubamba,. 1997). The wet smears also reveal empty cells left behind after the endospores were freed. Sometimes, the image of a central endospore can be seen, fenced in by endospores arranged in a shape, resembling a crown (Glusac, 1999).

With *Prototheca* the process of burgeoning is not seen; neither is the forming of pseudo mycelium as seen with yeasts (Lagneau, 1996; Rapuntean, 2002). Prototheca possesses a complex chemical composition. Muramic acid and glucosamine, aspect distinctive as to bacteria and fungi, are missing from the wall-cell structure. Within its composition cellulose is to be found (Lagneau, 1996).

The strains separated in our researches could be identified as belonging to the species of *Prototheca zopfii*, based on the cultural and morphological characters. In order to differentiate biotypes, biochemical exams can be carried

out. Padhye *et al*., 1979, report fast identification of Prototheca species by means of the API 20 C system. By utilizing the systems API 20 C and ID 32 C, the differentiation between yeast and algae is possible (Lagneau, 1996; Ramani *et al*., 1998).

The increased occurrence of disease in man and animal, in which etiologically differing species of *Prototheca* are involved, has led to amplified research works in many countries being implied aspects concerning ecology, cultivation, pathogenity, determination of susceptibility to antibiotics and antifungal, improved techniques of diagnosis and treatment. That the disease has become emergent in the cow affecting mainly the mammary gland (occurrence of mastitis) and discharge of *Prototheca* via milk, the risk of transmission to man, animal and surrounding environment is increased. Due to such reasons our research work has contributed to the clarification of some aspects regarding the biology of *Prototheca zopfii* strains sampled from mastitic cow milk.

Conclusions

1. Prototheca develops on liquid- and solid media. All of the strains have developed on glucose media (broth and agar); these may be taken as habitual media for the growth of these algae. Cultivation has been successful on other media too, such as: selective media containing antibiotics (Lincospectin); blood agar; potato medium; MacConkey agar; media of various pH values (2 to 9); Smith-Baskerville agar (not containing antibitotics) and agar with thin engine-oil film.

2 Most suiTab. cultivation conditions have been the following: incubation in aerobiosis at 37°C. The culture becomes visible within 36-48 hours of incubation. However, cultural aspects alter in time reason why we recommend surveillance for several days (at least 5-7).

3. A granular- like sediment is to be noticed in liquid media. The supernatant stays clear. The sedimentation homogenizes easily but sediments rapidly due to the weight of cells. Sometimes, there has been noticed a thin and flimsy pellicle formed on the surface and stretching over the walls of the tube.

4. On solid media, colonies form and become visible after 36-48 hours. First, the colonies are of small dimensions but gradually grow to reach 3-4 mm in diameter. They become white- or creamy in colour and the surface is irregular displaying a characteristic mulberry aspect. In order that all morphological characters of the colonies to be seen (dimensions, surface, the look of edges, color), examination should be done under stereo magnifying glass (x1.6; x4).

5. Prototheca morphology is readily seen within the frame of proper conditions in wet smears made ready in Lügol solution or, in some other staining solutions (methylene blue, malachite green, Congo red or fuchsine). The prevailing shape of Prototheca is the ovoid one, rarely round or kidney shape of variable extent, between 9.5/10.5 μm up to 27.5/30.4 μm.

6. The cultural and morphological characters of the studied strains, mostly the aspect of the colonies, revealing of cells having the aspect of sporangia with endospores, lack of burgeoning, have allowed for the distinction from other micro-organisms, mostly cocci (Micrococcus and Staphylococcus) and yeasts (Candida) and classifying the strains with Prototheca zopfii.

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