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A CYTOCHEMICAL STUDY OF ACID CARBOHYDRATES ON THE SURFACE OF *CANDIDA LIPOLYTICA* GROWN IN TWEEN 80-CONTAINING MEDIUM

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ABSTRACT

Carbohydrate-containing molecules were located on the surface of *Candida lipolytica* by using ruthenium red in a cytochemical study. The yeast was grown in media containing Tween 80. The surfactant, at 1.0% and 0.5%, was added to the culture medium in different intervals of time, correspondent to the beginning of exponential growth phase, mid of logarithimic phase and beginning of stationary growth phase. Control cultures were grown in a medium containing glucose. The growth of the yeast in media containing glucose and Tween 80 induced changes in the pattern of distribution and location of acid polyssacharides in the cell wall of the microorganism. In adition, the pattern also changed according to Tween 80 concentration. The influence of Tween 80 on cellular carbohydrate expression is discussed.

Key words: *Candida lipolytica*; carbohydrate cytochemistry; fungi ultrastructure

INTRODUCTION

Candida lipolytica has been object of various biochemical and physiological studies concerning its abilities to grow and utilize hydrocarbons, methanol, acetate and fatty acids as carbon sources. It produces extracellular proteases, lipases, isocitric and citric acids. Surfactants offer a potentially valuable model system for the study of extracellular metabolites production in lower eukaryotes and potential use in industry. In addition, genetic analysis is possible due to the discovery of its sexual cycle (12, 37, 38, 43, 44, 49, 60, 62).

Non-ionic surfactants of the Tween series have been used as culture additives to improve cellular growth of bacteria and filamentous fungi and the secretion of various microbial products, particularly enzymes. The maximal stimulation is observed with Tween 80, the only surfactant bearing an unsaturated fatty acid residue (oleate). It has been suggested that the effect of surfactants is on cell membrane permeability. On the other hand, surfactants of Tween serie could be used to supply fatty acid to the culture. However, the mechanism through which surfactants affect enzyme production and cellular growth is not clearly understood (3, 19, 39, 46, 52, 55).

Many of the properties of the cell surface, which include surface antigens, hormone binding, cell recognition, adhesiveness and adsortion can be ascribed to polysaccharides or may be influenced by them (13, 27, 34).

Acid polysaccharides contain negatively charged residues. Because of their negative charge they are highly hydrated molecules, which avidly bind cations and may form gels of varying pore size and charge density, acting in cell osmoregulation. Thus they work as sieves to regulate the traffic of ions in and out of cell and consequently in cell morphogenesis, cell differentiation and cellular growth (13).

Fungi cell wall is a prime station in the coding and decoding of regulatory signal. This cell component is involved in many cellular functions, such as morphogenetic events and cellular

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interaction. It is also residence for enzymes, determines cell sensibility to drugs and the arquitecture and composition of the cell wall may vary according to environmental conditions. It is important therefore to understand the structural organization, the molecular array, the composition, and the functional properties of all components of the cell wall (7, 15, 24, 25, 51).

This work was carried out to localize the acid polysaccharides *in situ* in *Candida lipolytica* cell wall and to evaluate the effects of Tween 80 on the ultrastructure by using a ultrastructural cytochemistry approach.

MATERIALS AND METHODS

Microorganism and growth medium

Candida lipolytica IA 1055 was grown in Cald Yeast Mold (CYM) medium described by Cirigliano and Carman (11) modified by Nascimento *et al.*, (43), over 96 h at 27°C on a reciprocal shaker (120 Hz). Tween 80 was added to the culture medium at intervals of 0, 8, 16 and 24 h during culture to final concentrations of 0.5% and 1.0%. These intervals corresponded to the beginning of the culture, beginning of exponential growth phase, intermediary exponential growth phase, and end of exponential growth phase/beginning of stationary phase, respectively. Control cultures corresponded to those grown in same medium but added of glucose.

Cytochemical analysis

Samples of *C. lipolytica* cultures grown in glucose and those grown in Tween 80 were collected from the culture medium at 8, 16 and 24 hours of growth, washed twice in phosphate buffer saline (PBS), pH 7.0 and fixed for 1h at room temperature in 2.5% glutaraldehyde in cacodylate buffer 0.1M, pH 7.2, containing 0.5mg/ml of ruthenium red. Samples were washed twice in cacodylate buffer containing ruthenium red in concentrations cited before. The post-fixation was performed in 1.0% osmium tetroxide in cacodylate buffer 0.1M, pH 7.2 containing 0.5mg/ml of ruthenium red during 1h at room temperature. After a new washing as before, dehydration was performed at acetone 50%, 70%, 90% and 100% and embedded in Epon resin. Samples for cytochemistry control were obtained by removing the ruthenium red of all preparatory steps. Ultrathin sections were obtained in a Reichert ultracut, collected on copper grids, and examined in JEOL CX-100 transmission electron microscope (32,33).

RESULTS

Thin sections of *C. lipolytica* showed a typical construction of eukaryotic cells with mitochondria, electrondense cytoplasm bodies rich in granules, and the plasma membrane exhibiting many invaginations. The thickness of the cell wall increased with cellular age (Fig. 1).

Ultrastructural cytochemistry using ruthenium red as marker of control samples (grown in presence of glucose) revealed that surface of cell wall of *C. lipolytica* presented a dense reaction product coat of fibrillar material covering the whole cell surface. The intensity of the reaction decreased with age of culture (Figs. 2A, 2B and 2C).

On the other hand, cells grown in presence of Tween 80 presented different cytochemical staining pattern according to the surfactant concentration. Cells treated with 0.5% exhibited a reduced cytochemical staining compared to control samples and a decreasing of cytochemical staining according to growth phase of cultive similar to control cells (Figs. 3A, 3B and 3C).

Cells of cultures treated with Tween 1.0% exhibited the lowest cytochemical labeling in the cell wall in spite of surfactant addition time in culture medium (Figs. 4A, 4B and 4C).

The presence of reaction product observed as a coat of fibrilar material was not observed in the cell wall of control samples, which were not submitted to both cytochemical staining and contrastation (Figs. 5, 6 and 7). In these samples the cell wall was an eletronlucent space without visualization of layers.

DISCUSSION

The ultrastructure of yeast cells was first studied in 1957 and the techniques used have advanced greatly in the 40 years since then. Information about yeast cell wall composition, synthesis, genetics and structure has come from studies involving saprophytic and medically important species such as *Candida albicans* and *Saccharomyces cerevisiae*. Genetic, biochemical and ultrastructural combined approaches are essential for furthering the knowledgement of various aspects of yeast cell wall. There is little understanding of the processes that result in joint of components into a wall. A start on the problem might be based on the type of polysaccharide present in a given cell wall (7, 29, 31, 48).

Ultrastructural characteristics of the fungal cell wall, septal pore, mitochondria, nucleus, organelles and plasma membrane of many taxonomic groups, including medically important species, have been extensively studied, and already reviewed (28, 40, 47, 53, 54, 57). However, there is no detailed analysis of the *C. lipolytica* ultrastructure is available.

The results of this work reveal that in *C. lipolytica* control culture, the plasmatic membrane presents invaginations, which become longer and deeper according to the growth phase. Indeed, the cell wall became thicker during cell growth, in which distinct layers were observed. Mitochondria, eletrondense bodies, vesicles and vacuoles and an eletrondense cytoplasm were observed in the micrographs.

In yeasts, the cell wall makes 15 up to 30% of the dry weight of the cell. The walls are composed mostly of mannoproteins and fibrous and branched glycans that link other components of the wall such as chitin, as demonstrated by biochemical and degradative techniques. Carbohydrate-containing molecules

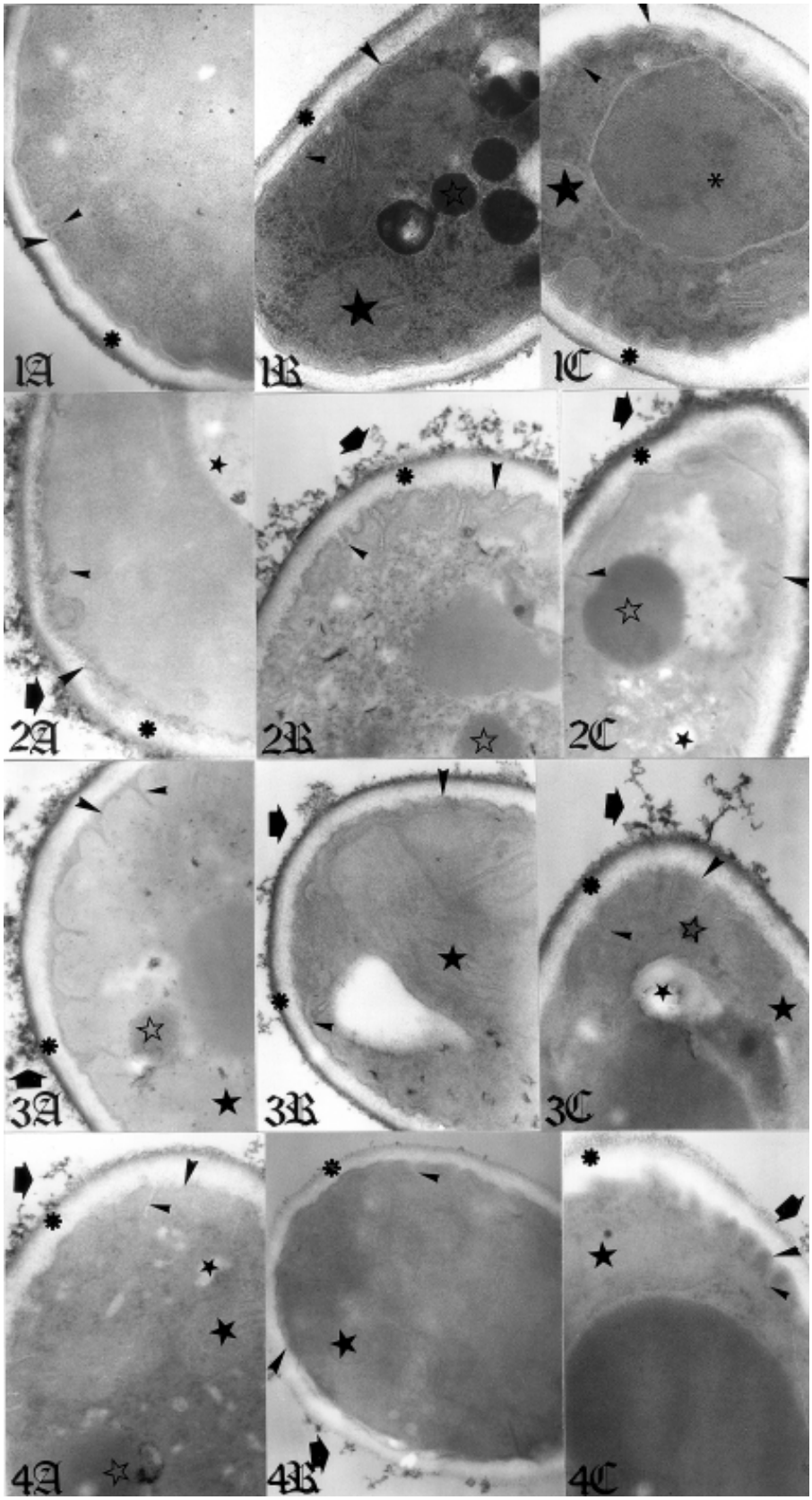

Figure 1. *Candida lipolytica* control culture. Cell wall (✹), Cell membrane (➤), Cell membrane invaginations (➢), Mitochondria (★), Nucleus (✻), Electron dense bodies (☆). A, B and C correspond to cells with 8, 16 and 24 hours of cultive. 20.000X.

Figure 2. *Candida lipolytica* control culture submitted to ruthenium red cytochemistry. Cell wall (✹), Cell membrane (➤), Cell membrane imaginations (➢), Vacuoles (★), Electron dense bodies (☆). Reaction products on cell wall outer surface (◗). A, B and C correspond to cells with 8, 16 and 24 hours of cultive. 20.000X.

Figure 3. *Candida lipolytica* grown in presence of tween 80 0,5% and submitted to ruthenium red cytochemistry. Cell wall (✹), Cell membrane (➤), Cell membrane invaginations (➢), Vacuoles (★), Electron dense bodies (☆), Mitochondria (★). Reaction products on cell wall outer surface (◗). A, B and C correspond to cells with 8, 16 and 24 hours of cultive. 20.000X.

Figure 4. *Candida lipolytica* grown in presence of tween 80 1,0% and submitted to ruthenium red cytochemistry. Cell wall (✹), Cell membrane (➤), Cell membrane invaginations (➢), Vacuoles (★), Electron dense bodies (☆), Mitochondria (★). Reaction products on cell wall outer surface (◗). A, B and C correspond to cells with 8, 16 and 24 hours of cultive. 20.000X.

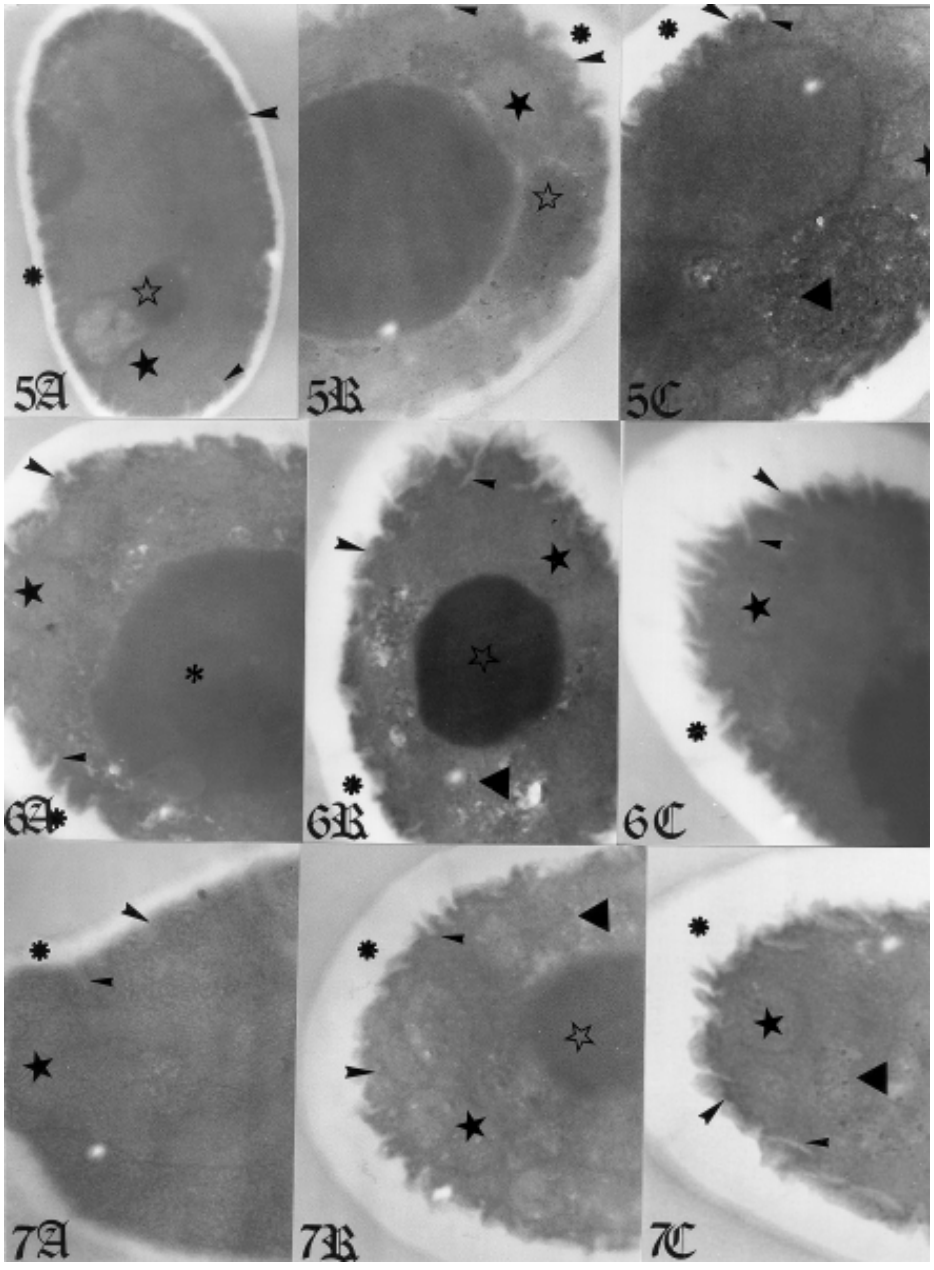

Figure 5. *Candida lipolytica* control culture of cytochemical labelling without constrastation. Cell wall (✹), Cell membrane (➤), Cell membrane invaginations (➢), Mitochondria (★), Electron dense bodies (☆), Multivesicular bodie (▲). A, B and C correspond to cells with 8, 16 and 24 hours of cultive. 20.000X.

Figure 6. *Candida lipolytica* grown in presence of tween 80 0,5% cytochemical control. Cell wall (✹), Cell membrane (➤), Cell membrane invaginations (➢), Multivesicular bodie (▲), Electron dense bodies (☆), Mitochondria (★), Nucleus (✻). A, B and C correspond to cells with 8, 16 and 24 hours of cultive. 20.000X.

Figure 7. *Candida lipolytica* grown in presence of tween 80 1,0% cytochemical control. Cell wall (✹), Cell membrane (➤), Cell membrane invaginations (➢), Multivesicular bodie (▲), Electron dense bodies (☆), Mitochondria (★) A, B and C correspond to cells with 8, 16 and 24 hours of cultive. 20.000X.

have been located on the yeast cell wall by ultrastructural cytochemistry (2, 5, 6, 8, 16, 20, 28, 30, 31, 48, 56).

Cytochemical observations of *C. lipolytica* collected from control cultures by using ruthenium red revealed an eletrondense coat of fibrillar and granular material distributed over the yeast cell wall surface. However, the intensity of the reaction changed according to the cell growth phase. The cytochemical labelling intensity was greater in control samples with 8 hours of growth compared to those with 16 and 24 hours suggesting a natural modification in the surface expression of carbohydrate during cell aging.

Although significant progress has been achieved in the knowledgement of the structure of fungal wall polysaccharides, little is known about their organization on the cell surface. It has been proposed that cellular polysaccharides and lipids distribution and localization in microorganisms may vary with cultural conditions, phase of cell cycle, oxigen tension and temperature. By manipulating cell wall construction a fungus

may assume a variety of functions (4, 7, 21, 26, 29, 30, 31, 50).

Cellular carbohydrate composition, content and distribution in the cell surface are very important in the cellular differentiation/maturation stages of an organism. Besides their structural role, the surface carbohydrates are associated to cell interaction and can act as receptors for extracellular substances and molecules, (1, 13, 14, 22, 23, 54). Alterations in the carbohydrate composition and distribution were observed in yeasts according to the growth phase and phase transition (dimorphism) by using biochemical degradative techniques (10, 24, 58, 59).

The acid polysaccharide presence was first demonstrated for *Coccidioides immitis* (45). Indeed, the presence of acidic carbohydrates in zygomycetes by using biochemical and enzimatic methods was also demonstrated (9).

Using ruthenium red, Edwards *et al.* (18) detected a dense cell coat in *Paracoccidioides brasiliensis* assexual spores. A thin coat was observed in the mycelium surface.

That the effects of surfactants on growth and lipase production by *C. lipolytica* were first observed using sodium dodecyl sulphate - a anionic surfactant (41, 42). Nascimento *et al.* (43) showed the effects of Tween 80 on protease production by *C. lipolytica*. The addition of Tween 80 to a *C. lipolytica* culture medium induced an increase in cellular viability, with maximum stimulation of enzyme production when added at the begining of culture. The highest stimulatory enzyme secretion was observed with Tween 80 1.0%.

This work revealed that *C. lipolytica* cells grown in medium with Tween 80 exhibited differences on the presence and distribution of acid polysaccharides according to addition time and concentration of the surfactant. Cells treated with Tween 80 1.0% at the beginning of culture presented a no reaction product in spite of surfactant time addition. Treatment with Tween 80 0.5% treatment at 8 hours of culture induced a reduced acid polysaccharides labeling compared to control cells.

The effects of surfactants were demonstrated on cellular lipids of bacteria, in which the oleic acid could be introduced in the lipid bilayer of the cell membrane. Cells grown in the presence of Tween 80 exhibited an increase in the cellular lipid content (55, 61). However, the effect of Tween 80 on cellular polysaccharides was not studied. This work demonstrated for the first time the effect of surfactant on the surface polysaccharide of *C. lipolytica*.

Studies on effects of surfactants on cell ultrastructure are rare. However, some ultrastructural alterations were observed in bacteria of MAC complex grown in presence of Tween 80 by using transmission and scanning electron microscopy. Cell elongation, inhibition of formation of cell coat, appearance of cytoplasmic inclusion-like bodies and reduction of cell eletrondensity are some of the cellular changes (17, 35, 36).

The results demonstrate for the first time the ultrastructural localization and distribution of acid polysaccharides in *Candida lipolytica* in different growth phases, and the effects of the addition of Tween 80 to the growth medium on the expression of these sugar residues. Also, this work demonstrated the effects of different carbon sources in the culture medium on the cellular carbohydrate content on the organism cell surface. The elucidation of the chemical nature of the cell wall is essential to infer the physiological and biochemical activities of the fungal cell.

By means of electron microscopy, it is possible to gain new insight into the organization and constitution of fungi cell wall at a level not revealed by routine methods. Much needs to be learned about fungi ultrastructure due to their importance, specially in medical and industrial fields.

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RESUMO

Estudo citoquímico dos carboidratos ácidos na superfície de *Candida lipolytica* crescida em meio contendo Tween 80

Moléculas contendo resíduos de açúcares foram localizadas sobre a superfície de *Candida lipolytica* utilizando-se o vermelho de rutênio como marcador citoquímico. A levedura foi semeada em meio contendo Tween 80. O surfactante, nas concentrações de 1,0% e 0,5%, foi adicionado ao meio de cultivo em diferentes intervalos de tempo, correspondentes ao início da fase exponencial de crescimento, meio da fase logarítmica e final da fase exponencial de crescimento, Culturas-controles foram cultivadas em meio contendo glicose como fonte de carbono. O crescimento da levedura em meios contendo glicose ou Tween 80 induziu o surgimento de alterações na distribuição e na localização de polissacarídeos ácidos na parede celular do organismo. Células crescidas em glicose ou Tween 80 exibiram variações citoquímicas em função de sua fase de crescimento. Adicionalmente, o padrão de marcação citoquímica também sofreu variações de acordo com a concentração do surfactante. A influência do surfactante na expressão de carboidratos é discutida.

Palavras-chave: *Candida lipolytica*; citoquímica de carboidratos; ultraestrutura de fungos.

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