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MINIREVIEW

Pyogenic Streptococci – Danger of Re-emerging Pathogens

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Abstract

β-hemolytic, pyogenic streptococci are classified according to type of major surface antigen into A (*Streptococcus pyogenes*), B (*Streptococcus agalactiae*), C (multiple species including *Streptococcus dysagalactiae*) and G (multiple species including *Streptococcus canis*) Lancefield groups. Group A *Streptococcus* causes each year hundreds of thousands deaths globally as a result of infections and post-infectional sequelae. An increasing number of severe, invasive infections is caused by selected, specialized pathogenic clones. Within the last 50 years, an increasing number of human infections caused by groups B, C and G Streptococcus (GBS, GCS, GGS) has been observed worldwide. GBS was first identified as animal pathogen but the spectrum of diseases caused by GBS quickly shifted to human infections. Groups C and G *Streptococcus* are still regarded mostly as animal pathogens, however, an increased number of severe infections caused by these groups is observed. The increasing number of human infections caused worldwide by GCS/GGS can be a sign of similar development from animal to human pathogen as observed in case of GBS and this group will gain much more clinical interest in the future.The situation in Poland regarding invasive infections caused by pyogenic streptococci is underestimated.

Key words: *Streptococcus* sp., GAS, GBS, GCS, GGS

Background

In the antibiotics era and with the development of vaccines, it was believed that bacterial infections could be easily managed, and prevented thanks to vaccination. However, the spread of antibiotic resistance and lack of vaccines for multiple pathogens have become a public health problem. Lack of efficient tools to combat infections promotes the emergence of strains that are more pathogenic, more difficult and expensive to eradicate.

Streptococci are major human and animal pathogens, divided into more than 40 species and multiple groups whose taxonomy changed several times over the years (Kohler, 2007). This mini review will concentrate on β-hemolytic group of pyogenic streptococci and is intended to give a broad overview of the group, stress common aspects of their pathogenicity and point out the health cost of infections and economic aspects.

Classification of *Streptococcus*

Streptococcus is a genus that groups catalase negative, gram-positive cocci. Due to single and parallel division plane, they form chains composed of two or more cells. First classifications of streptococci, in addition to cell type and biochemical properties, were based on the type of hemolysis: α – reduction of hemoglobin, resulting in greenish zone around colonies; β – complete lysis of erythrocytes and γ – lack of visible hemolysis.

The pioneering work of Rebecca Craighill Lancefield in the early 1930s (Lancefield and Todd, 1928; Lancefield, 1933) systematized the classification of streptococci based on the presence and type of surface antigen: cell wall carbohydrate or lipoteichoic acids. The Lancefield classification differentiates well the β-hemolytic group and subdivides it further into groups labeled with capital letters from A through W. Some streptococci that exhibit α or γ hemolysis, for example *Streptococcus pneumoniae*, do not encode Lancefield antigen. Major human streptococcal pathogens belong to so called "pyogenic" division of β-hemolytic streptococci and are classified as Lancefield groups A, B, C and G. For current and historic overview of classification of all streptococci see recent publications (Hardie and Whiley, 1997; Facklam, 2002; Kohler, 2007).

Group A *Streptococcus* – a major player

Streptococcus pyogenes belongs to Lancefield group A and is often called group A *Streptococcus* or simply GAS. Only a few other species such as *Streptococcus dysagalactiae* subsp. *equisimilis*, *Streptococcus*

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castoreus, Streptococcus anginosus, *Streptococcus constellatus* subsp. *constellatus* and *Streptococcus orisratti* can in very rare cases contain group antigen A (Kohler, 2007).

S. pyogenes is a causative agent of common suppurative, superficial infections of mucosal surfaces, skin and skin structures. The most common examples of GAS infections are streptococcal pharynghitis/tonsillitis, scarlet fever, impetigo, erysipelas, cellulitis, abscesses of various localization and pyoderma. However, in some cases these infections can lead to post-streptococcal non-suppurative sequelae as rheumatic fever, rheumatic heart diseases and glomerulonephritis. GAS is also able to cause severe, invasive, life threatening infections as streptococcal toxic shock syndrome (STSS), necrotizing fascitis (NF), rare cases of meningitis and pneumonia, puerperal sepsis and septicemia (for a review see (Cunningham, 2000) and references therein). Factors that predispose to invasive GAS infections are often related to immunological defects, metabolic diseases as diabetes, previous viral infections (chicken pox and influenza) and skin injuries (lesions, surgery, injecting drug use) (Lamagni *et al.*, 2008).

GAS is recognized as one of the most important bacterial pathogens. Based on WHO data (Carapetis *et al.*, 2005), it is estimated that GAS infections were in 2002 amongst the deadliest, just behind HIV, tuberculosis, malaria, pneumococcus, hepatitis B, *Haemophilus influenzae* type b, measles and rotavirus. Carapetis and co-workers (Carapetis *et al.*, 2005), based on systematic analysis of GAS epidemiological data, estimate that GAS causes 616 million new cases of pharyngitis each year and number of existing cases of pyoderma is estimated to exceed 110 millions. In addition, the number of severe cases (both invasive infections and post-streptococcal sequelae) is estimated as at least 18.1 million of existing cases, with 1.78 million new cases each year that result in over half a million deaths each year globally.

Classification of GAS is based on the type of major surface antigen: protein M (serotyping) (Cunningham, 2000). In the past, M type of the strain was determined using immunological reactions; recently most of the laboratories use sequencing of variable region of the gene encoding M protein (*emm* typing) (Johnson *et al.*, 2006). So far, over 150 M general serotypes (not including individual alleles of *emm* gene within each serotype) have been described (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm).

The geographical distribution of M types varies, the most prevalent types in high income countries belong to M12, M1, M28, M4 and M3. Moreover, in these countries over 90% of all strains belong to 25 serotypes while remaining 10% groups over 145 serotypes (Steer *et al.*, 2009). The distribution of strains in regions as Africa or Pacific region is remarkably different and 25 most prevalent serotypes contribute only 60% of all isolated serotypes (Steer *et al.*, 2009). Serotypes isolated from invasive cases in Poland are very diverse on the molecular level but the majority of strains belong to serotype M1 (Szczypa *et al.*, 2006). Currently, there is no commercially available vaccine that could prevent GAS infection. GAS is universally sensitive to penicillin and in cases of immediate allergy to penicillin, GAS infections are usually treated with macrolides as ertyhromycin. However, the increasing number of macrolide resistant strains in many parts of the world including Poland (Szczypa *et al.*, 2004) can make the treatment more expensive and less efficient. Antibiotic treatment of invasive diseases is very often unsuccessful due to very rapid onset of the disease that results in death rate as high as 50%. Based on success of pneumococcal vaccine (Whitney *et al.*, 2003; Pletz *et al.*, 2008; Isaacman *et al.*, 2010) it seems that the future direction in management of GAS is elaboration of vaccine. Because infection with certain M type does not protect from infection with other M types, formulation of currently tested vaccine is based on multiple M proteins (McNeil *et al.*, 2005).

GAS virulence factors. Group A *Streptococcus* encodes a set of sophisticated virulence factors that are involved in multiple aspects of pathogenesis, from adhesion to intimate modulation of human immune system.

Initial contact between GAS and human host cells is achieved via interactions between bacterial adhesins and cell receptors as integrins, fibrinogen, collagen and extracellular matrix proteins (fibronectin, laminin, vitronectin) (Cunningham, 2000; Nobbs *et al.*, 2009). Each strain of GAS encodes multiple, usually highly polymorphic, surface proteins that allows complex binding of host proteins (Nobbs *et al.*, 2009).

The major adhesin and virulence factor of GAS is M protein. The protein has a very characteristic coiled coil structure with conserved cell wall anchored C terminus and hyper variable N terminal part (Cunningham, 2000; Bisno *et al.*, 2003). Sequencing of the fragment encoding first 50 aa with 10 aa signal peptide is a base of molecular *emm* typing to determine serotype (Beall *et al.*, 1996). M and M-like proteins are also involved in interaction between patogen and immune system (Perez-Caballero *et al.*, 2004). They disrupt the classic complement cascade by binding of C4b factor and are able to disrupt the alternative cascade by binding factor H and factor H-like protein 1 (FHL-1) (Perez-Caballero *et al.*, 2004). M proteins also acts as anti-phagocytic factor by physical blocking of complement binding (Carlsson *et al.*, 2005) and is a potent

inducer of inflammation upon binding Toll-like receptor 2 (Pahlman *et al.*, 2006).

As a next step after initial contact, bacterial cells are released from the site and spread through tissues thanks to activity of multiple lytic enzymes. Host tissues are degraded by SpeB protease (Bohach *et al.*, 1988; Chiang-Ni and Wu, 2008), host protease – plasmin, activated by streptokinase produced by GAS (Bergmann and Hammerschmidt, 2007) and hyaluronidase (Hynes *et al.*, 2000).

In addition to host tissue degradation, GAS produces set of factors, including multiple proteases, involved in modulation of human immune response. SpeB protease cleaves component C3b of the complement and immunoglobulins (Collin and Olsen, 2003; Chiang-Ni and Wu, 2008); ScpA protease cleaves human C5a complement component and slows influx of inflammatory cells to the site of infection (Ji *et al.*, 1996); Mac/IdeS protease cleaves human IgG and blocks phagocyosis (Lei *et al.*, 2001; von Pawel-Rammingen *et al.*, 2002); SpyCEP protease cleaves chemokines as IL-8, granulocyte chemotactic protein 2, growth-related oncogene alpha, macrophage inflammatory protein 2-alpha and growth-related protein beta (Edwards *et al.*, 2005; Sumby *et al.*, 2008; Kurupati *et al.*, 2010); SIC blocks C5b-C9 complement complex (Akesson *et al.*, 1996). GAS also developed strategy to evade response of innate immune system by production of DNAses that allow to escape neutrophil extracellular traps (Sumby *et al.*, 2005a; Buchanan *et al.*, 2006).

Third large group of GAS virulence factors is composed of multiple toxins as pore forming streptolysins S and O (Nizet, 2002), and superantigens that are also factors interacting with immune system (Fraser *et al.*, 2000). Superantigens bind directly to MHC-II receptors and activate T-cells what leads to the uncontrolled release of pro-inflammatory cytokines (Fraser *et al.*, 2000).

Selection of highly virulent clonal lineages. One of the most important aspects of multiple M types is non-random association of M type with manifestation of the disease. For example serotype M12 predominantly causes throat infections (Luca-Harari *et al.*, 2009), while M3s are causing relatively more severe infections with higher mortality (Davies *et al.*, 1996; Daneman *et al.*, 2007), M18 serotype is correlated with rheumatic fever (Smoot *et al.*, 2002a; Smoot *et al.*, 2002b), and M28 serotype with puerperal sepsis (Green *et al.*, 2005a; Green *et al.*, 2005b).

The severity of the disease is associated with the emergence of particular lineages within certain serotypes that evolved as a result of acquisition of new genes/virulence factors that improve their fitness. The first well documented example of hyper virulent GAS clone is M1T1 lineage (Cleary *et al.*, 1992). Strain classified as M1T1 are the most frequent isolated strains from severe invasive GAS infections (Aziz and Kotb, 2008). The unique features of M1T1 clone are related to the presence of 36 kb genomic island, prophages, acquisition of *speA2* allele encoding superantigen SpeA and *sda1* encoding DNase (Aziz *et al.*, 2005; Sumby *et al.*, 2005b). The activity of Sda1 in particular was shown as major factor responsible for selection of highly virulent line (Walker *et al.*, 2007).

Similar selection of virulent clone can be observed in case of M3 strains (Beres *et al.*, 2002; Beres *et al.*, 2004) where the process is also driven by acquisition of phage encoded new virulence factor – phospholipase A2 named SlaA (Sitkiewicz *et al.*, 2006).

Group B Streptococcus – *Streptococcus agalactiae* (GBS)

Streptococcus agalactiae is classified as Lancefield group B (GBS). GBS is a major bovine pathogen that is one of the causes of bovine mastitis. Bovine infections still have big economic impact, as GBS might infect over 40% of the herd and influence milk quality and quantity (Keefe *et al.*, 1997). GBS infecting humans was first isolated and described in 1930s in vaginal samples and samples from puerperal sepsis (Lancefield and Hare, 1935). Today, GBS colonizes gastrointestinal tract and genitourinary tract of about 30% of healthy individuals, without any symptoms (Badri *et al.*, 1977; Foxman *et al.*, 2006; van der Mee-Marquet *et al.*, 2008).

Until mid 1960s, human infections caused by GBS were described infrequently. However, from late 1960s to 1970s GBS became predominant pathogen in neonates and children younger than 3 months (Phares *et al.*, 2008). In children, GBS causes two major types of infections named early – and late onset disease. Early onset disease is caused by direct vertical transmission from colonized birth canal, usually develops within first few days of live and manifests as pneumonia, meningitis and/or sepsis. Fatality of early onset disease can be as high as 50% (Shet and Ferrieri, 2004). Late onset disease can develop up to third month of live and direct mode of transmission is still unknown. The late onset disease manifests as sepsis, meningitis or osteomyelitis. In addition, GBS is able to cause infections of amniotic fluid during pregnancy and cause septic abortions (Daugaard *et al.*, 1988; McDonald and Chambers, 2000). Following recommendations issued first by Centers for Disease Control in the United States (CDC, 2010) and later in many countries including Poland (Kotarski *et al.*, 2008), the number of neonatal cases dropped in USA from 1.7 per 1000 live births in 1993 to 0.34 per 1000 live births in 2005. Unfortunately, antibiotic (penicillin)

prophylaxis in pregnant individuals responsible for dramatic drop in early onset cases does not prevent late onset of the disease (Phares *et al.*, 2008).

Like GAS, GBS is able to cause invasive diseases in non-pregnant individuals. The surveillance data shows that on the contrary to decline in neonatal infections, number of adult infections, especially among people older than 65 years, is high (25.3 cases per 100 000) (Phares *et al.*, 2008). Adult infections in non-pregnant individuals are very often correlated with underlying medical conditions as diabetes mellitus, heart disease, cancer, obesity, neurologic disorders, immunosuppressive diseases *etc.* According to surveillance studies, 88% of adult cases have at least one underlying medical condition (Phares *et al.*, 2008). Unfortunately, the success of penicillin treatment in GBS infection prevention and treatment delayed the development of GBS vaccine

GBS virulence factors. GBS virulence factors are less described than those of GAS, but similar classes of virulence factors can be distinguish in both pathogens. GBS encodes multiple adhesins responsible for interaction with eukaryotic cells: fibrin, fibrinogen and laminin binding proteins (Maisey *et al.*, 2008); Srr (**s**erine **r**ich p**r**oteins) proteins that allow binding to keratin (Samen *et al.*, 2007); surface pilli-like structures (Lauer *et al.*, 2005); and large Alp/Rib family of adhesins that groups αC (ACP), βC (BCP), epsilon/Alp1, Alp2, Alp3, and Rib proteins (Bolduc *et al.*, 2002; Baron *et al.*, 2004; Creti *et al.*, 2004). GBS also encodes pore forming toxins as hemolysin (Nizet, 2002). Production of hemolysin is correlated with the production of orange pigment protecting GBS from free radicals. Second pore forming toxin is called CAMP factor (Lang and Palmer, 2003) but its role in pathogenesis process remains unclear (Hensler *et al.*, 2008). Finally, GBS produces set of factors that are involved in modulation of immune response as ScpB protease, homolog of GAS ScpA, that cleaves C5a component of complement (Bohnsack *et al.*, 1997) and abolishes the activity of polymorphonuclear leukocytes (Takahashi *et al.*, 1995). In addition, ScpB influences adhesion by cutting host proteins (Cheng *et al.*, 2002). CspA encoded by GBS has similar sequence and function to SpyCEP (Harris *et al*., 2003). It cleaves fibrinogen and extracellular matrix proteins, but also degrades chemokines as growth-related oncogenes alpha, beta and gamma, granulocyte chemotactic protein 2 and neutrophil-activating peptide 2, but on the contrary to GAS it does not cleave interleukin 8 (Bryan and Shelver, 2009).

Clonal structure of GBS population. Similarly to GAS, infections with GBS exhibit non random association with serotype, neonatal infections are predominantly caused by serotypes Ia and III, while infections in non pregnant adults are caused more frequently by serotypes Ib, II and V, with small percentage of infections caused by serotype III (Shet and Ferrieri, 2004).

Multiple studies on population structure of GBS conducted worldwide show that GBS infecting and colonizing humans has a highly clonal structure (Bohnsack *et al.*, 2008; Springman *et al.*, 2009). The clonal structure was also detected in Poland (Sadowy *et al.*, 2010). Based on MLST (*multi locus sequence typing*) analysis, one particular clonal complex (CC 17) distinguished within serotype III is associated with neonatal invasive disease (Luan *et al.*, 2005; Jones *et al.*, 2006). Interestingly, analysis of population structure clearly shows the emergence of highly virulent human-associated CC17 complex from the bovine-associated CC67 (Sorensen *et al.*, 2010).

Groups C and G *Streptococcus* – an underestimated problem

Groups C and G *Streptococcus* (GCS and GGS) are pathogens of animal origin, many are still classified as opportunistic pathogens. Similarly to GAS and GBS they can be carried by humans and are able to cause similar diseases as GAS, such as pharyngitis or impetigo. Human infections related to animal origin are often milk-borne and can have outbreak characteristics with severe complications as glomerulonephritis (Bordes-Benitez *et al.*, 2006).

The classification of C and G groups of streptococci has changed over the last 40 years, and often various species designations are used by different authors. Bergey's Manual of Determinative Bacteriology from 1974, lists four species of group C Streptococcus: *Streptococcus equisimilis*, *Streptococcus dysagalactiae*, *Streptococcus equi* and *Streptococcus zooepidermicus*. However, some *S. equisimilis* strains contain the group G antigen. More recently, the introduction of subspecies was proposed to better distinguishes between Lancefield groups (Vandamme *et al.*, 1996). *S. equi* was subdivided into *S. equi* subsp *equi* (GCS) and *S. equi* subsp *zooepidermicus* (GCS); and *S. dysagalactiae* was subdivided into *S. dysagalactiae* subsp *dysagalactiae* (GCS or rarely group L) and *S. dysagalactiae* subsp *equisimilis*. (GCS/GGS and rarely GAS or group L). The other species classified as GCS is *Streptococcus canis*.

Until the 1970s only rare cases of GCS/GGS invasive infections were described. For example, among 150 000 blood cultures obtained at the Mayo Clinic from 1968 to 1977, only 8 revealed signs of GCS infection (Mohr *et al.*, 1979). Multiple case reports from 1980-90s showed that most infected patients have some underlying diseases such as cardiovascular disease or malignancy, similar to GAS and GBS. In the case of GCS, the most common clinical manifestations were bacteremia and endocarditis, but also puerperal

sepsis, pleuropulmonary infections, skin and soft-tissue infection, central nervous system infection, urinary tract infection, intra-abdominal abscess, epidural abscess, and dialysis-associated infection (Quevedo *et al.*, 1987; Salata *et al.*, 1989; Bradley *et al.*, 1991; Marchandin *et al.*, 2007).

In last 20 years an increase in number of human, invasive and often fatal diseases caused by GCS and GGS has been observed and GCS and GGS are being recognized as an important and emerging pathogens (Brandt and Spellerberg, 2009). According to epidemiological data from 2003–2004, burden and death rate parallels that of GAS (XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases, presentation 013.3 "Genotypic analysis of invasive, *emm*-typeable *Streptococcus dysagalactiae* subsp. *equisimilis* and *Streptococcus canis*" Sakota V. *et al.*) (Efstratiou, 1989; Efstratiou, 1997; Ikebe *et al.*, 2010).

GAS, GBS, GCS and GGS epidemiology in Poland

Based on published epidemiological data, increasing number of severe infections caused by pyogenic streptococci is observed worldwide. In addition, selection of highly virulent clones and dangerous shift from zoonotic infections to humans should be carefully monitored. Therefore, there is constant need of surveillance to trace of sources of infections, clonality and antibiotic resistance spread.

The situation in Poland regarding invasive diseases caused by pyogenic streptococci is greatly underestimated. National Institute of Public Health (PZH) collects epidemiological data about infections in Poland (http://www.pzh.gov.pl/oldpage/epimeld/index_p.html). Until 2008 only cases of scarlet fever and erysipelas caused by GAS and cases of bacterial non meningococcal meningitis (presumably caused by GBS) were required by law to be reported to the authorities. According to legislation changed in 2008, the reported data includes other invasive infections caused by GAS. Cases of invasive GBS infections in non pregnant adults and infections caused by GCS and GGS are not reported. Collection of strains causing invasive infections is not required by law. However, the material is often sent to reference and academic centers for microbiological evaluation.

Knowledge about infections caused by GAS and GBS in Poland and clonal structure of populations is mostly related to the ongoing activity of two research groups (Szczypa *et al.*, 2004; Szczypa *et al.*, 2006; Skoczynska *et al.*, 2007; Brzychczy-Wloch *et al.*, 2008; Strus *et al.*, 2009a; Strus *et al.*, 2009b; Brzychczy-Wloch *et al.*, 2010; Sadowy *et al.*, 2010). The rate of infections caused by GCS and GGS in Poland is currently not recognized.

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MINIREVIEW

Gemini Alkylammonium Salts as Biodeterioration Inhibitors

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Abstract

To protect materials against biodeterioration, physical, biological or chemical methods can be used. Chemical inhibitors of biodeterioration are the most common and effective. A new class of chemical inhibitors-gemini alkylammonium salts-shows excellent biocidal properties and good ecological profile. These compounds can be applied as biodeterioration inhibitors in a wide variety of materials.

K e y w o r d s: gemini alkylammonium salts, quaternary ammonium salts, microbial activity in biodeterioration

Introduction

Microorganisms, the first inhabitants of the biosphere, possess the ability to survive and adapt to almost any challenge. This ability must have been laid down in their genomes during their long and successful sojourn on our Earth. In many cases microorganisms are essential for normal metabolic and biotechnology processes. However, they also cause disease and demises. Moreover, the microbial spoilage-biodeterioration – of wood, paper, textiles, paints, stonework, steel, costs many millions of euro each year. To protect hard materials against biodeterioration, biological, physical and chemical methods are used (Allsopp *et al*., 2004). Physical methods exploit mostly UV or γ radiation, high or low temperature and strong electric or magnetic field. In turn, biological methods use some kind of safe microorganisms like *Bacillus fluorescens* or proteinaceous toxins-bacteriocins-produced by bacteria to inhibit the growth of similar or closely related bacterial strains. The chemical methods are based on microbiocides, *i.e.* chemical compounds with biocidal activity. Microbiocides include some phenols and their derivatives, organic and inorganic halogen compounds, oxidizing substances, quaternary ammonium compounds, alcohols, aldehydes and organic and inorganic acids (Block, 2001; Fraise *et al*., 2004; Manivannan, 2008; Paulus, 2005; Cross *et al*., 1994). The most important group of microbiocides are quaternary ammonium compounds (QAC) because of their wide spectrum of biocidal activity, the safety of application and low costs. Quaternary ammonium compounds were introduced as antimicrobial agents by Domagk over seventy years ago (Domagk, 1935). The first generation of QAC was standard benzalkonium chloride, *i.e.* alkylbenzyldimethylammonium chloride, with specific alkyl distribution, namely C_{12}, 40%; C_{14}, 50% and C_{16}, 10% (Fig. 1a) (Block, 2001). The second generation of QAC was obtained by substitution of the aromatic ring in alkylbenzyldimethylammonium chloride by chlorine

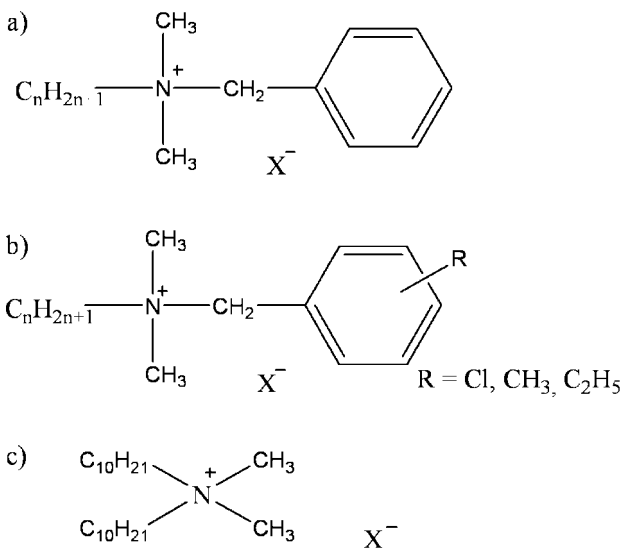

Fig. 1. Structures of quaternary alkylammonium salts (QAC).

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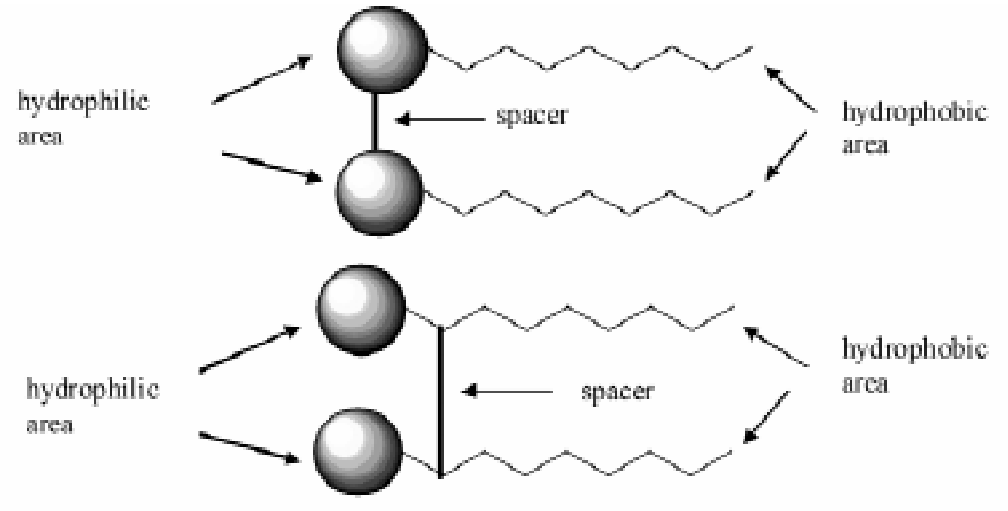

Fig. 2. Schematic representation of gemini alkylammonium salts.

or alkyl group to get a product like alkyldimethylethylbenzylammonium chloride (Fig. 1b) with alkyl distribution C_{12}, 50%; C_{14}, 30%; C_{16}, 17% and C_{18}, 3%. The dual quaternary ammonium salts are the third generation of QAC. This product is a mixture of equal proportions of alkyldimethylbenzylammonium chloride with alkyl distribution C_{12}, 68%; C_{14}, 32% and alkyldimethylethylbenzylammonium chloride with alkyl distribution C_{12}, 50%; C_{14}, 30%; C_{16}, 17% and C_{18}, 3%. The twin chain quaternary ammonium salts, like didecyldimethylammonium chloride, are the fourth generation of QAC (Fig. 1c). The concept of synergistic combination in the dual QAC has been applied to twin chain quaternary ammonium salts. The mixture of dialkyldimethylamoonium chloride (dioctyl, 25%; didecyl, 25%, octyldecyl, 50%) with benzalkonium chloride (C_{12}, 40%; C_{14}, 50%; C_{16}, 10%) is the newest blend of quaternary ammonium salts which represents the fifth generation of QAC's (Block, 2001).

Structures and properties of gemini surfactants. Gemini alkylammonium salts represent a new class of dimeric surfactants made up of two identical or different amphiphilic moieties having the structure of monomeric

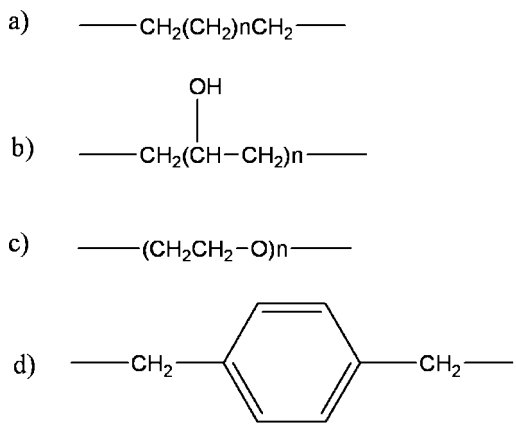

Fig. 3. Types of spacers in gemini surfactants.

quaternary alkylammonium salts connected by a spacer group (Zoller, 2009; Menger *et al*., 2000; Zana *et al.*, 2004) (Fig. 2). This class of quaternary ammonium salts can be considered the sixth generation of QAC.

The spacer may be hydrophobic (aliphatic or aromatic) (Fig. 3a and 3d) or hydrophilic (polyether, hydroxyalkyl) (Fig. 3b and 3c). It can also be rigid (stilbene) or flexible (polymethylene chain). The length of hydrocarbon spacer chain can vary from two methylene groups up to 20 methylene groups. The spacer group must connect the two amphiphilic moieties at the level of, or in close vicinity to, the head group. The symmetric gemini alkylammonium salts can be depicted as [m-s-m], where m is the number of carbon atoms in the hydrophobic chain and s is the number of methylene groups in the spacer (Fig. 4a).

The gemini alkylammonium salts show unique micelle-forming and surface-adsorbing properties in aqueous solution. Critical micelle concentration (cmc) for gemini surfactants is usually two orders lower than for corresponding monomeric surfactants. For example, the cmc value of dodecyltrimethylammonium bromie (DTAB) (Fig. 4b), which is a typical monomeric cationic surfactant, is 1.5×10^{-2} M, whereas the cmc value for trimethylene-1,3-bis-(*N*,*N*-dimethyl-*N*-dodecyloammonium)bromide [12–3–12] is $9.1–9.6 \times 10^{-4}$ M (Zana *et al.*, 2004). Critical micelle concentrations are very sensitive to the structure of surfactant. For gemini surfactants of [m-s-m] type cmc values decrease with an increase of spacer length (Table I). Moreover, the thermodynamic data for gemini surfactants, enthalpy (ΔH^o) an free energy (ΔG^o), are much lower then those for monomeric alkylammonium salt (Table I) (Zana, 2004). It clearly indicates that stability of gemini alkylammonium salts is much higher in comparison to monomeric alkylammonium salt, like DTAB. Increases stability of gemini surfactants *vs.* monomeric salts is also observed in the solid state.

Table I
Critical micelle concentrations (cmc) and thermodynamic data for DTAB and [12-s-12] gemini surfactants.

Surfactant	cmc (mM) (25°C)	$\Delta H°_M$ (kJ/mol) (25°C)	$\Delta G°_M$ (kJ/mol) (25°C)
DTAB*	15	–1.7	–19.1
12–2–12	0.84	–22	–47.3
12–4–12	1.17	–9.3	–45.1
12–6–12	1.03	–8.5	–44.6
12–8–12	0.83	–9.0	–44.2
12–10–12	0.63	–11.6	–45.5
12–12–12	0.37	–12.2	–46.8

* DTAB – dodecyltrimethylammonium bromide

The melting points of ethylene-1,2-bis-(*N*,*N*-dimethyl-*N*-alkyloammonium)iodides [12–2–12] increase with increased length of hydrocarbon chain, what indicates strong hydrophobic interactions between hydrocarbon chains and better packaging in the crystals (Fig. 5) (Brycki *et al*., 2010). In the contrary, melting points of monomeric alkylammonium salts decrease as the length of hydrocarbon chain increase, this being in accordance with an increase of conformational freedom as hydrocarbon chain become longer. One of the most important parameters of surface activity is the ability to decrease the surface tension of water, what strongly depend on the area of surfactant at the air/water interface (Broze, 1999; Lai, 1999; Holmberg *et al*., 2003). The area per molecule in a saturated monolayer at the water-air interface, made by gemini surfactant is bigger than that for the corresponding monomeric surfactants. For ethylene-1,2-bis-(*N*,*N*-dimethyl-*N*-dodecyloammonim)bromide [12–2–12] the area is 0.72 nm^2 whereas for DTAB this area is 0.49 nm^2 per molecule (Zana *et al.*, 2004). The efficiency of decreasing of

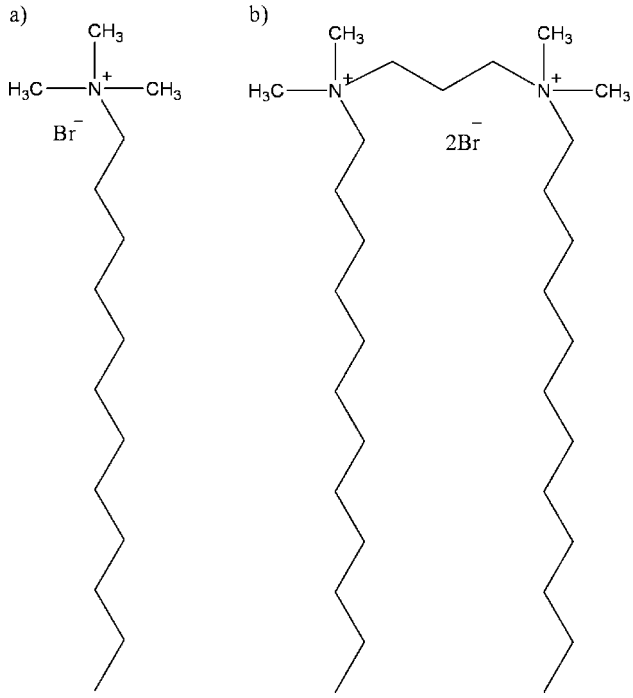

Fig. 4. Structures of trimethylene-1,3-bis-(*N*,*N*-dimethyl-*N*-dodecyloammonium) bromide [12–3–12] (**a**) and trimethyldodecyl ammonium bromide (DTAB) (**b**).

the surface tension of water is often characterized by the concentration C_{20}, *i.e.*, the surfactant concentration required for lowering the surface tension of water by 0.02N/m (Holmberg *et al*., 2003). For [12–2–12] and DTAB these values are 0.0083 and 0.21 wt.%, respectively (Zana *et al.*, 2004). It means that gemini surfactant [12–2–12] is over 25 times more effective then DTAB to decrease the surface tension of water.

Antimicrobial activity of gemini surfactants. The mechanism of biocidal activity of quaternary ammonium

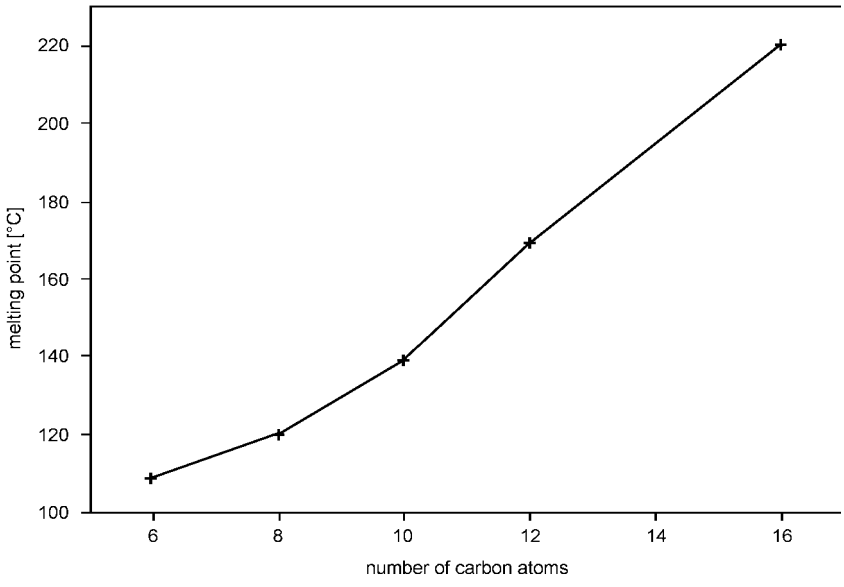

Fig. 5. Relationship of melting points of trimethylene-1,3-bis-[(*N*,*N*-dimethyl-*N*-alkylammonium)iodide] [m-3-m] *vs.* number of carbons in hydrocarbon chain.

Table II
MIC (μg/ml) for gemini alkylammonium surfactants [12-s-12] (Laatiris, 2008).

Microorganisms	MIC (μg/mL)		
	12–2–12	12–3–2	12–4–12
Staphylococcus aureus (ATCC 9144)	6	6	1.5
Pseudomonas aeruginosa (ATCC 27857)	200	200	200
Escherichia coli (ATCC 9637)	50	50	50

Table III
MIC (μg/ml) of geminis (Pérez, 1996).

Microorganisms	$C_4(CA)_2$	$C_2(LA)_2$	$C_3(LA)_2$
Gram-negative			
Alcaligenes faecalis ATCC 8750	64	128	64
Streptococcus faecalis ATCC 1054	4	32	16
Escherichia coli ATCC 1054	128	64	>128
Pseudomonas aeruginosa ATCC 9721	32	128	64
Gram-positive			
Bacillus cereus var. mycoides ATCC 11778	64	64	64
Bacillus subtilis ATCC 6633	64	64	32
Staphylococcus aureus ATCC 2518	4	32	16
Staphylococcus epidermidis ATCC 155–1	4	32	16
Micrococcus luteus ATCC 9341	32	64	64

$C_4(CA)_2$: N^{α}, N^{ω}-bis (N^{α}-caprylarginine)-1,2-diaminebutylamide
$C_2(LA)_2$: N^{α}, N^{ω}-bis (N^{α}-laurylarginine)-1,2-diamineethylamide
$C_3(LA)_2$: N^{α}, N^{ω}-bis (N^{α}-laurylarginine)-1,3-diaminepropylamide

salts is based on the adsorption of compound on the bacterial cell surface, diffusion through the cell wall and then binding and disruption of cytoplasmic membrane (Block S., 2001). Damage to the membrane results in the release of potassium ions and other cytoplasmic constituents, precipitation of cell contents and finally the death of the cells. The antibacterial activity (MIC) of quaternary ammonium salts strongly depends on their hydrophilic-lipophilic balance (HLB), according to the equation:

$$\text{Log1/MIC} = a + b\log P + C[\log P]^2$$

where P is an octanol-water partition coefficient, which characterizes HLB of the molecule. The levels of antimicrobial activity are parabolically related to the alkyl chain length, and thereby to logP (Hansch *et al.*, 1973; Hansch *et al.*, 1964). The lower chain lengths, C_{10}-C_{12}, are more active against yeast and fungi, whereas Gram-negative organisms are most susceptible toward the more lipophilic C_{16} compounds. This is probable a consequence of the lipophilic nature of the Gram-negative cell wall and of the difficulties often encountered by hydrophilic molecules to traversing it. The bacterial cell surfaces are usually negatively charged and that adsorption of QAC onto surface is expected to be facilitated by polyammonium cations (Block, 2001). Gemini alkylammonium salts, due to their structures, possess not only double positive charge on two nitrogen atoms but also have higher lipophilic character. Therefore, gemini surfactants in some cases show even hundreds times higher biocidal activity in comparison to monomeric quaternary alkylammonium salts (Zana *et al*., 2004). This means that the same biocidal effect can be reached using much smaller amounts of biocide, what is of fundamental importance from toxicological and ecological point of view (Zoller, 2004). Symmetrical gemini alkylammonium surfactants [12-s-12] show very good antibacterial activity against both Gram-positive and Gram-negative bacteria (Table II) (Laatiris, 2008). An average minimal inhibitory concentration of [12-s-12] for Gram-positive bacteria is 6 μg/mL and decrease to 1.5 μg/mL for longer spacer. The MIC for Gram-negative bacteria, *Pseudomonas aeruginosa*, is 100 μg/mL. The higher concentration of microbiocide necessary to destroy *Pseudomonas aeruginosa* is a typical feature for almost all kind of microbiocides (Laatiris, 2008).

Applications of new biodeterioration inhibitors depend on several variables. The most important is an antimicrobial efficacy and ecological profile, including biodegradability, bioconcentration and bioaccumulation factors. In addition, biodeterioration inhibitors have to be safe for hard surfaces. From this point of view special interest is focused on gemini alkylammonium salts based on amino acid and sugar derivatives. This group of biodeterioration inhibitors show not only excellent antimicrobial activity against Gram-positive and Gram-negative bacteria but also easily undergoes biodegradation in dilute solutions (Table III) (Pérez, 1996). The efficacy of gemini alkylammonium salts as biodeterioration inhibitors is additionally enhanced by hydrophobisation of surfaces making the settlement of microorganism on this surface difficult. The bigger the gemini surfactant, the higher the degree of hydrophobisation observed. For gemini alkylammonium salts of type [m-s-m], which also prevent corrosion, the most effective is [14–2–14] and then [12–2–12] and [10–2–10] (El Achouri, 2001). The best biocidal activity and effect of hydrophobisation of surface can be reached for gemini surfactants with optimized hydrophilic-lipophilic balance. HLB can be modified by introducing sugar or oxyethylene derivatives to alkylammonium molecules (Fig. 6) These compounds have not only a very good antimicrobial activity, but also can be exploited as micellar hydrosolubilizers for other microbiocides. In case of 2,4,4'-trichloro-2-hydroxydiphenyl ether (triclosan), which is in water practically insoluble, micellar hydrosolubilization can enhanced its solubility over 10 000 times (van Doren *et al*., 2000; Chiapetta *et al*., 2008).

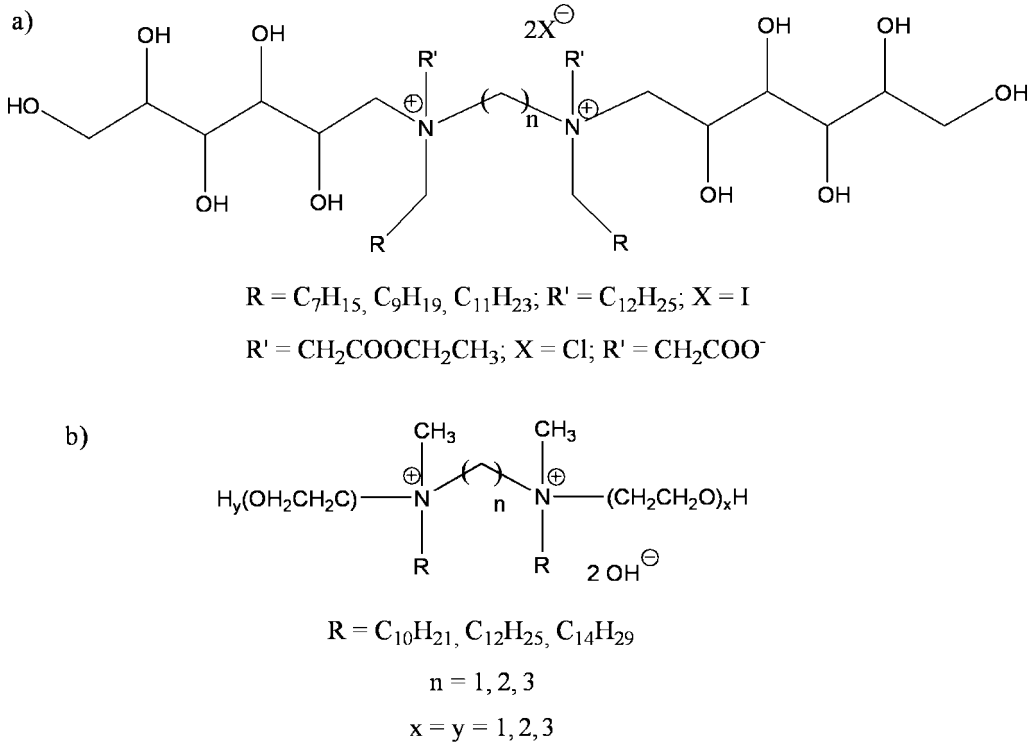

Fig. 6. Polyoxoethylene (a) and glucityl (b) derivatives of gemini alkyl ammonium salts.

Gemini alkylammonium salts are new group of biodeterioration inhibitors. These compounds show excellent antimicrobial activity and possess not only the ability to hydrophobize surfaces but also can act as micellar hydrosolubilizers. The properties of gemini alkylammonium salts make them very competitive compared to other microbiocides.

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ORIGINAL PAPER

Biofilm Forming Multi Drug Resistant *Staphylococcus* spp. Among Patients with Conjunctivitis

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Abstract

Biofilm forming multidrug resistant *Staphylococcus* spp. are major reservoirs for transmission of ophthalmic infections. They were isolated from ocular patients suffering from conjunctivitis. In this study we analyzed biofilm forming ability, antibiotic resistance profile of the *Staphylococcus* spp. isolated from clinical ocular patients, and their phylogenetic relationship with other community MRSA. Sixty *Staphylococcus* spp. strains isolated from clinical subjects were evaluated for their ability to form biofilm and express biofilm encoding ica gene. Among them 93% were slime producers and 87% were slime positive. *Staphylococcus aureus* and *S. epidermidis* were dominant strains among the isolates obtained from ocular patients. The strains also exhibited a differential biofilm formation quantitatively. Antibiotic susceptibility of the strains tested with Penicillin G, Ciprofloxacin, Ofloxacin, Methicillin, Amikacin, and Gentamicin indicated that they were resistant to more than one antibiotic. The amplicon of ica gene of strong biofilm producing *S. aureus* strains, obtained by polymerase chain reaction, was sequenced and their close genetic relationship with community acquired MRSA was analyzed based on phylogenetic tree. Our results indicate that they are genetically close to other community acquired MRSA.

Key words: *Staphylococcus* spp., biofilm, conjunctivitis, *ica* gene

Introduction

The surface of the eye is rich in nutrients and, consequently, supports a diverse range of microorganisms that constitute the normal ocular flora, the growth of which is regulated and thus infection is prevented (Armstrong, 2000). The eye, though protected by number of natural defense mechanisms, suffers from number of infections caused by adapted microorganisms. Bacteria involved in ophthalmic infections principally infect the conjunctiva, cornea, and the uveal tract (Sankaridurg *et al.*, 1996). Conjunctivitis caused by bacteria occurs worldwide and affects people of all ages and both sexes. It has been cited as one of the most frequent causes of self-referral in the practice of comprehensive ophthalmology. According to the American Academy of Ophthalmology, conjunctivitis infrequently causes permanent visual loss or structural damage but the economic impact of the disease in terms of lost work time, although undocumented, is doubtless considerable (Schlech and Blondeau, 2005). Bacterial conjunctivitis is typically self-limiting though treatment with topical ophthalmic antibacterials can reduce symptoms, recovery time, contagious spread, possible reinfection, and risk of complications. In fact, reports of outbreaks of bacterial conjunctivitis underscore the benefit of controlling the spread of disease with immediate treatment (McDonald *et al.*, 2008). Prolonged use of older and previous-generation antibiotics only facilitate the development of resistant strains (Schlech and Blondeau, 2005). Chronic bacterial infections were reported to persist over a long duration, as many as six decades, and the causative agent was shown to be susceptible to antibiotics used in high and sustained therapeutic doses. Biofilm forming bacteria are one among those that cause serious infections. Costerton *et al.*, (1999) defined biofilm as a structural community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to inert or living surfaces. Once a biofilm has formed, the bacteria within them are protected from phagocytosis and antibiotics (Hoyle *et al.*, 1992). The various definitions

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of biofilm (Carpentier *et al.*, 1993; Costerton and Lappin Scott, 1999; Elder *et al.*, 1995) encompass three basic ingredients namely the microbes, slime exopolysaccharide, and the surface. The biofilm does not develop if any of these components is removed from the mix. The current concept is that biofilm bacteria can usually survive the sterilants and/or antibiotics in concentrations that are 1000–1500 times higher than the concentrations that kill floating (planktonic) cells of the same species (Costerton *et al.*, 1999). Hence, it is of utmost importance to understand the mode of infection, proliferation, and survival of pathogens towards the control of eye infections. Vasudevan *et al.*, (2003) concluded that adherent cells within a biofilm are significantly more resistant to antimicrobial agents compared with planktonic organisms.

Early identification and evolving effective control strategies against potentially pathogenic biofilm-forming *Staphylococci* can be one of the essential steps towards the prevention and management of the most problematic eye infection. The classic method most often used to phenotypically detect slime production among bacterial species is the Congo red agar (CRA) plate test (Freeman *et al.*, 1989). Newly developed molecular methods recently provided a direct evidence of the genetic basis of slime production complementary to the CRA test. They became available with the discovery that slime synthesis is controlled by the ica operon (Mack *et al.*, 1996; Gerke *et al.*, 1998). Polysaccharide intercellular adhesin (PIA), a main slime component consisting of linear β-1,6-linked glucosaminylglycans, is synthesized *in vitro* from UDP-*N*-acetyl glucosamine by the enzyme *N*-acetyl glucosaminyl transferase. This is encoded by the *icaA* gene. Slime production is considered to be a significant virulence factor for some strains of Staphylococci (Christensen *et al.*, 1982). The challenge in eradicating a chronic infection associated with slime formation is mainly due to the fact that the slime producing bacteria resist higher antibiotic concentrations than non slime producing strains (Gristina *et al.*, 1987). Hence, a precise approach is to use PCR technology for the detection of adhesion genes (Tristan *et al.*, 2003). Antibiotic resistance in bacteria is currently a major public health problem (Fishman, 2006). Recent reports indicate that resistance to earlier generation ocular antibiotics among clinical bacterial isolates is becoming more prevalent. Antibiotic resistance has been noted among ocular isolates, necessitating treatment with medications such as fortified vancomycin (Kim *et al.*, 2005). Multidrug-resistant MRSA is proliferating in serious ocular infections. Based on the rate of increase in the "The Surveillance Network" (TSN) database USA, it was predicted that MRSA cultures from serious ocular infections could be more common than methicillin-susceptible *S. aureus.* Hence large-scale national surveillance programs are required to monitor *in vitro* antimicrobial resistance trends in ocular isolates (Asbell *et al.*, 2008) towards efficient management of the problem.

The increasing number of reports concerning ocular bacterial resistance to currently used antibiotics warrants a detailed study on the possible factor conferring antibiotic resistance and the chances of acquiring same from other non clinical organisms possibly due to horizontal gene transfer (HGT). In this context we investigated biofilm forming ability and their associated role in antibiotic resistance profile of the *Staphylococcus* spp. isolated from clinical ocular patients and their phylogenetic relationship with other community MRSA.

Experimental

Material and Methods

Bacterial strains. Two hundred samples (from 88 males and 112 females) were collected from conjunctivitis infected patients (cataract, red eye, keratitis and contact lens infection) from patients undergoing treatment at the Dr. Agarwal Eye Hospital, Salem, Tamil Nadu, India during the period from June 2008 to June 2009. The patients were asked to look up and the inferior conjuctival sac was swabbed in a single swab for secretions. All the collected samples were processed within two hours. The swabs obtained were inoculated on to Nutrient Agar, MacConkey Agar, Blood agar and Mannitol Salt Agar plates and incubated at 37°C for 24–48 hours. Isolates obtained from plates were identified using conventional microbiological methods. Colonies showing Gram-positive cocci in clusters, which were catalase positive, oxidase negative and bacitracin resistant, were presumptively identified and labelled as *Staphylococcus* sp. Identification of staphylococcal isolates to the species level was carried out by detection of enzyme production (coagulase, phosphatase, ornithine, and urease), L-pyrolidonyl-β-naphthylamide hydrolysis, hemolytic properties on sheep blood agar, acid production from mannitol, mannose and trehalose and resistance to novobiocin by using a published protocol (Kloos and Bannerman, 1999).

Antibiotic sensitivity test. The antibiotic resistance/sensitivity profile of the conjunctivitis infection isolates against various antimicrobial agents commonly used to treat or prevent ocular infections were assessed *in vitro* by the disk diffusion method according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2006). The inoculum for each confirmatory isolate was prepared using a 0.5 McFarland standard, which was then swabbed onto a Mueller Hinton (HiMedia, Mumbai, India) agar plate supplemented with 2% NaCl. The commercial antibiotic disks

(HiMedia, Mumbai, India) were placed in Petri plates and seeded with 10^8 CFU/mL (0.5 McFarland) of bacterial isolates. Penicillin G (10 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Methicillin (5 µg), Amikacin (5 µg), Gentamicin (30 µg) antibiotic discs were placed in Petri dish maintaining equal distance (4 mm) with the help of forceps which was flame sterilized intermittently. Following incubation at 37°C for 18 h, the bacterial growth inhibition zone around the disks was analysed and compared with standard chart.

Slime producing ability of all the isolates were evaluated by two different methods.

Tube adherence (Christensen *et al.*, 1982). A loopful of the isolate from agar plate was inoculated into a glass tube containing 5 ml of trypticase soya broth (TSB) and incubated at 37°C for 48 h. Each tube was decanted, stained with 0.25% safranin, and then gently rotated to ensure uniform staining and the contents were gently decanted. The tubes were then placed upside-down to drain. The color of the inner surfaces of the tubes was observed. An adherent film on the surface of the glass tube was taken as an evidence of slime formation. The absence of a film or the mere presence of a ring at the liquid-air interface was interrupted as a negative result (–). Based on slime production, the positive results were recorded as strong (+++), moderate (++), week (+). Each test was interpreted by two different observers.

Congo red agar method (Freeman *et al.*, 1989). Slime production by the isolates was determined by CRA method as described by Freeman *et al.*, (1989). The media used contained brain heart infusion broth 37 g/l, sucrose 0.8 g/l, agar-agar 10 g/l, and Congo red stain 0.8 g/l. The Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media when the agar was cooled to 55°C. Plates of the medium were inoculated and incubated aerobically for 24 h at 37°C. All the chemicals and reagents were procured from HiMedia, Mumbai, India. The appearance of reddish black colonies with a rough, dry, and crystalline consistency was considered to be indicative of slime production. Non slime isolates produced pinkish red, smooth colonies with a darkening at the centre.

Biofilm assay (Cucarella *et al.*, 2001). The isolates were individually grown overnight in TSB at 37°C, and diluted 1:40 in the same broth incorporated with 0.25% glucose. Sterile 96 well 'U' bottom polystyrene tissue culture plates (Tarsons, Mumbai, India) were inoculated with 200 µl of the bacterial suspension and incubated overnight (24 hours) at 37°C without agitation and visualized by staining with 1% crystal violet for 15 minutes after washing thrice with 200 µl of sterile phosphate buffer saline (pH 7.4) and drying. After rinsing three times with distilled water and subsequent drying, the formed biofilm was quantified in duplicate by a micro plate reader (model 680, Bio-Rad, Hercules, CA) at 570 nm. Uninoculated wells containing TSB with glucose served as blanks. *S. epidermidis* ATCC 35983, a known slime producer was used as positive control for slime production. Blank corrected absorbance values of strains were used for reporting biofilm production. Strains producing a blank corrected mean absorbance value of >0.1 were considered biofilm producers. Each strain was tested for biofilm production in duplicate and the assay was repeated three times.

Isolation of genomic DNA. Bacterial isolates grown overnight in a 50 ml LB broth, at 37°C in a rotary shaker (200 rpm) were used for genomic DNA isolation employing standard protocols outlined by Sambrook *et al.*, (1989). Two ml of overnight bacterial culture was transferred to an Eppendorff tube and spun at 6000 rpm for 4 minutes. The supernatant was discarded and drained well on to tissue paper. The bacterial pellet was re-suspended in 400 µl of TE buffer. Vortex rapidly then, 600 µl of 1M NaCl was added and allowed to heat at 65°C for 10 minutes. After 10 minutes, cooling it to room temperature 1000 µl of saturated phenol solution was added, mixed well centrifuged at 10,000 rpm for 10 minutes. (In the case of very dense aqueous phase, it was diluted with sterile distilled water). The aqueous phase was collected and mixed with 2 volume of absolute ice cold ethanol. Later it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded, the pellet was allowed to air dry completely. The pellet was washed with 70% ethanol and allowed to dry at room temperature. After drying, the pellet was stored in 20–80 µl of TE at –20°C. The extracted DNA was visualized by electrophoresis in 0.7% agarose gel and viewed under the Gel Documentation/Imaging Systems (Alpha Innotech, San Leandro, CA, USA) at 262 nm.

Detection and sequencing of *ica*A gene. The nucleotide sequences of the *ica*A gene available in the GenBank Sequence Database of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov) were utilized for designing primer sequences using genetyx version7 software. The primers used were: Forward: AAGTCATACACTTGCTGGCG and reverse: CTGTCTGGGCTTCACCATGT.

DNA from strong biofilm producing isolates was screened for *ica*A gene by the polymerase chain reaction (PCR) in a DNA thermal cycler (Eppendorf, Hamburg, Germany) (Vasudevan *et al.*, 2003). The reaction was carried out in a 25 µl volume containing 3 mmol of the primers, together with 25 ng of DNA, 1 U Takara ExTaq HS and buffer (containing 20 mmol MgCl2), 200 mmol each of dATP, dCTP, dGTP and dTTP. Thirty cycles of amplification, each consisting of denaturation at 92°C for 40 seconds, annealing at 49°C for 45 seconds and elongation at 72°C for 1 min,

along with a final extension at 72°C for 7 min was carried out. The presence and size of the amplicons were confirmed by electrophoresis on 1.5% agarose gel. Further nucleotide sequence of the amplicons were determined by sequencing which was carried at Ocimum Biosolution, MCV Chennai, India using the instrument ABI 3130, Applied Biosystems, CA, USA. The obtained sequences were submitted to NCBI (Accession numbers GQ 214387 and GQ214388).

Phylogenetic tree construction. The evolutionary relationships among the isolates were determined by phylogenetic analysis. The sequences were aligned first using CLUSTAL W that calculate a crude similarity measure between all pairs of sequences by using a fast and approximate alignment algorithm described by Wilbur and Lipman (1983) and then determined the order of sequences to be aligned in the final multiple alignment. The resulting distances were used to calculate a phylogenetic guide tree which uses pairwise sequence distance calculation to perform multiple sequence alignment The guide tree was calculated with the MEGA 4 method (Saitou *et al.*, 1987; Tamura *et al.*, 2007).

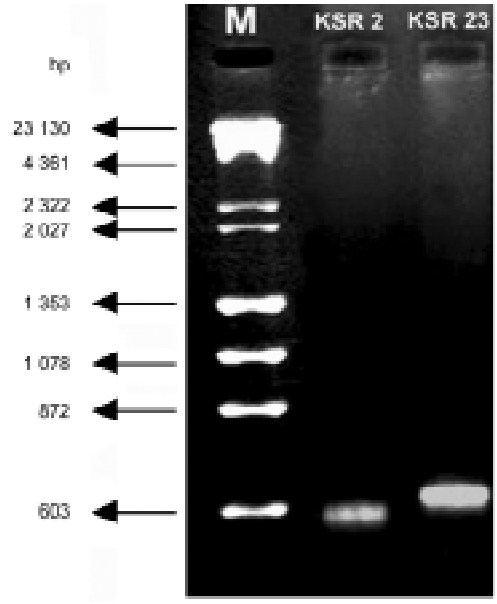

Fig. 1. PCR amplified product of ica A gene sequences of biofilm forming conjunctivitis Staphylococcus aureus isolates.

Results

Conjuctival specimens were examined from 200 patients and 108 bacterial isolates were obtained from them. All the isolates were presumptively identified as *Staphylococcus* spp., *Pseudomonas* spp., *Escherichia coli* and *Proteus* spp. Further it was observed that *Staphylococcus aureus* (36 isolates) and *S. epidermidis* (24 isolates) were dominant among the isolates obtained from ocular patients.

The biofilm forming ability of *S. aureus* and *S. epidermidis*, the dominant group among the isolates, was determined using tube adherence test, congo red plate method and confirmed using microtitre plate assay. It was observed that 38 out of 60 isolates were weakly positive (+1), 16 moderately positive (+2), two strongly positive (+3) and four isolates were biofilm negative. Further, the biofilm formed was quantified by microtitre plate adherence assay and their results indicated that isolates KSR 2, and KSR23 belonging to *S. aureus* were more prone to biofilm formation recording 0.564–1.157 OD, an indicative of higher activity.

The antibiogram study revealed that all the isolates of *S. aureus* and *S. epidermidis* were resistant to methicillin (Table I). Out of 60 *Staphylococcus* isolates 11 were penicillin resistant, 3 ciprofloxacin resistant, 49 ofloxacin resistant, 13 amikacin resistant and 13 gentamicin resistant. In total, 13 strains were resistant to three or more antibiotics. The antibiotic susceptibility data showed diminished activity of number of antibiotics on this conjunctivitis causing isolates which

Table I
Shows the antibiotic susceptibility and biofilm forming ability of the ophthalmic isolates

Strain No	Name of the isolate	No of isolates	Antibiogram of the isolates																		Biofilm Forming Ability Score					
			Penicillin (10 µg)			Ciprofloxacin (5 µg)			Ofloxacin (5 µg)			Methycillin (5 µg)			Amikacin (5 µg)			Gentamycin (30 µg)			Congo Red			Christenson		
			R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	+++	++	+	+++	++	+
1	*Staphylococcus aureus*	36	7	2	27	2	26	8	33	3	–	35	1	–	3	3	30	7	5	24	1	10	22	1	9	22
2	*S. epidermis*	24	4	3	17	1	18	5	16	5	3	22	2		10	11	3	6	3	15	1	6	16	1	9	12
3	*Pseudomonas* sp.	17	2	3	7	3	9	6	1	3	13	3	8	7	2	6	9	3	9	6	2	9	6	2	7	6
4	*Proteus* sp.	8	5	2	1	1	3	6	–	2	6	2	2	4	–	2	6	1	2	5	–	1	7	–	0	7
5	*Escherichia coli*	15	4	3	8	1	6	8	1	4	10	2	4	9	1	4	10	2	3	10		6	8	–	5	8
6	Others	8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	–	2	5	–	0	5

Note: R – Resistant; I – Intermediate; S – Sensitive; +++ Strong; ++ Moderate; + Weak; * Not Determined

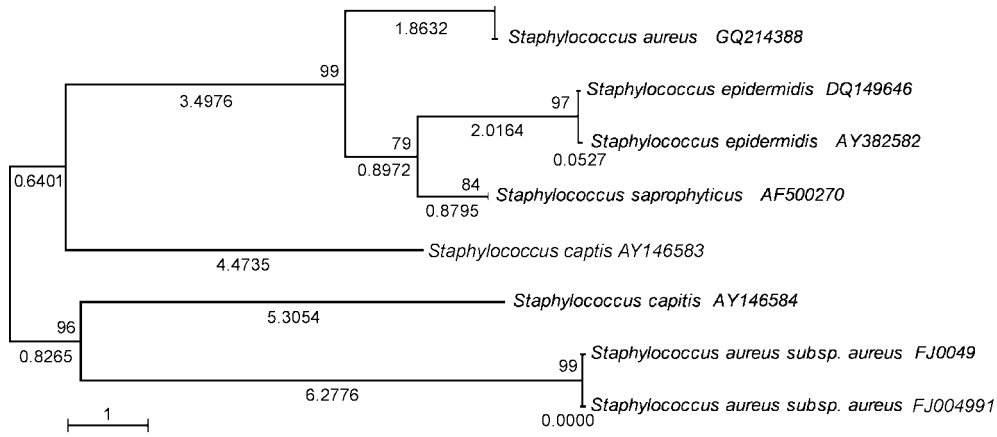

Fig. 2. Phylogenetic relationship of ica gene sequences among biofilm forming conjunctivitis *Staphylococcus aureus* isolated from ocular patients in Tamilnadu, India with reference sequences obtained through BLAST analysis.

increases with their biofilm forming ability. Though the other isolates *Pseudomonas* spp., *Proteus* spp., *E. coli etc.*, showed moderate antibiotic resistance; it acquires less significance because of their low biofilm forming ability.

The gene responsible for biofilm formation inter cellular adhesion (*ica*) was detected in 20 isolates of *S. aureus* and 16 of *S. epidermidis* by PCR amplification. Amplicon with 630 bp and 580 bp for *S. aureus* was obtained (Fig. 1). Automated sequencing of these amplicons provided partial sequences which were submitted to GenBank and were assigned the name and accession (GQ 214387 and GQ214388) (www.ncbi.nih.gov.). The phylogram presented in figure 2 indicates close relatedness of isolates obtained from the ocular patients with the sequences available in the genome database. The optimal tree with the sum of branch length equal to 26.69712461 as shown (Fig. 2) was obtained. Sequence analysis comparison of the *ica*A gene from these species revealed very high sequence similarity, suggesting the possibility of horizontal gene transfer of biofilm encoding genes.

Discussion

Coagulase negative Staphylococci (CoNS) commonly isolated mixed with more typical ocular flora lead to major infections including keratitis, conjunctivitis and endophthalmitis. CoNS, were considered as harmless skin commensal flora and dismissed as culture contaminants. But in recent years, they are increasingly being recognized as important human pathogens. The American Society of Cataract and Refractive Surgery survey for the year 2004 revealed that sixty-one percent of cases reported were due to *Staphylococci* (Donnenfeld *et al.*, 2005). *S. aureus* is the most common pathogen recovered from conjunctivitis (Knauf *et al.*, 1996) and its role in the pathogenesis of chronic allergic conjunctivitis due to colonisation has been suggested (Tuft *et al.*, 1992). *Staphylococcus epidermidis*, an opportunist microorganism, is now recognised as a real "new" pathogen, in particular as etiologic agent of infections associated bacterial colonies on the surface. *Staphylococcus aureus* was shown to undergo physiological changes in the early stages of biofilm formation (Williams *et al.*, 1999). In the present study both *S. aureus* and *S. epidermis* were isolated as dominant species from the conjunctivitis patients corroborating with the earlier reports.

Ophthalmologists believe that excessive and inadequate systemic use of antibiotics is one of the most important factors causing antibiotic resistance and that resistance among ocular isolates is a reflection of the practice pattern of this community. A study conducted between 1996 and 2001 showed that number of conjuctivitis causing isolates susceptible to methicillin decreases and the number of MRSA isolates increased from 8.5% in 1990 to 27.9% in 2001. It also revealed a 160% increase in ciprofloxacin resistance among keratitis and conjuctivitis *S. aureus* isolates (Marangon *et al.*, 2004). Goldstein *et al.* (1999) reported that resistance of *Staphylococcus aureus* to ciprofloxacin and ofloxacin increased 7-fold from 1993 to 1997. Hwang (2004) showed that several microorganism causing ophthalmic infections had developed resistance to ciprofloxacin and its sister fluoroquinolones, ofloxacin and levofloxacin, more quickly than imagined, and resistance levels are increasing each year.

Both *S. aureus* and *S. epidermidis* the well recognised etiologic agents of ophthalmic infections exploits the production of a polysaccharide biofilm for wrapping up and armouring their colonies on the surface as one pathogenic mechanism. Studies conducted using animal models have shown that biofilm-producing *S. epidermidis* strains are more virulent in causing

infections than biofilm negative strain (Deighthon *et al.*, 1996; Gelosia *et al.*, 2001). Though a number of tests are available to detect slime production by Staphylococci, all those methods including TM and CRA are often subject to severe analytical limitations and hence unable to detect bacterial adherence accurately (Mathur *et al.*, 2006). It is reported that *S. epidermidis* enters the eye during and after intraocular surgery and causes postoperative suppurative endophthalmitis. However, the factors contributing to the virulence of *S. epidermidis* are not well understood. It has been suggested that the ability to form biofilms on polymer surfaces greatly contributes to the virulence of *S. epidermidis*. This ability depends on the production of polysaccharide intercellular adhesion (PIA) molecules, encoded by the intercellular adhesion (*ica*) locus including the *ica*A gene, *ica*B gene, *ica*C gene, and *ica*D gene However, the prevalence of biofilm-forming strains of *S. epidermidis* in the conjunctival microflora has not yet been determined (Takashi Suzuki *et al.*, 2005). Results obtained in the present study and reports made in earlier studies prove that *S. aureus* and *S. epidermidis* are common pathogens causing eye infections. Their high prevalence and antibiotic resistance may be due to their biofilm forming nature. The PCR amplification of the *ica*A gene demonstrates the inherent biofilm producing nature of the isolates.

The evolutionary relationship of the strains *Staphylococcus aureu*s KSR2 and *S. aureus* KSR23 obtained from ocular patients in the present study was analyzed by comparison with sequence data available with National Center for Biotechnology Information (NCBI). Several studies have shown that the presence of genes encoding intra cellular adhesion (*ica*) is associated with the formation of slime and biofilm in *S. aureus* and *S. epidermidis* (Ammendolia *et al.*, 1999; Arciola *et al.*, 2001; Cramton *et al.*, 1999; Vasudevan *et al.*, 2003). In the community, hospital strains of methicillin resistant staphylococci may contribute to the emergence of methicillin resistant staphylococci *de novo*, through horizontal acquisition of the methicillin resistant encoding gene (Salmenlinna *et al.*, 2002). It has been reported that Methicillin Resistant (MR) staphylococci in haemodialysis units in hospitals, which were resistant to multiple antibiotics, probably contributed to the overall increase in the incidence of staphylococcal infections rather than simply replacing the more susceptible strains. The majority of the MR *Staphylococcus epidermidis* isolates from patients belonged to one main clone. The clonal relatedness of isolates strongly suggests that CoNS infections were probably contracted from a common source in the haemodialysis unit and that this clone was transferred by patient-to-patient transmission leading to infections (Liakopoulos *et al.*, 2008). In the present study, the antibiotic resistant *S. aureus* strains recovered from clinically significant conjunctivitis infection, as well as among colonizing isolates from community, were found to carry the ica operon and produce biofilms. Hence, it is presumed that there was a possible horizontal transfer of genes encoding biofilm among conjunctivitis isolates. Of course, the biofilm forming ability, their antibiotic resistance, the presence of *ica* gene and their close relationship with other community associated counterparts and probable HGT warrants an in depth study to draw final conclusions. Efficient detection of biofilm forming ability and expression of the encoding *ica*A gene, as well as the effective suppression of PIA or PS/A synthesis would facilitate development of feasible strategies for the treatment of conjunctivitis and prevention of the further transmission of their infection.

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ORIGINAL PAPER

Simultaneous Degradation of Waste Phosphogypsum and Liquid Manure from Industrial Pig Farm by a Mixed Community of Sulfate-Reducing Bacteria

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A b s t r a c t

The utilization of pig manure as a source of nutrients for the dissimilatory reduction of sulfates present in phosphogypsum was investigated. In both types of media used (synthetic medium and raw pig manure) increased utilization of sulfates with growing COD/SO_4^{2-} ratio in the medium was observed. The percent of sulfate reduction obtained in synthetic medium was from 18 to 99%, whereas the value for cultures set up in raw liquid manure was from 12% (at COD/SO_4^{2-} of 0.3) up to as high as 98% (at COD/SO_4^{2-} equal 3.80). Even with almost complete reduction of sulfates the percent of COD reduction did not exceed 55%. Based on the results obtained it was concluded that the effectiveness of removal of sulfates and organic matter by sulfate-reducing bacteria (SRB) depends to a considerable degree on the proportion between organic matter and sulfates in the purified wastewaters. The optimal COD/SO_4^{2-} ratio for the removal of organic matter was between 0.6 and 1.2 whereas the optimal ratio for the removal of sulfates was between 2.4 and 4.8.

K e y w o r d s: COD reduction, phosphogypsum, pig manure, sulfate-reducing bacteria (SRB)

Introduction

In recent years several papers have focused on the possibility of using sulfate reducing bacteria (SRB) for the anaerobic purification of industrial wastewaters, mining waters, drainage from the metallurgic industry, wastewater sediments and drainage from communal and industrial waste heaps (Dvorak *et al*., 1992; Hammeck and Edenborn 1992; Jong and Parry, 2006; Lens *et al*., 1998; 2002; Logan *et al*., 2005; Look *et al*., 1998; Silva *et al*., 2002; Hulshof, 2006, Alfaya *et al*., 2009).

Studies have also been conducted involving the use of SRB for the biodegradation of organic matter combined with: 1 – transformation of flue gas desulfurization gypsum (FGD), 2 – transformation of $FeSO_4$ produced during the manufacture of titanium white from the flotation concentrate of titanic iron by the sulfate method, 3 – transformation of technical $FeSO_4 \times H_2O$ from ferrous metallurgy and 4 – transformation of phosphogypsum (FG) (Dvorak *et al.*, 1992; Juszczak *et al*., 2002; Kaufman *et al.*, 1996; Kowalski *et al*., 1990; Przytocka-Jusiak *et al*., 1995; 1997; Kosińska and Miśkiewicz, 2000; Rzeczycka *et al*., 2001, 2004; Rzeczycka and Błaszczyk, 2005).

Phosphogypsum is a hazardous waste material formed during the processing of apatites and phosphorites to yield phosphoric acids (Kowalski *et al*., 1990, 1996; Arocena *et al.*, 1995; Azabou *et al*., 2005, 2007; Papastefanou *et al.*, 2006). The chemical plants that apply these technologies in Poland are located in Wizów (near Boleslawiec), Police (near Szczecin) and Gdańsk. Apatite concentrates at Wizów Chemical Plant are exclusively processed from the rocks of the Khibiny massif in Kola Peninsula, Russia. Other plants process phosphorite concentrates mainly from North Africa.

The main components of phosphogypsum are hydrated calcium sulfates: gypsum – $CaSO_4 \times 2H_2O$, halfhydrate – $CaSO_4 \times 0.5H_2O$ and anhydrite – $CaSO_4$ which make up 95% of the weight of phosphogypsum. Sulfate ions account for approximately 50% of the weight of phosphogypsum. The remainder includes: phosphoric acid, sulfuric acid, small amounts of Fe_2O_3, Al_2O_3, MgO, Na_2O, K_2O and the rare earth elements, the lanthanides.

Sulfate reducing bacteria are ubiquitous in anaerobic environments where organic substrates and sulfates are available. They can be encountered in aqueous and soil environments: hot springs, crude oil, sulfur deposits, natural gas outlets, estuary sludges,

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salt water reservoirs, corroding iron, the alimentary tract of animals and humans, industrial wastewaters rich in sulfates (*e.g.* from the chemical, metallurgic or paper industries), mining waters as well as in bioreactors (Postgate, 1984; Widdel, 1988; Hammeck and Edenborn, 1992; Deswaef *et al*., 1996; Hamilton, 1998; Hass and Polpraset, 1993; Cotta *et al*., 2003; Lens *et al*., 2003).

SRB are obligatory anaerobes, which obtain the energy they require from the oxidation of organic substrates, with sulfates being the terminal electron acceptors. SRB can also utilize thiosulfates or sulfites, and even elementary sulfur as electron acceptors. The preferred carbon sources for this group of microorganisms are compounds that are derived from fermentation processes, which are formed during the anaerobic degradation of organic matter: organic acids (*e.g.* lactate, pyruvate, formate and acetate) as well as alcohols (ethanol, propanol and butanol) (Postgate 1984; Hao *et al*., 1996; Rhabus *et al*., 2006).

The pathway of the degradation of carbon compounds by SRB depends on the interaction between this group of microorganisms and other bacteria. The addition of sulfates in the form of phosphogypsum, for example, favours domination of the system by sulfate-reducing bacteria (appropriate choice of COD/SO_4^{2-} ratio). The activity of these bacteria results not only in the removal of sulfates (by their conversion to sulfides), but also in the breakdown of organic matter.

Phosphogypsum can be the sole source of sulfate, phosphorus and biogenic elements for these bacteria. Like other microorganisms, SRB, besides sources of carbon and energy, also require a source of nitrogen. Therefore, besides phosphogypsum, a source of organic carbon and nitrogen is also necessary. One of the waste products that could be utilized as an inexpensive source of carbon and nitrogen for SRB is raw liquid manure.

Pig manure is a production waste that arises on pig farms during animal production. For many years both aerobic and anaerobic biological manure purification methods, with accompanying production of methane, have been used. Manure is not, however, a good medium for growing bacteria because of unfavorable proportions between the basic biogens. The biotransformation of phosphogypsum, combined with anaerobic breakdown of manure could be one of the ways of reducing the amounts of both these arduous waste matters. Phosphogypsum is rich in calcium and phosphorus and poor in nitrogen, but it should be kept in mind that the introduction of large amounts of sulfates together with phosphogypsum will alter the direction of the selection of bacteria in the anaerobic purification of liquid manure and that the leading role in the last stages of purification will be played by sulfate reducing bacteria.

The end products formed in the course of the purification process are: a calcium-organic deposit (which has potential use as a fertilizer) and elemental sulfur obtained in chemical or biological pathway.

The aim of the current study was to investigate the possibility of using a community of sulfate reducing bacteria for the simultaneous biotransformation of phosphogypsum and manure from industrial pig farming, thus removing two onerous waste products at one time.

Experimental

Materials and Methods

Microorganisms. A mixed community of sulfate-reducing bacteria isolated from various environments, as described elsewhere (Przytocka-Jusiak, 1995) was used.

Growth media. Sulfate-reducing bacteria were cultured in: A – synthetic minimal medium containing (in g/l distilled water): 1.0 NH_4Cl; 1.0–10.0 phosphogypsum (in the form of a deposit), the source of carbon used was (g/l): sodium lactate (4.7). B – non-sterile pig manure (average chemical composition – Table I) used in this study was obtained from an industrial pig farm and stored in 5 l bottles at 4–5°C. It was diluted 3 or 6 times prior to before use. C – Liquid manure containing (g/l in distilled water) 1.0–10.0 phosphogypsum (in the form of a deposit).

Phosphogypsum (Table II) was from a waste dump located in Wizów near Bolesławiec (Kowalski *et al*., 1990). The solubility of phosphogypsum in the above medium was approx. 36% and the concentration of phosphogypsum in the solution was therefore approx. 1.8 g/l (930 mg SO_4^{2-}/l). The pH of the medium at the time the culture was set up was 7.4. The growth of sulfate reducing bacteria was studied in the COD/SO_4^{2-} range from 0.3 to 4.8.

Culture conditions. The cultures were set up and maintained in 0.33 dm^3 bottles with rubber stoppers, 30 ml of active mother culture was introduced through the rubber stopper using a syringe into 270 ml medium. The cultures were incubated in thermostat

Table I
Mean chemical composition of raw pig manure.

Dry weight (total) mg/l	30.00
COD (mg O_2/l)	15.00
BZT_5 (mg O_2/l)	10.50
Total nitrogen (Kjeldahl) (mg N/l)	3000.00
Ammonia nitrogen (mg N/l)	1600.00
Total phosphorus (mg P/l)	650.00
pH	7.60

Table II
Chemical composition of phosphogypsum from Wizów.*

Component	Sample 9/2	Ranges of variation of the chemical composition of phosphogypsum from Wizów
CaO	29.23	29.23 – 30.49
SO_3	41.95	41.95 – 43.20
Fe_2O_3	0.13	0.08 – 0.20
Al_2O_3	0.20	0.20 – 0.29
MgO	0.05	0.03 – 0.06
SrO	1.53	1.53 – 1.82
BaO	0.04	0.01 – 0.06
Na_2O	0.31	0.31 – 0.56
K_2O	0.10	0.08 – 0.11
P_2O_5	2.20	1.26 – 3.03
Ln_2O_3	0.61	0.11 – 0.65
H_2O	20.40	19.80 – 20.50
F_2	0.50	0.30 – 0.65

* major components, data given as weight %

for 8–15 days at 30°C. When the culture reached its maximum activity it was introduced into fresh medium with the same composition. Three consecutive passages in the same medium (minimal medium or pig manure) were made.

Determinations. Sulfides were determined using the iodometric method with Lugol's solution (0.05 M) and sodium thiosulfate (0.05 M) against starch (0.5%) as an indicator. Chemical oxygen demand (COD) was determined as described in (Malina, 1967).

Calculations. Reduction of sulfates was calculated according to the formula:

$$R_{SO4} = (HS^-_{tmax} - HS^-_{to}) \times 2{,}91 / t_{max} - t_o,$$

where: – R – maximum reduction of sulfates (mg SO_4^{2-}/l); HS^-t_{max} – concentration of sulphites at time t_{max}; HS^- – concentration of sulfites at time t_o.

The COD/SO_4^{2-} ratio was calculated as the ratio of COD and concentration of sulfates in the medium. The coefficient of carbon oxidized was calculated from the proportion between COD reduction and amount of reduced sulfates. The maximal rate of sulfate reduction (V_{max} SO_4^{2-}) was calculated based on the maximum rate of sulfate reduction (mg SO_4^{2-}/l x day).

Results and Discussion

The growth of a community of sulfate reducing bacteria (SRB) in minimal medium and raw manure was examined. The source of the sulfates used was phosphogypsum: an industrial waste that is formed in large quantities during the production of phosphoric acid. Phosphogypsum has been shown to be a good electron acceptor for SRB (Kowalski *et al.*, 1996). The solubility of phosphogypsum in the above medium was approx. 36% and the concentration of phosphogypsum in the solution was therefore approx. 1.8 g/l (930 mg SO_4^{2-}/l). Such a high amount of sulfate ions allows for the high activity of sulfate reducing bacteria (*ca.* 400 mg SO_4^{2-}/l × day) (Fig. 1). The carbon source for these bacteria in minimal medium was sodium lactate and in the case of liquid manure the organic compounds present in it.

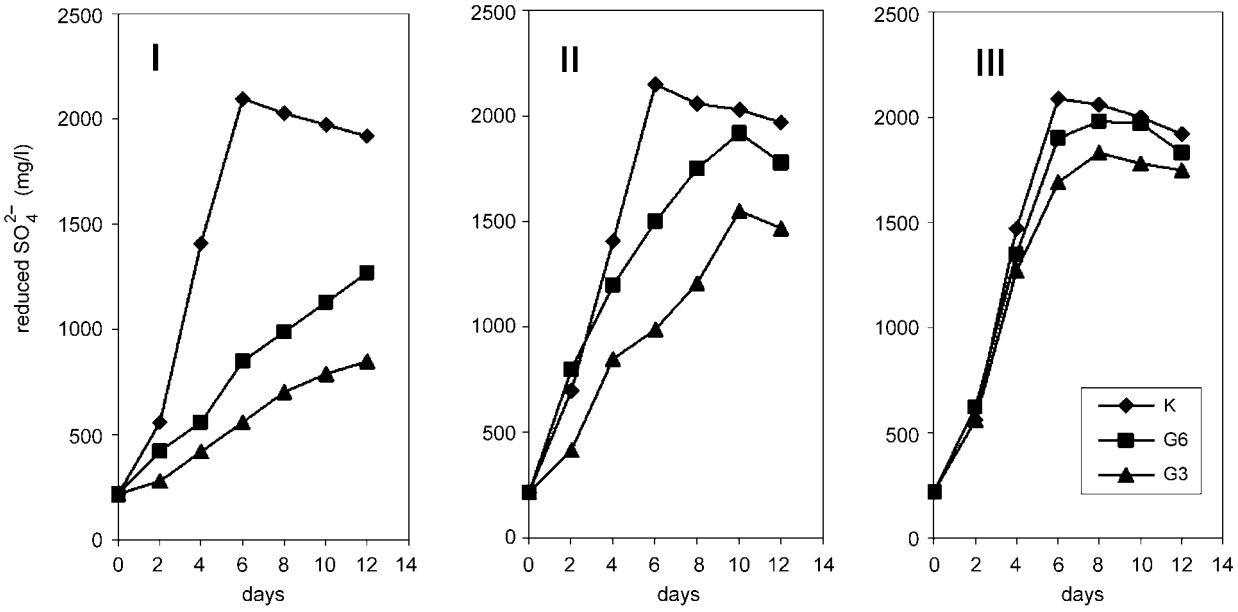

Fig. 1. Reduction of sulfates in medium with phosphogypsum and sodium lactate (K) and in liquid manure diluted six-fold (G6) or three-fold (G3) in consecutive passages of sulfate-reducing bacteria. I, II, III – consecutive passages.

Considerable differences in the development of SRB in medium minimal and diluted manure were observed (Fig. 1). In the first passage the reduction of sulfates in pig manure diluted six-fold (COD approx. 2500 mg O_2/l) and three-fold (COD approx. 5000 mg O_2/l) reached its maximal value only after 12 days of incubation (1270 and 850 mg SO_4^{2-}/l, respectively) whereas in parallel culture in minimal medium 2100 mg SO_4^{2-}/l were reduced after only 6 days of incubation. This is easy to explain because the studied community of bacteria had been repeatedly grown in minimal medium. The necessity of considerably diluting liquid manure in order to achieve simultaneous phosphogypsum biodegradation would be economically unprofitable and for that reason an attempt was made to adapt the bacteria to the raw manure. The activity of the bacteria grew considerably in the second and third passages (Fig. 1B and C). In three-fold diluted pig manure the reduction of sulfates in the second passage after 10 days was 1550 mg SO_4^{2-}/l, and after a consecutive (third) passage it was comparable to that obtained in culture in minimal medium and raw manure diluted six-fold (Fig. 1C).

It is thus possible, when using pig manure as a culture medium for bacteria reducing sulfates in phosphogypsum, to strongly limit the dilution of the manure with water.

The use of pig manure as a source of carbon for SRB also requires determining the appropriate proportion between the concentration of electron donor and concentration of electron acceptor in the form of sulfates in phosphogypsum. In the subsequent stage of our studies the development of a community of sulfate reducing bacteria in minimal medium and raw manure diluted three-fold, with COD/SO_4^{2-} ratio between 0.3 and 4.8, was examined.

The amount of sulfates reduced depended on the COD/SO_4^{2-} ratio in the medium (Fig. 2). It was the highest when the ratio was 0.8–1.2 and successively dropped as the ratio increased. Reduction in the amount of reduced sulfates with increase of COD/SO_4^{2-} ratio (from 0.30 to 4.70) was also observed by Kosińska and Miśkiewicz (1997, 1999) in cultures of *Desulfovibrio desulfuricans* maintained in liquid pig manure. Studies by Song *et al.* (1998) on electroplating wastewaters inoculated with a community of SRB showed that the amount of sulfates reduced dropped with increasing COD/SO_4^{2-} ratio in the medium from 0.33 to 1.21.

The rate of the reduction of sulfates (Fig. 3) in minimal medium and raw manure was the highest when the COD/SO_4^{2-} ratio in both studied media was 0.3 (300 and 180 mg SO_4^{2-}/l x day, respectively). With increasing COD/SO_4^{2-} ratio in the medium the rate gradually declined. Song *et al*. (1998) observed accelerated rate of reduction of sulfates with increase of COD/SO_4^{2-} ratio from 0.33 to 1.21, but the rate of violently decreased when the latter value was exceeded. Kosińska and Miśkiewicz (1997, 1999) in their studies on the growth of *Desulfovibrio desulfuricans* in raw liquid manure observed that the rate of reduction of sulfates was the highest at COD/SO_4^{2-} = 0.8–1.3.

Sulfate reducing bacteria are able to utilize various carbon sources. However, sodium lactate, which we used as the sole carbon source in minimal medium, is considered the best carbon source for these bacteria (Hao *et al*., 1996; Postgate, 1984; Rhabus *et al*., 2006). The second of the growth media used – fermented pig manure – contains various organic compounds, both high and low molecular weight ones. The specific chemical composition of pig manure is the outcome of the action of different groups of microorganisms, especially of bacteria conducting hydrolysis and fermentation processes (Cotta *et al*., 2003; Zhu, 2000; Logan *et al*., 2005). Pig manure is also rich in mineral compounds and contains high concentrations of inorganic nitrogen, phosphorus and magnesium as well as microelements. Bacteria growing in pig

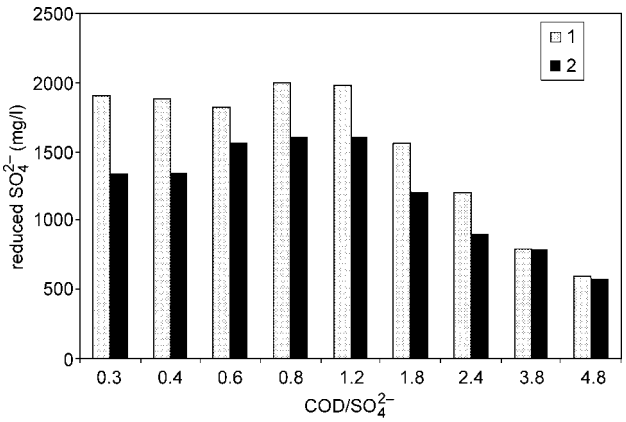

Fig. 2. Maximum reduction of SO_4^{2-} in cultures of a community of sulfate-reducing bacteria in minimal medium (1) and raw manure (2) with varying COD/SO_4^{2-} ratio.

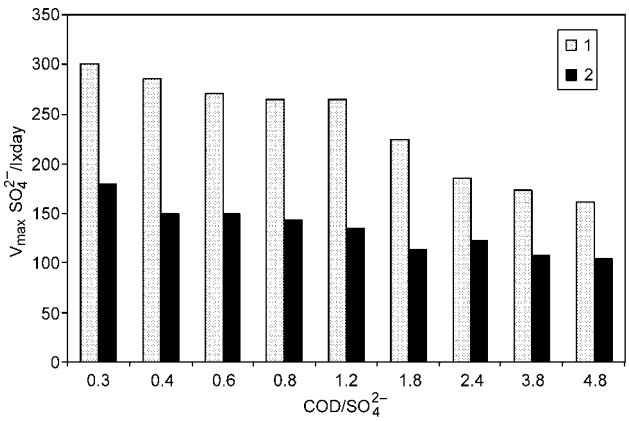

Fig. 3. Rate of sulfate reduction ($V_{max}SO_4^{2-}$) in cultures of a community of sulfate-reducing bacteria in minimal medium (1) and raw manure (2) with varying COD/SO_4^{2-} ratio.

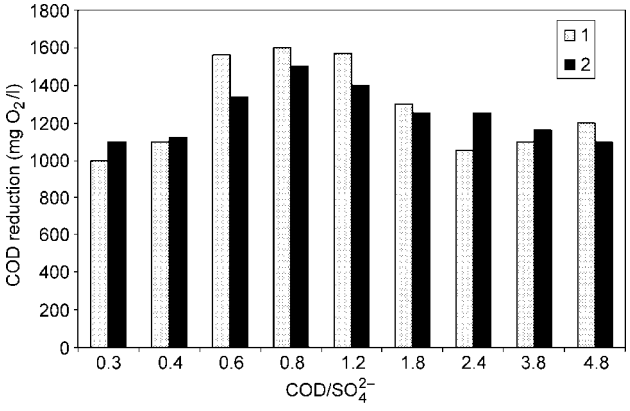

Fig. 4. COD reduction (mg O_2/l) in cultures of a community of sulfate-reducing bacteria in minimal medium (1) and raw manure (2) with varying COD/SO_4^{2-} ratio.

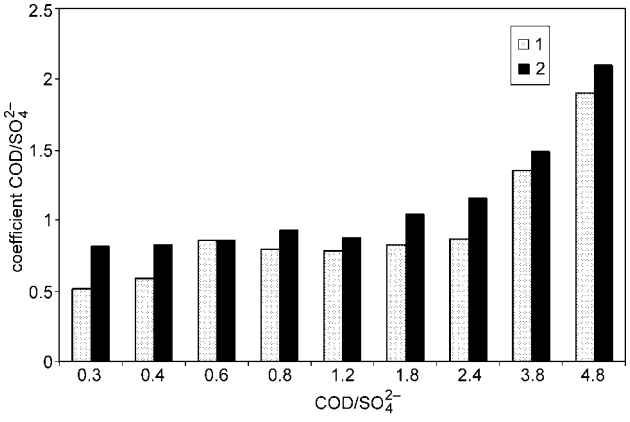

Fig. 5. Value of COD/SO_4^{2-} coefficient in cultures of a community of sulfate-reducing bacteria in minimal medium (1) and raw manure (2) with varying COD/SO_4^{2-} ratio.

manure utilize first of all those compounds that are the simplest and easiest to assimilate. The studied cultures in our experiments were maintained under non-sterile conditions and moreover, they were inoculated with a community of bacteria in which accompanying microflora, competing with sulfate reducing bacteria for carbon sources, was always present.

Literature data indicate that the growth and domination of individual groups of microorganisms in such communities depends not only on the carbon source used, but also on the COD/SO_4^{2-} ratio in the medium. The main group competing with SRB are methanogenic archeons. At COD/SO_4^{2-} ratio lower than 1.7 SRB dominate, within the range 1.7–2.7 both groups of organisms co-dominate, and when the ratio exceeds 2.7 methanogenic archeons take over. According to Hao *et al*. (1996) the only group present in the medium at COD/SO_4^{2-} ratio lower than 0.4 are sulfate-reducing bacteria.

The COD/SO_4^{2-} ratio in the medium in the studies presented in this paper was 0.3–4.8. Consequently, in cultures with COD/SO_4^{2-} ratio higher than 1.7, the significant role of methanogenic archeons could be anticipated. The highest COD reduction in cultures in minimal medium or pig manure was observed at the COD/SO_4^{2-} ratios of 0.6 and 1.2, respectively (Fig. 4). The effectiveness of the removal or organic carbon (similarly to the rate of sulfate reduction) dropped with increase of COD/SO_4^{2-} ratio in the medium to values above 1.2. This is understandable because the concentration of sulfates in the medium that could serve as electron acceptors for SRB decreases. Somewhat different results were obtained by Kosińska and Miśkiewicz (1999) for *Desulfovibrio desulfuricans* cultures in raw liquid manure and wastewaters from a yeast production plant. The authors obtained the best results for the removal of organic matter at COD/SO_4^{2-} ratio in the medium of 0.4–0.8. Vossoughi *et al*. (2003) demonstrated that a decrease of COD/SO_4^{2-} ratio (from 16.7 to 6.0) had little positive effect on removal efficiency. This behavior could be attributed to the presence of the numerous sulfate-reducing bacteria, which are able to utilize organic carbon.

Based on the reaction: $2C + SO_4^{2-} + H_2O \rightarrow H_2S + 2HCO_3^-$ Maree and Strydom (1987), Hao *et al*. (1996) calculated that the theoretical COD/ SO_4^{2-} ratio should be 0.67 mg COD/mg SO_4^{2-}. Stoichometrically, 1200 mg COD is required for the reduction of 1800 mg sulfate. However, this coefficient is usually higher and depending on the carbon source and type of culture, it ranged from 0.7 to 1.5. In the studies presented in this paper the value of the coefficient of oxidized carbon to reduced sulfates in minimal medium was from 0.52 to 1.8, whereas in pig manure it was higher, from 0.82 to 2.0 (Fig. 5). The high value of the discussed coefficient explicitly indicates the utilization of the organic compounds present in the medium by accompanying microflora that occurs in the pig manure in large numbers.

In the studied cultures on mineral medium and three-fold diluted raw pig manure an increase in COD/SO_4^{2-} ratio in the medium was observed to be accompanied by increased utilization of sulfates (Fig. 6). The reduction of sulfates in cultures set up in raw liquid manure with COD/SO_4^{2-} of 0.3 was 12% and with COD/SO_4^{2-} of 3.8 was as high as 97%. Similar results were obtained by Kosińska and Miśkiewicz (2000; 2005) for *Desulfovibrio desulfuricans* grown in medium with pig manure. The authors observed, however, a somewhat higher percentage of sulfate reduction (approx. 46%) at low COD/SO_4^{2-} ratio in the medium. It should be pointed out, however, that even with almost complete reduction of sulfates (*e.g.* 97% for pig manure and 99% in minimal medium) COD reduction never exceeded 55% (Fig. 6).

Based on the data obtained in this study it can be concluded that the effectiveness of the removal of sulfates and organic compounds by a mixed community

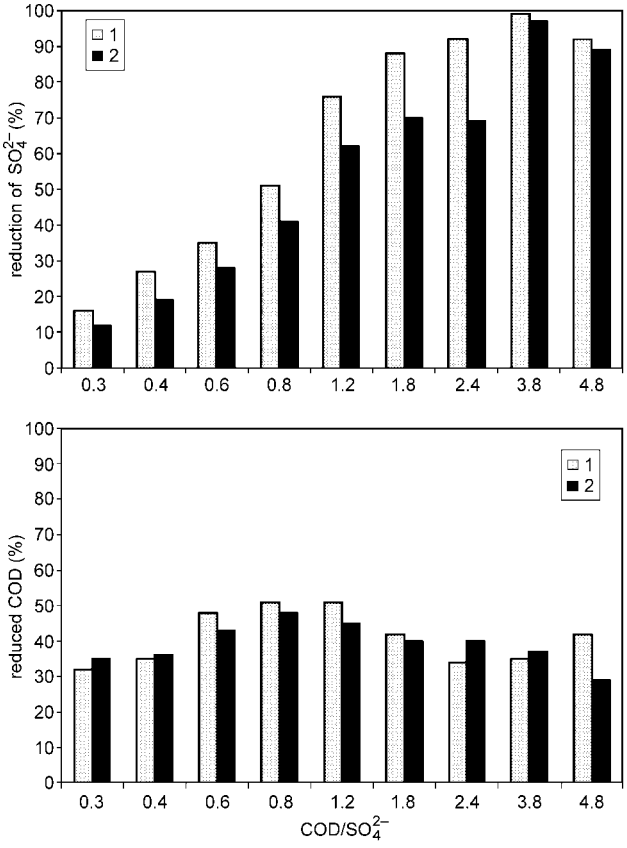

Fig. 6. Reduction of SO_4^{2-} (%) and COD reduction (%) in cultures of a community of sulfate-reducing bacteria in minimal medium (1) and raw manure (2) with varying COD/SO_4^{2-} ratio.

of sulfate reducing bacteria depends to a significant degree on the proportion between organic matter and sulfates in the purified wastes. The COD/SO_4^{2-} ratio that is optimal for the removal of organic matter differs, however, from that being optimal for the removal of sulfates and is 0.8–1.2 and 2.4–4.8, respectively.

The studies presented here, conducted using synthetic medium and raw liquid manure, are consistent with earlier observations regarding the possibility of using sulfate reducing bacteria for simultaneously removing sulfate ions and organic matter from varied wastewaters. Wastewaters are rarely characterized by adequate ratio of organic matter content to that of sulfates from the viewpoint of effective course of purification. Consequently, besides appropriate pH, salinity, temperature or C:N ratio, also the COD/SO_4^{2-} ratio is an indicator of crucial importance. However, our results are in strong support of the future potential of employing SRB for the removal of hazardous pig manure and phosphogypsum in a single process.

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ORIGINAL PAPER

Peroxidase Activity in the Sulfate-Reducing Bacterium *Desulfotomaculum acetoxidans* DSM 771

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A b s t r a c t

Earlier research demonstrated the secretion of benzoate, which must be oxygenated to its 4-hydroxy derivative in order to be included in further sulfate uptake processes. The present study on *Desulfotomaculum acetoxidans* DSM 771 was designed to determine the activity and catalytic specificity of the enzyme (most probably peroxidase) catalyzing the hydroxylation of secreted benzoate. Peroxidase activity measured with ABTS (2,2'-azino-bis (3-ethylbenzathiazoline-6-sulfonic acid) during cultivation indicated the greatest activity on the third and thirteen days (3.4 and 2.3 nkat per ml sample respectively). The highest (0.7979) correlation coefficient was calculated between peroxidase activity and hydrogen peroxide levels. The cell walls from 3- and 13-day cultures were subjected to an isolation procedure, PIPES (piperazine-N,N'-bis (2-ethane-sulfonic acid) extract followed by preparative electrophoresis. The extracts of a ~30 kDa band on the gel were analyzed by Western blotting and the membrane was stained with TMB (3,3',5,5'-tetramethylbenzidine-specific for the presence of peroxidase). This same protein was incubated for 6 h with benzoate, H_2O_2, Na_2SO_4. The product formed a complex with Fe^{3+}, whose maximum absorption spectra (501.7 nm) corresponded with a ferric complex of synthetic 4-hydroxy-3-sulfo-benzoate. The H_2S level during the cultivation was higher in culture grown with 15.5 mM 4-hydroxy-3-sulfo-benzoate than in culture with lactate supplemented with 15.5 mM sulfate. The role of peroxidase in oxygen utilization and sulfate uptake is discussed.

K e y w o r d s: *Desulfotomaculum acetoxidans*, specificity and activity of peroxidase

Introduction

Sulfate-reducing bacteria (SRB) are capable not only of sulfur assimilation but also of the dissimilative reduction of sulfate or sulfur. Consequently, the mode of sulfate fixation and transport processes is very important for their metabolism. On the other hand, it is very interesting and essential for environmental biotechnology, that sulfate-reducing bacteria are capable of the biodegradation and catabolic utilization of monoaryls under anaerobic conditions (Schink *et al.*, 1992; Rabus and Widdel, 1995; Noh *et al.*, 2003). Kuever *et al.* (1993) found very rapid growth of an isolated SRB strain, especially on benzoate and 4-hydroxy-benzoate. However, it is still not clear in what way those obligatory anaerobes can oxidize benzoate, which is required for such biodegradation (Schink *et al.*, 1992).

Admittedly, among the proteins that were more abundant under oxygen exposure of *Desulfovibrio vulgaris* Hildenborough, thiol-peroxidase was identified but its function was not established (Fournier *et al*., 2006). Moreover, the authors suggested the cytoplasmic location of this protein.

The finding of benzoate secretion by *Desulfotomaculum acetoxidans* should change opinion regarding the metabolic role of this aryl in cell walls (Pawłowska-Ćwięk and Pado, 2005). The authors proposed the following sequence of sulfate uptake processes occurring, especially during lag phase: 1. Secretion of benzoate; 2. Oxidation of the ring in position 4; 3. Sulfonation of 4-hydroxybenzoate.

Since the second step requires enzymatic participation, because the product of the spontaneous benzoate oxydation is 3-hydroxy-benzoate, we pursued further research on this putative cell wall enzyme, which is described herein.

Experimental

Materials and Methods

Growth of organism. *Desulfotomaculum acetoxidans* strain DSM 771 was cultivated at room temperature (about 20°C) in a medium with 42 mM sodium lactate as a catabolic substrate (Pawłowska-Ćwięk and Pado, 2007). Other medium components were as

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previously (Pado and Pawłowska-Ćwięk, 2004; Pawłowska-Ćwięk and Pado, 2005). After autoclaving and inoculation, the media were immediately covered with a liquid paraffin layer (about 5 mm thick). Moreover, the independent stationary cultures of this strain were carried out under anoxic conditions in 15.5 mM synthetic 4-hydroxy-3-sulfo-benzoate or 31.0 mM lactate plus 15.5 mM sulfate-containing media. The synthesis of 4-hydroxy-3-sulfo-benzoate was obtained by sulfonation of 4-hydroxy-benzoate and purified by HPLC and a detailed chemical analysis of this compound will be carried out separately.

Measurements during cultivation. Peroxidase activity was determined spectrophotometrically by using 2,5 mM ABTS (2,2'-azino-bis (3-ethylbenzathiazoline-6-sulfonic acid) (Szutowicz *et al.*, 1984) in 10 min aerated samples. The samples were aerated, because of content of reducers, *e.g.* reduced glutatione (GSH) and sulfide (Bartosz and Bartosz, 1999) which inactivated the colored positive radical of ABTS. Monitoring of the change in absorbance at 419 nm was performed from 15 min to 4 h. Activity (in nkatales) was calculated assuming $E_{ABTS} \approx 24600$ (Luterek *et al.*, 1997) and recalculated for mg proteins. Because most peroxidases require hydrogen peroxide and frequently oxygen for their activity, H_2O_2 and O_2 levels were measured in these cultures as well. The hydrogen peroxide level was determined according to Pick (1986). The oxygen concentration was measured by potentiometric method by using CTN-980R oxygen detector (ELSENT Poland) coupled to a CX-315 microcomputer pH/oxygenmeter (ELMETRON Poland); the assay method was described previously (Pawłowska-Ćwięk and Pado, 2007). Moreover, in additional cultures with 4-hydroxy-3-sulfo-benzoate or lactate plus sulfate, the concentration of hydrogen sulfide was measured using the methylene blue method (Fago and Popowsky, 1949). The results of triplicate assays are presented as arithmetic mean for oxygen and hydrogen sulfide. The correlation coefficients were calculated using Excel program commonly.

Preparation of cell wall extract. After 3 days of cultivation, 1 l culture was aerated (O_2 total amount was approximately 450 mmoles) for 12 h and then centrifuged at 2,500×*g* for 25 min. The cell biomass pallet was retained while the supernatant was evaporated to about 50 ml at 8°C. The shaken suspension was centrifuged at 10,000×*g* for 15 min and a lysozyme solution (10^5 U/1 ml 10 mM Tris buffer, pH 6.9) was added to the pellet, to degrade peptidoglycan. The suspension was shaken and incubated for 48 h and re-centrifuged. The pellets were than extracted with 2 mM PIPES (piperazine-N,N'-bis (2-ethane-sulfonic acid) buffer (pH 5.5) for 24 h. This operation was repeated three times and the extracts were pooled. An identical procedure was carried out for a 13-day culture. On the other hand, the pellet from the 10-day culture (which was not aerated) after the above procedure was extracted with phosphate buffer (pH 7.6) for the presence of catalase.

Purification of peroxidase. The crude PIPES extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue G-250. Since it was assumed that this peroxidase too (like almost all of those known so far) is a hemoprotein, in order to locate it on the gel, staining with 3,3'-diaminobenzidine (McDonnel and Staehelin, 1981) and 3,3'-dimetoxybenzidine (Francis and Becker, 1984) was employed. The final PIPES extract was purification by non-denaturing preparative polyacrylamide gel (15%) electrophoresis (PAGE). The ~30 kDa band containing hemoprotein, eluting at 10 mM Tris-HCl (pH 6.1), was collected. The peroxidase activity in final extract was determined and calculated as above.

Activity staining. The final Tris-HCl extracts from 3- and 13-days cultures containing 75 mg proteins after Western blotting onto PVDF – Immobilon^{TM}-P^{SQ} membrane were stained with TMB (3,3',5,5'-tetramethylbenzidine) in accordance with SIGMA instruction.

Testing catalytic specificity of peroxidase. The final Tris-HCl extract (2 ml) of 3-day culture was incubated with equal volumes of 5 mM benzoate, 400 mM hydrogen peroxide and 80 mM sodium sulfate for 6 h at room temperature. The product of this incubation formed a colored complex with 207 mM $Fe(NH_4)(SO_4)_2$ in 1 M HCl; this complex was analyzed spectrophotometrically.

Test of catalase presence. The collected phosphate extract from 10-day culture was analyzed by non-denaturing electrophoresis and gel staining according to Woodbury *et al.* (1971).

Spectrophotometric analysis. The enzymatic assays and spectral analyses were performed using a CECIL 8020 spectrophotometer (bandwidth 2 nm and path length 10 mm).

The chemicals and reagents (except for 4-hydroxy-3-sulfo-benzoate) were obtained from Merck, Fluka or Sigma Chemical Companies.

Results

The obtained results showed highest peroxidase activity on days 3 and 13 (3.423 and 2.338 nkatales per ml sample, respectively) (Fig. 1). The calculated correlation coefficient between peroxidase activity and hydrogen peroxide or oxygen level was –0.0516 and –0.10579, respectively, for the entire cultivation period (Table I). However, if the assumption is made that hydrogen peroxide induces peroxidase then in the calculation hydrogen peroxide level should precede

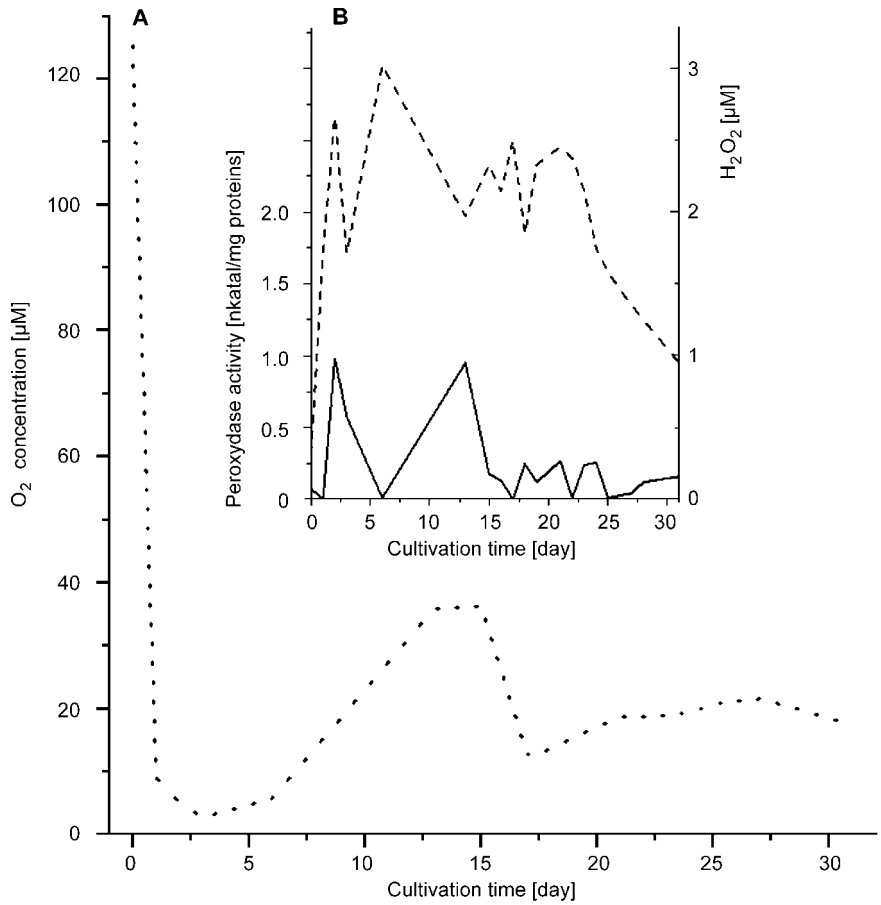

Fig. 1. Oxygen concentration (**A** – dotted line), peroxidase activity (**B** – solid line) and hydrogen peroxide level (**B** – dashed line) during cultivation of *Desulfotomaculum acetoxidans* in lactate plus sulfate containing medium.

peroxidase activity by one day. Thus the following correlation coefficients were calculated: 0.55406 – if hydrogen peroxide level from 2 to 30 day and peroxidase activity from 3 to 31 day and –0.236 for peroxidase activity *vs* oxygen level. The highest coefficient 0.79794 was obtained when the values of peroxidase activity from days 3–17 were assigned to the values for hydrogen peroxide concentration from days 2–16. While, negative correlation coefficients peroxidase activity *vs* oxygen level (–0.253 or –0.285) were obtained when peroxidase activity values from 2 to 31 day were assigned of oxygen level values from start to 29 day or peroxidase activity from days 2–19 and oxygen level from days start to 17, respectively.

Table I
Correlation coefficients.

Cultivation period $t_{start\ day} - t_{last\ day}$	Peroxidase activity (P.a.) *vs* H_2O_2 level	Peroxidase activity (P.a.) *vs* O_2 level	H_2O_2 level *vs* O_2 level
Both factors $t_0 - t_{31}$	–0.05162	–0.10579	–0.16188
Both factors $t_2 - t_{31}$	–0.12478		
P.a. $t_3 - t_{31}$ and H_2O_2 $t_2 - t_{30}$	0.55406		
P.a. $t_3 - t_{24}$ and H_2O_2 $t_2 - t_{23}$	0.64652		
P.a. $t_3 - t_{17}$ and H_2O_2 $t_2 - t_{16}$	0.79794		
P.a. $t_1 - t_{31}$ and O_2 $t_0 - t_{30}$		–0.236	
P.a. $t_2 - t_{31}$ and O_2 $t_0 - t_{29}$		–0.25282	
P.a. $t_2 - t_{24}$ and O_2 $t_0 - t_{22}$		–0.28475	
Both factors $t_1 - t_{31}$			–0.18063
H_2O_2 $t_1 - t_{31}$ and O_2 $t_0 - t_{30}$			–0.18587
Both factors $t_1 - t_{23}$			–0.7937

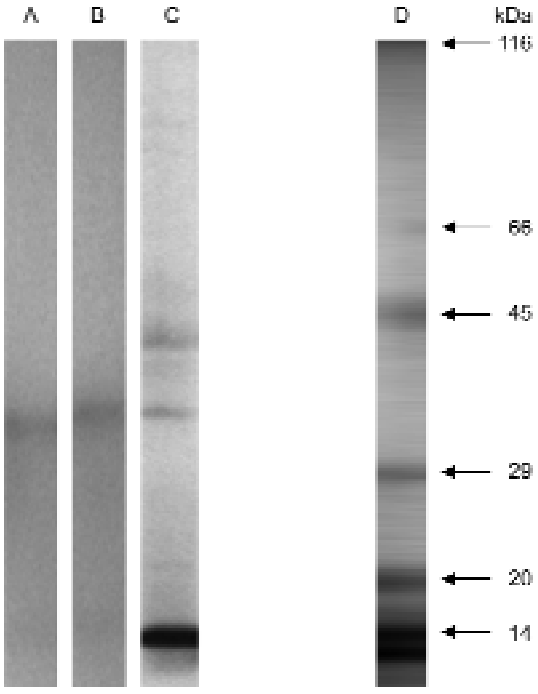

Fig. 2. PAGE-SDS gels (15%) stained: A – 3,3'-diaminobenzidine; B – 3,3'-dimetoxybenzidine; C – Coomassie blue G-250; D – molecular weight markers.

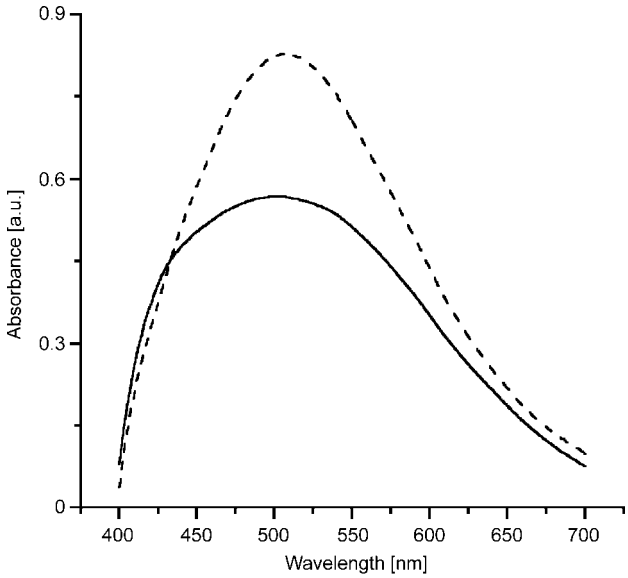

Fig. 4. Absorption spectra of ferrous complexes: TRIS extract from preparative gel (band ~30 kDa – peroxidase) after incubation with benzoate, hydrogen peroxide and sodium sulfate (solid line) (l_{max} = 501.7 nm) from 3-day culture of *D. acetoxidans* and synthetic 4-hydrohy3-sulfo-benzoate (dotted line) ($_{max}$=503,8 nm).

Based on the assumption that peroxidase is a hemoprotein, it was located on the gel by using 3,3'-dimetoxybenzidine. The molecular mass of this hemoprotein (~30 kDa) was determined by SDS-PAGE and compared with standard proteins (Fig. 2). After Western blotting this purified hemoprotein was transferred onto a membrane which was stained with TMB. The obtained result showed a blue spot characteristic for peroxidase in both the day 3 and day 13 cultures (Fig. 3). The peroxidase activity was 51.3 ncatales per ml sample.

The product of the incubation (purified peroxidase with benzoate, hydrogen peroxide and sodium sulfate) formed a colored complex with iron present in $Fe(NH_4)(SO_4)_2$ solution. The maximum absorption at 501.7 nm of this complex was almost identical with the ferrous complex (503.8 nm) of synthetic 4-hydroxy-3-sulfo-benzoate (Fig. 4).

Monitoring measurements showed an increase of oxygen level in the second week of the cultivation (Fig. 1).

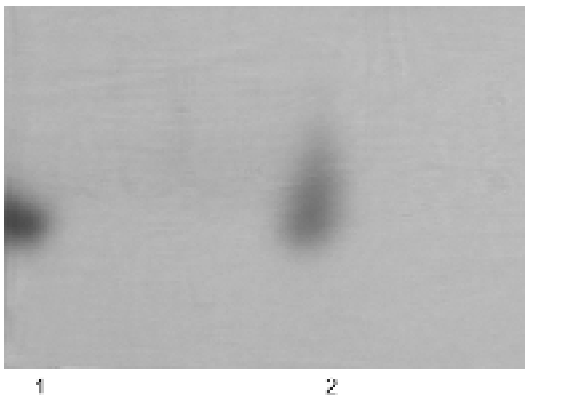

Fig. 3. Purified peroxydase from aerated 3-day **(1)** and 13-day **(2)** cultures of *Desulfotomaculum acetoxidans* after Western blotting onto PVDF – Immobilon^{TM}-P^{SQ} membrane stained 3,3',5,5'-tetramethylbenzidine (TMB) in accordance with SIGMA instruction.

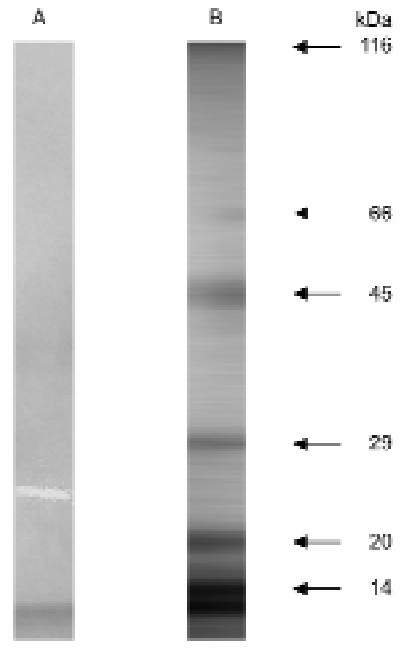

Fig. 5. Gel after non-denaturing electrophoresis and staining the mixture ferric chloride and potassium ferrocyanide (A); B – molecular weight markers.

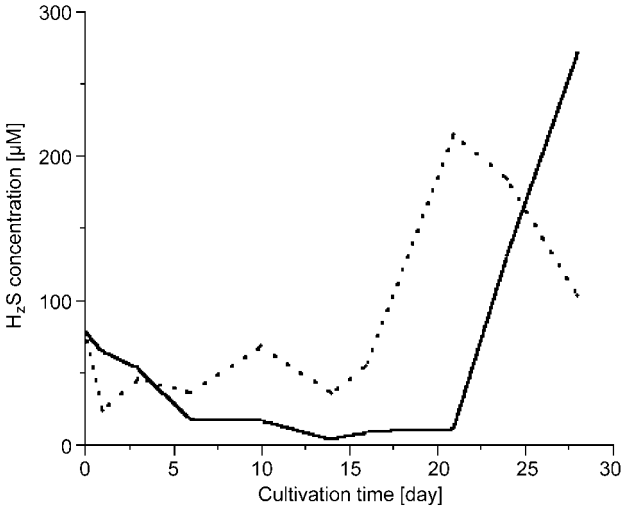

Fig. 6. Hydrogen sulfide level during cultivation of *D. acetoxidans* with 15.5 mM sulfate (plus 31 mM lactate) (solid line) or 15.5 mM synthetic 3-sulfo-4-hydroxybenzoate (dotted line).

One of the causes could be catalase activity so an attempt was made to prove this by non-denaturing electrophoresis and staining the gel with ferric chloride and potassium ferrocyanide.

The yellow band on the gel (~25 kDa) indicated of catalase activity in phosphate extract from the cell wall of cells from a 10-day culture (Fig. 5).

Additional confirmation of the essential role played by peroxidase in sulfate fixation and transportation into the cytosol was provided by cultures of the studied strain grown in a medium containing synthetic 4-hydroxy-3-sulfo-benzoate or sulfate of the same concentration, besides lactate at the proper molar ratio. Sulfate uptake processes greatly affect both sulfate reduction pathways, but it is easier to ascertain this for the dissimilatory one, by determining its end product-hydrogen sulfide. The results prove that between early third day of cultivation up to the 24 day the H_2S level was greater in the culture containing synthetic 4-hydroxy-3-sulfo-benzoate than in the sulfate containing one (Fig. 6).

Discussion

Until now, bacteria of the genus *Desulfotomaculum*, like other sulfate-reducing bacteria (SRB), were classified among the so-called strictly anaerobic organisms, However, it is a well-known fact that they can survive exposure to oxygen (Fuseler *et al.*, 1996; Johnson *et al.*, 1997; Eschemann *et al.*, 1999; Brune *et al.*, 2000). Long-term experiments carried out in a laboratory system mimicking proved that oxygen did not exhibit any toxic effect on SRB and did not cause a cessation in their sulfide production (Gutierrez *et al.*, 2008). Taking into consideration that wastewater and oxygen injection was loaded in cycles (up to 16 on a daily), this system acted permanently in the initial stages.

In other SRB, especially the genus *Desulfovibrio* the antioxidant enzymes: neelaredoxin (Silva *et al.*, 1999), superoxide dismutase (SOD) and catalase (Santos *et al.*, 2000; Brioukhanov and Netrusov, 2004) were found. Also in the examined strain, the presence of neelaredoxin was determined (Pawłowska-Ćwięk, 2006). Moreover, the strain also revealed antioxidative factors, such as GSH (Pawłowska-Ćwięk and Pado, 2007). However, benzoate and 4-hydroxybenzoate could be found as well (Pawłowska-Ćwięk and Pado, 2005; Pawłowska-Ćwięk, 2006). Nevertheless, the participation of peroxidase is essential to transform benzoate into 4-hydroxy derivative. A supposition on existence of the enzyme suggested that benzoate secretion should have a physiological basis, even regarding the high cost of its biosynthesis (~−200 kJ/mol benzoate). An essential reason to secrete the monoaryl might be its antioxidative function, especially at the initial stage of growth, but other factors cannot be excluded.

Gutierrez *et al.* (2008) showed the detoxification of oxygen injected after 2–4 h yet and more interestingly, this injection did not cause a cessation in sulfide production by SRB in the biofilm. The present results indicate that most of the oxygen dissolved in media is utilized during the initial period of cultivation, which involves an increase in H_2O_2 level (Fig. 1), and is reflected by negative values of the correlation coefficient between oxygen and hydrogen peroxide, also during later period of cultivation (Table I). In SRB, H_2O_2 production can result from the neutralization of oxygen anion-radical (*e.g.* occurring by molecular oxygen) catalyzed by low-molecular (~14 kDa) neelaredoxin and SOD (Silva *et al.*, 1999; Santos *et al.*, 2000; Brioukhanov and Netrusov, 2004). However, SOD and catalase are cytoplasmic or periplasmic proteins while neelaredoxin is an outer membrane protein.

The present results prove that peroxidase occurs in the studied strain, its highest activity falling on days 3 and 13 of cultivation (compare Fig. 1B and Fig. 3). The calculated correlation coefficients indicate that the enzyme activity first of all depends on the concentration of hydrogen peroxide (Table I). It should, however, be pointed out that high values of the coefficient were obtained when the values of H_2O_2 from a specific period of the culture were correlated with the values of peroxidase activity from a period shifted one day later. This points to the inductive function of hydrogen peroxide and thus explains high peroxidase activity on the third day of cultivation.

A product of incubation of purified peroxidase, benzoate, hydrogen peroxide and sodium sulfate

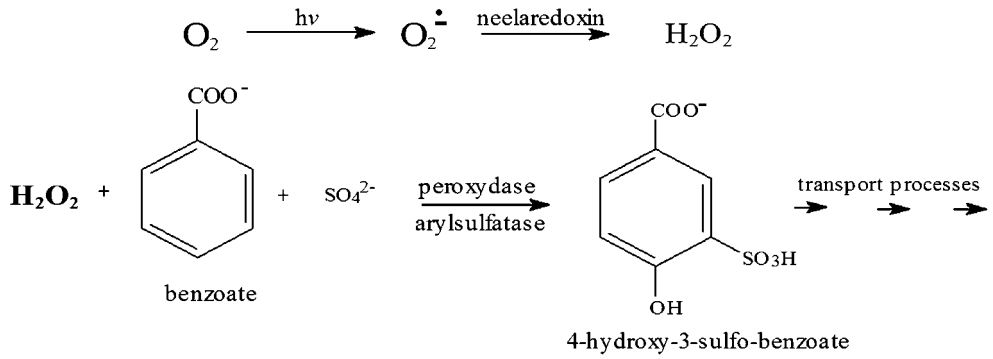

Fig. 7. Proposed sequence reactions in cell wall during initial period of *D. acetoxidans* cultivation.

formed complexes with Fe^{3+} that displayed absorption maximum at 501.7 nm, which is almost identical with the maximum of an analogous complex formed by synthetic 4-hydroxy-3-sulfo-benzoate (Fig. 4). This is proof that the suggestion of an essential role played by peroxidase is correct, especially in sulfate transport into cytosol as a result of sulfonation of its activity product (Pawłowska-Ćwięk and Pado, 2005). The higher concentration of dissimilated hydrogen sulfide found between days 3 and 24 of cultivation in 4-hydroxy-3-sulfo-benzoate culture than in lactate/sulfate ones proves that sulfonated 4-hydroxy-benzoate is the proper substrate in the dissimilatory sulfate reduction pathway (Fig. 6). Therefore, the process with the participation of peroxidase is not only an antioxidant defense, but also it affords possibilities of the efficient course of sulfate transport into cytosol in the studied strain (and probably in other SRB). To summarize, the following sequence of reactions in the initial stages of the culture can be suggested (Fig. 7).

Nevertheless, the question rises why peroxidase activity increases on day 13 of cultivation. A certain explanation of the phenomenon might be the occurrence of catalase in the 10 day culture – which produces O_2, and that causes an oxygen level increase in the second week of cultivation (compare Fig. 1 and Fig 5). However, this raises another question – where does hydrogen peroxide, the catalase substrate, come from. H_2O_2 production by neelaredoxin and SOD is unlikely at low $O^{\bullet-}_2$ level. Therefore, answering this question needs further research.

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ORIGINAL PAPER

Probiotic Properties of Yeasts Isolated from Chicken Feces and Kefirs

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A b s t r a c t

The probiotic potential of 3 yeasts strains of *Saccharomyces cerevisiae* isolated from kefirs and feces was investigated and compared with 3 isolates from medicines and 2 collection strains (ATCC) of *Saccharomyces cerevisiae* var. *boulardii*. Genetic identification of yeasts based on karyotypes indicated their affiliation to *Saccharomyces* spp. although chromosomal polymorphism was observed. Concerning probiotic characteristics survival in simulated gastric and intestinal environment were examined. The survival of all tested yeasts in medium of pH 2.5 was comparable and equaled 86.8–97.1% after 8 hours of incubation at 37°C. The fecal isolate, probiotic and collection yeasts showed also high resistance to pH 1.5 and their survival was 85.3–92.1%, whereas for kefir strains it amounted to 33.1 and 38.9%. All yeasts tested demonstrated high resistance to synthetic bile salts as well. In the presence of 0.1% sodium cholate and sodium deoxycholate the reduction of cell number by only 1 log unit after 4 hours of incubation at 37°C was observed. However, 1.0% addition of ox bile did not affect their viability. In simulated gastric and intestinal environment survival of fecal, probiotic and collection strains was 86.3–93.7% after 4 hours of incubation in media with addition of 3 g/l pepsin and 1 g/l pancreatin. Kefir isolates were more sensitive to these conditions and a further 10% reduction of cell number in relation to probiotic yeasts was observed. The tested strains, except for kefir isolates, were able to grow at 37°C. All the tested strains survived in sufficient number to create the possibility of proper action in the human body, although fecal, probiotic and collection strains tolerated the conditions of the human gastrointestinal tract better than food-borne yeasts.

K e y w o r d s: karyotype, probiotic yeasts, survival in human gastrointestinal tract

Introduction

Probiotic yeasts are non-pathogenic strains belonging to the species *Saccharomyces cerevisiae* var. *boulardii* (Ouwehand *et al*., 2002). Probiotic cultures have been used as both a preventive and therapeutic agent for the treatment of a variety of diarrheal diseases. *S. cerevisiae* var. *boulardii* is reported to be effective in the treatment for diarrhea in adults and children infected with *Clostridium difficile*, for diarrhea in human immunodeficiency virus-infected patients and for acute and chronic diarrhea in children and adults (McFarland and Bernasconi, 1993; Ouwehand *et al*., 2002). Potential mechanisms of their probiotic activity are based on secretion of proteases or inhibitory proteins, stimulation of immunoglobulin A, acquisition and elimination of secreted toxins (Fooks and Gibson, 2002; McFarland and Bernasconi, 1993).

A set of selection criteria considered to be relevant for any probiotic microorganism has been proposed by Ouwehand *et al*. (1999). Tolerance to low pH and bile salts is seen as a prerequisite for strain survival through the gastrointestinal tract. Probiotics must be also able to resist to certain local stresses such as the presence of gastrointestinal enzymes, organic acids and temperature 37°C (Conway, 1996; Ouwehand *et al*., 1999).

The taxonomic characterization of *Saccharomyces cerevisiae* var. *boulardii* is still controversial. On the basis of molecular typing techniques this group of yeasts was initially identified as a separate species of the genus *Saccharomyces i.e. Saccharomyces boulardii* (McFarland, 1996). However, the development of molecular phylogenetics has led to changes in the classification of many yeast species (Liti *et al*., 2006; Vaughan-Martini, 2003). Using comparative genomic hybridizations for whole-genome analysis *S. boulardii* and *S. cerevisiae* were reported as members of the same species (Edwards-Ingram *et al*., 2004). In this context, the question appears if other *S. cerevisiae* strains demonstrate probiotic features as well.

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In the present study, yeasts isolated from fermented milk products and chicken feces have been examined to evaluate their potentially probiotic properties. They have been classified taxonomically and compared to other *Saccharomyces* spp. strains of evidenced probiotic activities.

Experimental

Materials and Methods

Yeast strains. Two strains isolated from two different kefirs (S11, S12) and one strain isolated from chicken faces (S10) were examined and compared with three probiotic strains isolated from medicines: Enterol®250 Biocodex, Hamadin®N Dr. Willmar Schwabe, Omniflora®Akut Novartis and *Saccharomyces cerevisiae* var. *boulardii* MYA-796, MYA-797 originated from the American Type Culture Collection. Yeasts were kept on YPD (1% yeast extract, 2% peptone, 2% dextrose) agar slants and maintained at –20°C in YPD broth containing 20% (v/v) glycerol.

Analysis of chromosomal DNA. Chromosomes of yeast strains were isolated and separated by pulsed-field gel electrophoresis PFGE. The typical conditions for preparation of chromosomal DNA from the *Saccharomyces* yeasts were followed (Schwarz and Cantor, 1984). Yeast chromosomes were separated with a CHEF-DR II apparatus (Bio-Rad) in TBE buffer (Sigma) at 10°C and an interpolation of pulsed time of 110–120 s for 26 hours. The gel was stained in ethidium bromide for visualization of nuclear DNA. A standard set of *S. cerevisiae* YNN 295 chromosomes was obtained commercially (Bio-Rad) and used for comparison.

Tolerance to low pH. Cultures activated by two transfers in liquid YPD were centrifuged (3000 × g, 10 min, 4°C) and cells were resuspended in sterile phosphate buffered saline PBS (0.8% sodium chloride, 0.02% potassium chloride, 0.144% disodium phosphate, 0.024% potassium phosphate, pH 7.4). Low pH tolerance was estimated by inoculating (10^6 cfu/ml) activated cultivates into PBS with pH adjusted at 1.5 and 2.5 with 3N HCl. The samples were incubated at 37°C and the number of viable cells was determined by the count plate method after 1, 2, 3, 4, 6 and 8 hours of incubation. The results are given as the mean value of three replicates and expressed as percentage log survival, calculated according to Williamson and Johnson (1981):

Percentage log survival = (log N / log N_0) × 100

where N – count (cfu/ml) after incubation, N_0 – count at time 0 (cfu/ml).

Bile tolerance. Cultures activated as described above were inoculated into liquid YPD medium supplemented with 0.1% and 1.0% mixture of synthetic bile salts (50% sodium cholate and 50% sodium deoxycholate). Bile tolerance was also evaluated in liquid YPD medium with addition of 0.1% and 1.0% ox gall. The number of yeast cells was estimated by the count plate method after 20, 40, 60, 120, 180 and 240 minutes of incubation at 37°C. The results are given as the mean value of three replicates and presented as log cfu/ml. Results are presented in graphic form.

***In vitro* survival in gastric and intestinal environment.** Activated cells were harvested by centrifugation at 3000 × g for 10 min and inoculated at the level 10^6 cfu/ml in a simulated gastric environment aqueous solution containing 3 g/l pepsin (3260 U/mg) and 5 g/l NaCl, pH 2.0 (Charteris *et al*., 1998). Yeast cultures were also inoculated into medium reproducing human intestine conditions, constituted by an aqueous solution containing 1 g/l pancreatin (903 U/mg) and 5 g/l NaCl, pH 8.0. The cell viability was determined by the count plate method after 20, 40, 60, 120, 180 and 240 minutes of incubation at 37°C. The results are given as the mean value of three replicates and presented as percentage log survival (Williamson and Johnson, 1981).

Growth at 28 and 37°C. Growth at different temperatures was estimated by inoculating (10^6 cfu/ml) activated cultures into YPD liquid medium. The samples were incubated at 28 and 37°C for 48 hours and the number of cells was determined by the count plate method. In order to compare growth curves the Gompertz equation in conjuction with DMFit program was used (Baranyi and Roberts, 1994). The Gompertz parameter values (A, B, C, M) were used to calculate exponential growth rates EGRs, generation times GTs, lag phase durations LPDs as described by Zaika *et al.* (1998).

Statistical analysis. Results were analysed using 3-way ANOVA at the confidence level of $p<0.05$. Results of the population viability were presented as the arithmetic mean of three assays with standard deviation not exceeding 0.2 logarithmic units.

Results

Fecal and kefir isolates were identified as *Saccharomyces cerevisiae* according to their morphological characteristics, ability to assimilate carbon and nitrogen compounds and sugar fermentation patterns (data not shown). On the basis of karyotypes the tested strains were classified to the genus *Saccharomyces*, since they show 11–12 bands of a size varying between 200 and 2.300 kb (Fig. 1). There were differences in both the number and length of bands in electrophoregram of yeast S10–S12 chromosomal DNA. The fecal isolate S10 and one of the kefir strain S11 showed

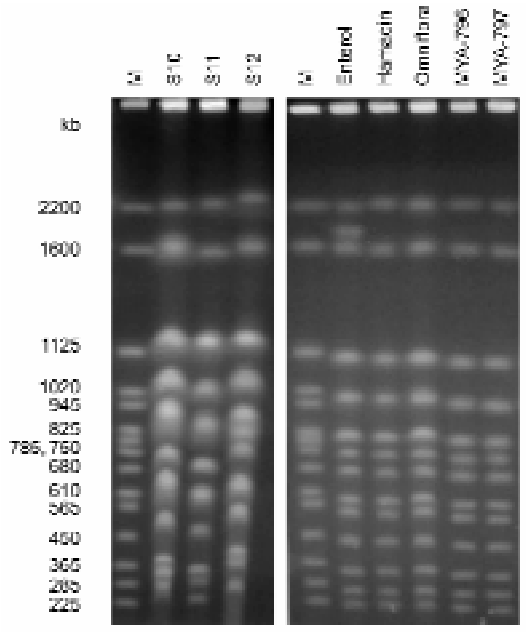

Fig. 1. Electrophoretic patterns of yeast chromosomal DNA
M – marker *S. cerevisiae* YNN295 (Bio-Rad)

11 bands whereas the other strain derived from kefir S12 had 12 chromosomes. The electrophoretic patterns confirmed that probiotic and collection yeasts belong to the genus *Saccharomyces*. The chromosomal patterns of four *S. cerevisiae* var. *boulardii* strains (Hamadin®, Omniflora®, MYA-796, MYA-797) did not show polymorphism and consisted of 13 bands sized 222–2251 kb (Fig. 1). Only the karyotype of the probiotic strain isolated from Enterol® was distinctive with additional chromosome 1884 kb.

The yeasts were characterized by high adaptation to conditions of the human gastrointestinal tract. The survival of all tested strains in medium of pH 2.5 was comparable and equaled 86.8–97.1% after 8 hours of incubation at 37°C (Table I). Decrease in pH to 1.5 led to further reduction in cell viability and the survival was 85.3–92.1% in the case of fecal, probiotic and collection strains. The kefir isolates showed higher sensitivity to pH 1.5 and their survival was 33.1 and 38.9% after 8 hours of incubation.

Fecal and kefir isolates as well as probiotic and collection strains demonstrated high resistance to bile salts. The addition of 0.1 and 1.0% ox bile did not restrict viability of microorganisms (Fig. 2). In medium with 1.0% synthetic bile salts reduction of cell numbers by 1 log unit after four hours of incubation was observed for the fecal isolate S10, probiotic and collection yeasts. The least bile salts tolerant were kefir

Table I
Percentage survival of yeasts at low pH

Strain	pH 2.5						pH 1.5					
	Incubation time [h]											
	1	2	3	4	6	8	1	2	3	4	6	8
S10	99.6	99.1	98.3	98.0	97.4	97.1	98.8	96.1	95.8	94.6	93.3	92.1
S11	99.5	98.8	97.3	95.3	90.8	86.8	85.9	70.0	58.8	56.5	47.6	38.9
S12	99.6	97.8	96.6	95.0	91.3	87.7	77.2	64.6	57.5	50.0	41.6	33.1
Enterol®	98.8	98.3	97.4	97.0	96.5	96.3	99.0	97.0	95.2	92.4	87.4	85.3
Hamadin®	99.3	98.5	97.8	97.4	97.2	97.0	99.3	98.3	97.0	94.1	92.1	90.8
Omniflora®	99.8	99.3	98.9	98.4	97.4	95.4	98.5	97.6	96.6	94.9	91.6	87.4
MYA-796	99.4	98.8	98.5	98.2	97.7	96.7	98.5	97.4	95.2	93.0	90.4	87.4
MYA-797	99.7	99.2	98.2	97.8	97.4	97.0	98.6	97.0	95.4	93.7	88.8	87.5

Table II
Percentage survival of yeasts in simulated gastric and intestinal environment

Strain	Gastric juice						Intestine					
	Incubation time [min]											
	20	40	60	120	180	240	20	40	60	120	180	240
S10	99.6	98.9	98.2	93.4	88.2	86.6	99.7	99.4	98.9	97.9	97.0	93.7
S11	99.5	97.9	96.1	88.6	83.5	75.5	98.8	97.7	97.0	93.5	87.1	83.1
S12	98.8	97.9	96.5	89.3	84.0	76.0	98.7	97.9	97.0	92.6	87.4	84.9
Enterol®	99.4	98.9	98.3	94.4	89.6	86.3	99.9	99.7	99.3	97.7	94.7	92.6
Hamadin®	99.7	99.4	99.2	97.6	93.5	88.5	99.9	99.8	99.7	99.1	98.4	97.3
Omniflora®	99.7	99.5	98.8	97.6	95.7	93.0	99.0	98.8	98.7	97.2	96.0	94.1
MYA-796	99.5	99.1	98.7	97.5	95.8	93.2	99.6	99.3	99.1	98.3	97.5	96.4
MYA-797	99.7	99.3	99.1	96.9	93.3	88.4	99.8	99.7	99.5	98.5	96.2	94.8

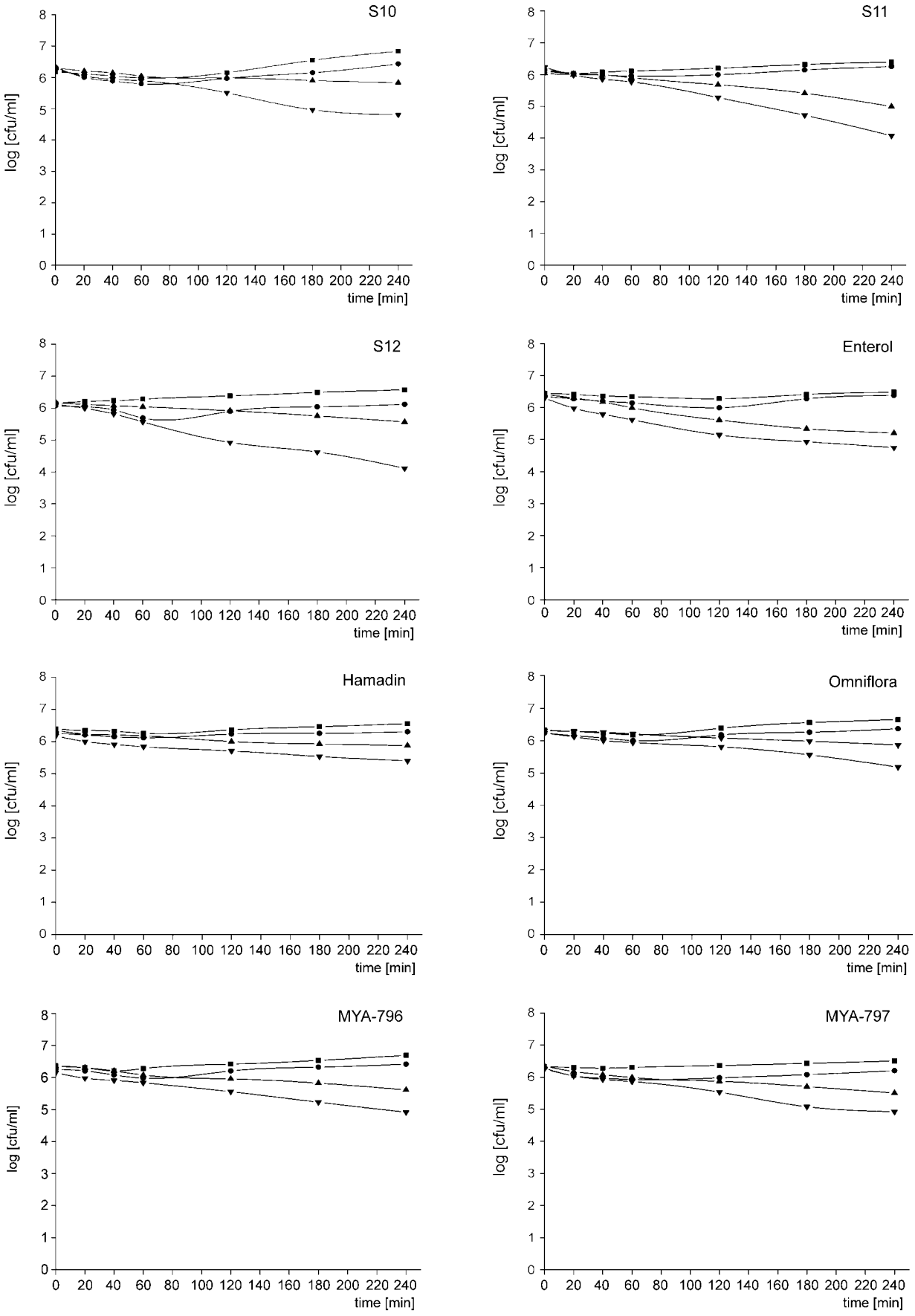

Fig. 2. Viability of yeasts in the presence of ox gall and bile salts.

–■– 0.1% ox gall, – ●–1% ox gall, –▲– 0.1% synthetic bile salts, –▼–1% synthetic bile salts

Table III
Gompertz equation parameters and derived growth kinetics values for yeast at different temperatures

Strain	Temperature [°C]	Gompertz parameters				EGR [(log cfu/ml)/h]	GT [h]	LPD [h]
		A	C	B	M			
S10	28	6.45	7.89	0.0157	10.25	0.194	0.66	3.89
	37	6.51	7.77	0.0592	16.88	0.169	1.78	1.41
S11	28	6.64	7.85	0.0637	19.74	0.184	1.64	4.04
	37	6.68	NG	–	–	–	–	–
S12	28	6.72	7.92	0.0531	22.98	0.154	1.94	4.16
	37	6.65	NG	–	–	–	–	–
Enterol®	28	6.57	7.96	0.0664	18.61	0.194	1.55	3.54
	37	6.61	7.35	0.0339	31.28	0.092	3.28	1.84
Hamadin®	28	6.59	8.20	0.0759	16.69	0.229	1.31	3.53
	37	6.63	7.73	0.0548	21.07	0.156	1.93	2.83
Omniflora®	28	6.59	8.20	0.0897	14.89	0.269	1.12	3.73
	37	6.64	7.73	0.1053	13.17	0.287	1.05	3.68
MYA-796	28	6.53	7.92	0.0817	16.09	0.238	1.27	3.85
	37	6.59	7.82	0.0896	13.30	0.258	1.17	2.13
MYA-797	28	6.44	7.98	0.0736	17.73	0.216	1.40	4.14
	37	6.42	7.47	0.0625	19.94	0.172	1.75	3.95

NG – no growth

yeasts (S11 and S12) but even their populations decreased by only 2 log units in the course of experiment.

In simulated gastric and intestinal environment the survival of all tested yeasts equaled 75.5–93.2% and 83.1–97.3% after 4 hours of incubation in media with pepsin and pancreatin, respectively (Table II). It was found that the fecal isolate S10, probiotic and collection yeasts expressed comparable tolerance to the presence of gastrointestinal enzymes and the differences among strains amounted to less than 7%. Compared to these yeasts the kefir isolates S11 and S12 were more affected by exposure to the enzymes and the low pH. It seems that factor which stronger restricted the viability of yeasts was pH, much lower in medium reproducing conditions in human stomach than in human intestine.

The fecal isolate S10, the probiotic yeasts and the collection strains were able to grow at 37°C. In most cases decrease in the rate of growth and lower yield compared to the growth at optimal temperature 28°C were noted (Table III). Interestingly, except for the probiotic Omniflora® strain, 42–80% shortening of lag phase was observed at 28°C. The kefir isolates S11 and S12 were incapable of growth at 37°C, but nevertheless their survival after 48 hours of incubation at 37°C was 92.5%.

Discussion

On the basis of chromosomal patterns the kefir and fecal isolates were identified as *Saccharomyces* spp. and the classification of probiotic and collection strains to the genus *Saccharomyces* was confirmed. Similar results were obtained for dozens of yeasts belonging to *Saccharomyces* sensu stricto group (Cardinali and Martini, 1994; Naumov *et al*., 2001; Vaughan-Martini *et al*., 1993). However, positive yeast identification at the species level is very difficult because of chromosomal length polymorphism (Fietto *et al*., 2004; Naumov *et al*., 2001; Pataro *et al*., 2000). Vaughan-Martini *et al.* (1993) concluded that none of the species of genus *Saccharomyces* could be distinguished by the consistent presence or absence of a unique band or cluster of bands. Some taxonomic studies (Mitterdorfer *et al*., 2002; Van der Aa Kühle *et al*., 2001) have indicated that *S. boulardii* should be considered as a strain of *S. cerevisiae*. This conclusion was confirmed by analysis of DNA polymorphism (Molnar *et al*., 1995) and reports of 95% DNA homology between *S. cerevisiae* and *S. boulardii* (Vaughan-Martini and Martini, 1987). These findings are reflected in current nomenclature and according to the International Code of Botanical Nomenclature strains *S. boulardii* comprise the subtype of the *S. cerevisiae* species and should be referred as *S. cerevisiae* var. *boulardii*.

Interestingly, in several studies no variations in the genotype of *S. cerevisiae* var. *boulardii* have been observed (Cardinali and Martini, 1994; Hannequin *et al*., 2001; Malgoire *et al*., 2005; McFarland, 1996), which is in disagreement with the results of present work. Recently, Klingberg *et al.* (2008) found that probiotic strains isolated from medicines Levucell®SB and Precosa® had identical profiles but clustered together with the probiotic isolates get from Ultra-Levure® at a similarity of about 90%.

Most definitions of probiotics emphasize that the microorganisms should be viable and reach their site of action alive (Ouwehand *et al*., 1999). The primary barrier in the stomach is the gastric acid of inhibitory action being related to low pH and enzyme presence. All the tested yeasts showed high tolerance to these conditions, however kefir isolates were more sensitive than the probiotic, collection and fecal strains. It seems that the factor which could influence stronger the growth of yeasts was pH. There were significant differences between yeast survival in medium of pH 2.5 and 1.5 for food-borne isolates S11 and S12 (47.9 and 54.6%, respectively). Normal values for human gastric pH are 1–3 fasting and up to 5 after a meal (Cummings *et al*., 2004) and average stomach transit time is 2.5–4 hours (Camilleri *et al*., 1989). We assumed stricter conditions in our investigation and examined yeasts survival during 8 hours of incubation in medium of very low pH. In these harsh conditions sufficient viable cells of all the tested strains could enter the small intestine indicating the possibility of their survival and proper activity in the human intestine.

Besides tolerance to acid conditions, all the tested yeasts demonstrated the ability to withstand 1.0% ox gall. Similar results have been reported previously for *S. cerevisiae* strains isolated from infant feces, feta cheese and beverages (Psomas *et al.*, 2001; Van der Aa Kühle *et al.*, 2005). Bile tolerance is important for allowing a microorganism to survive in the intestinal tract (Gilliland *et al*., 1984).

Growth at 37°C seems to be a variable characteristic of *S. cerevisiae* (Barnett *et al*., 2000). In this study the fecal isolate S10, all probiotic and collection yeasts were able to grow at this temperature, in contrast to two strains isolated from kefirs. Nevertheless, the survival of these strains equaled 97.9% for S11 and 86.9% for S12 after 48 hours of incubation at 37°C.

Generally, isolates from feces, probiotic strains derived from medications and collection strains are able to grow at the temperature of 37°C and tolerate better the low pH, presence of gastrointestinal enzymes and bile salts than kefir yeasts. This finding is consistent with the conclusion of the importance of yeast origins for probiotic properties (Ouwehand *et al*., 1999). However, irrespective of strain origins, all the tested yeasts may survive passage throughout the upper gastrointestinal tract and be viable at their sites of action in the gut environment.

The results of the present study suggest that all tested yeasts may survive in the human gastrointestinal tract and thus create the possibility of proper activity in the human body.

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ORIGINAL PAPER

β-glucuronidase and β-glucosidase Activity of *Lactobacillus* and *Enterococcus* Isolated from Human Feces

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Abstract

The domination of microorganisms characterized by excessive activity of the so-called fecal enzymes may be one of the reasons of the large intestine cancers. These enzymes are mainly those that belong to the hydrolase and reductase classes and their excessive activity may lead to disorders in the functioning of the digestive tract. The aim of tise research was to determine the activity of β-glucuronidase and β-glucosidase of *Lactobacillus* and *Enterococcus* strains isolated from the feces of healthy children, aged 1 and 8, and adults, aged 30 and 80. The analysis included 10 strains isolated from the feces of individuals in each of the age groups. β-glucuronidase activity in the case of the isolates from children, depending on the strain, equaled from about 0.15 mM/h/mg of protein to 0.26 mM/h/mg of protein and was lower, respectively, by 52.35% and 57.81%, than the β-glucosidase activity. Simultaneously, the activity of the *Lactobacillus* enzymes from children was 2.4 times higher, and in case of the isolates obtained from adults they were 4.6 and 2.7 times higher than the activity of the *Enterococcus* enzymes. The highest β-glucuronidase activity was observed in *Lactobacillus* isolates coming from an 80-year-old subject. The differences between the activity of *Enterococcus* β-glucuronidase isolated from the feces of 1 and 8 year old children were statistically insignificant. On the other hand, in the case of the subjects aged 30 and 8 the isolates were characterized by activity lower by, respectively, 48% and 37% than the isolates coming from children. The highest β-glucosidase activity was discovered in the case of *Lactobacillus* and *Enterococcus* coming from children, which was higher by 32% than the activity of the isolates from adult persons. Therefore, it was determined that the activity of β-glucuronidase of *Lactobacillus* strains isolated from feces from people aged 80 was the highest, and the isolates of the examined microorganisms coming from children were characterized by the highest β-glucosidase activity.

Key words: β-glucuronidase, β-glucosidase, intestinal bacteria

Introduction

The large intestine is a complex ecosystem of microorganisms that performs a very important immunological function since it takes part in inhibiting the growth of harmful bacteria and also in processing food components supplied to the organism. The collection of microorganisms of the large intestine is dominated by absolute anaerobes that belong to *Bacteroides*, *Clostridium*, *Ruminococcus*, *Butyrovibrio*, *Fusobacterium*, *Eubacterium*, *Peptostreptococcus* and *Bifidobacterium* (Ouwehand *et al.*, 2002). It also includes bacteria species that may induce disorders in the functioning of the digestive tract, especially when they become the dominant ones (McGarr *et al.*, 2005). Excessive activity of the so-called fecal enzymes, *i.e.* the enzymes of bacteria present in the large intestine, generates many genotoxic, mutagenic and carcinogenic products, or it transforms pro-carcinogenic substances into carcinogenic ones. The enzymatic activity of intestinal microorganisms may therefore induce the formation of digestive tract cancer, including mainly the cancer of the large intestine (Burns *et al.*, 2000). The presence of bacterial strains characterized by high activity of β-glucuronidase (EC 3.2.1.31) or β-glucosidase (EC 3.2.1.21) in the large intestine may be a risk factor leading to the formation of a tumor (De Preter *et al.*, 2008).

β-glucuronidase (Glucuronohydrolase of β-D-glucuronides) hydrolyzes β-D-glucuronides to glucuronic acid and aglycone that may have the form of an alcohol, rest of organic acid, amine, imine or a thiol compound. The formation of glucuronides is catalyzed by UDP-glucuronylotransferase. From the liver, where their synthesis takes place, they are partially removed with bile to the large intestine. There, under

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the influence of bacterial β-glucuronidase, they are subject to hydrolysis to aglycones (Beaud *et al.*, 2005; De Moreno de leBlanc *et al.*, 2005). In patients with diagnosed tumors of the large intestine high activity of β-glucuronidase was observed, which suggests that this enzyme plays an important role in promoting large intestine tumors (Kim *et al.*, 2001). Among the intestinal microflora the highest activity of β-glucuronidase is shown by *Escherichia coli*, *Clostridium paraputrificum*, *Clostridium clostridioforme*, *Clostridium perfringens*, *Bacteroides fragillis*, *Bacteroides vulgatus*, *Bacteroides uniformie*, *Ruminococcus gnavus* and species that belong to the genera *Peptostreptococcus*, *Staphylococcus* and *Eubacterium* (De Moreno de leBlanc *et al.*, 2005; Nakamura *et al.*, 2002).

β-glucosidase (β-D-glucoside glucohydrolase, amygdalase) hydrolyzes glycosides to sugar groups, *i.e.* glycones, and to non-sugar groups, *i.e.* aglycones (Rafter *et al.*, 2002). A diet composed of large amounts of glycosides of plant origin (*e.g.* flavonoids) may be the reason for the formation of harmful substances in the large intestine. Glycosides that have not been digested are transported to the large intestine where they are hydrolyzed under the influence of bacterial β-glucosidase. The aglycones that are formed in such a way often manifest toxic and carcinogenic properties, *e.g.* querecin that is one of the promoters of the large intestine tumors and is formed in the process of rutin transformation involving β-glucosidase (De Preter *et al.*, 2008). This enzyme is present in the cells of some of the microorganisms located in the large intestine and the highest activity is shown by *Bacteroides uniformis*, *Clostridium paraputrificum*, *Clostrdium clostridioforme* and *Enterococcus faecalis* (De Preter *et al.*, 2008; Nakamura *et al.*, 2002). The qualitative and quantitative composition of the intestinal microflora in healthy human beings contains a dominant number of microorganisms favorable to their health and it is relatively balanced. The dominance of some of the intestinal bacteria species and an increase in the activity of the so-called fecal enzymes may lead to increased production of carcinogenic compounds.

The aim of the research was to determine the activity of β-glucuronidase and β-glucosidase of *Lactobacillus* and *Enterococcus* bacteria. *Enterococcus*, although usually are not the dominant microorganisms in the large intestine, consist a constant and typical element of this ecosystem. On the other hand, *Lactobacillus* that belongs to the intestinal endogenic microorganisms is considered to have a favorable influence on the activity of the digestive tract (Russel *et al.*, 2001). The present article describes the changes in the activity of the large intestine bacteria enzymes isolated from healthy persons, including children aged 1 and 8 and adults aged 30 and 80.

Experimental

Material and Methods

Bacterial *Lactobacillus* and *Enterococcus* strains used for the purpose of the research were isolated from the feces of 5 healthy children (aged 1 and 8) and 5 adults (aged 30 and 80). The above-mentioned genera were isolated on selective media: *Lactobacillus* on Rogosa medium, and *Enterococcus* on BE medium with bile and esculin. The material for the determination of the enzymatic activity was a 24-hour culture of the tested bacteria. The enzymatic activity of β-glucuronidase and β-glucosidase was determined spectrophotometrically using the wavelengths 540 nm and 450 nm. The method was based on color reaction between a substrate and the examined enzyme. The activity of β-glucuronidase and β-glucosidase was determined with the method described by Freeman (1986), using as a substrate phenolphthalein-β-D-glucuronide (Sigma) for β-β-glucuronidase and p-nitrophenyl-β-D-glucopiranoside (Sigma) for β-glucosidase. The adopted activity unit equaled such amount of phenolphthalein (for β-glucuronidase) and p-nitrophenyl (for β-glucosidase) expressed in mM that was released during the reaction in 1 hour, calculated per 1 mg of protein. The total concentration of protein in bacterial cells was determined with the use of Lowry method as described (Lipińska *et al.*, 1999). The results were elaborated based on statistical analysis.

Results

The aim of the conducted research was to determine the activity of β-glucuronidase and β-glucosidase of *Lactobacillus* and *Enterococcus* isolated from the feces of healthy children, aged 1 and 8, and adults aged 30 and 80. The analysis included 10 strains isolated from human feces in each of the age groups.

The activity of β-glucuronidase of *Lactobacillus* coming from children, depending on the strain, in interval equaled from 0.135 to 0.275 mM/h/mg of protein. In the case of the adults, the activity of this enzyme ranged from 0.159 mM/h/mg of protein to 0.313 mM/h/mg of protein. *Enterococcus* strains coming from children were characterized by β-glucuronidase activity within the range 0.07–0.114 mM/h/mg of protein, and in the case of the subjects aged 30 and 80, the activity of the enzyme equaled from 0.032 to 0.064 mM/h/mg of protein. The activity of β-glucosidase of *Lactobacillus* strains obtained from children equaled from 0.021 to 0.048 mM/h/mg of protein, while in case of adults it equaled from 0.018 to 0.032 mM/h/mg of protein. These differences were statistically significant. The isolates of *Enterococcus*

Table I
The activity of β-glucuronidase and β-glucosidase of bacteria isolated from feces of person in different age

Bacterium	Age (years)	Activity*	
		β-glucuronidase	β-glucosidase
Lactobacillus	1	0.135–0.275	0.021–0.048
	8	0.164–0.237	0.019–0.042
	30	0.159–0.217	0.018–0.029
	80	0.257–0.313	0.020–0.032
Enterococcus	1	0.073–0.103	0.013–0.015
	8	0.070–0.114	0.015–0.017
	30	0.032–0.053	0.007–0.010
	80	0.044–0.064	0.006–0.009

* activity of β-glucuronidase (mM phenolphtalein /h/mg of protein), β-glucosidase (mM p-nitrophenyl /h/mg of protein)

from children were characterized by β-glucosidase activity at the level from 0.013 to 0.017 mM/h/mg of protein, and in adults the activity of this enzyme was very low and equaled from 0.006 to 0.01 mM/h/mg of protein (Table I).

It was discovered that the average activity of both β-glucuronidase and β-glucosidase of *Lactobacillus* isolates coming from children was 2.3 and 2.4 times higher, respectively, and in the case of the isolates coming from adults it was 4.6 and 2.7 times higher than the activity of these enzymes from *Enterococcus* (Figure 1 and 2). The highest *Lactobacillus* β-glucuronidase activity was observed in isolates from an 80-year-old subject (0.313 mM/h/mg of protein) (Table I). The differences between the activity of β-glucuronidase of *Enterococcus* isolated from the feces of 1 and 8-year-old children were statistically insignificant. In the case of children the activity of the *Enterococcus* isolates was higher by 48% in comparison to 30-year-old subjects and by 37% in comparison to the oldest group. *Lactobacillus* bacteria, depending on the strain, were characterized by varied β-glucuronidase activity. The differences for the activity of this enzyme in isolates coming from children was 2-fold, while in the case of the 80-year-olds this difference equaled only 18%. A similarly varied

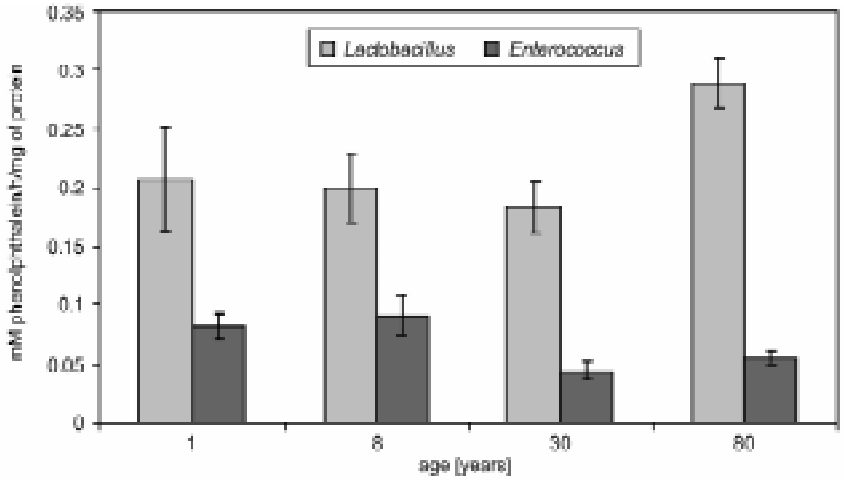

Fig. 1. Average activity of β-glucuronidase of *Lactobacillus* and *Enterococcus* isolates

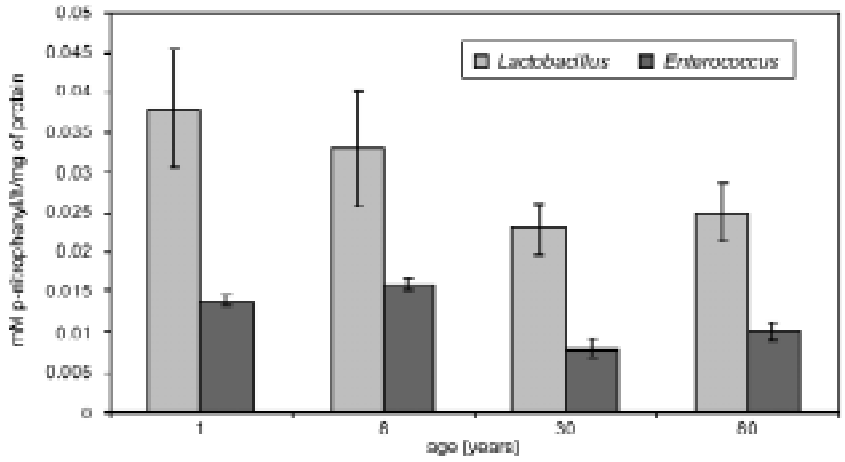

Fig. 2. Average activity of β-glucosidase of *Lactobacillus* and *Enterococcus* isolates.

activity of β-glucosidase was observed in the isolates of *Enterococcus* coming from children (2.2-fold), whereas in the adult groups this difference equaled 37.5% (Table I). The highest enzymatic activity of β-glucosidase both regarding *Lactobacillus* and *Enterococcus* was discovered in the isolates obtained from the feces of children, *i.e.* 0.048 mM/h/mg and 0.017 mM/h/mg respectively. It was determined that the activity of β-glucosidase of the *Lactobacillus* isolates coming from children was by 32% higher than the activity of the isolates from adults. It was also observed that the differences between the activity of β-glucosidase of the *Lactobacillus* and *Enterococcus* isolates coming from a one-year-old and an eight-year-old were statistically insignificant (Fig. 2). Moreover, it was shown that the activity of β-glucuronidase of *Lactobacillus* strains isolated from the feces of the 80-year-olds was the highest, and the isolates of the examined microorganisms coming from children were characterized by the highest activity of β-glucosidase.

Discussion

The age of a person may have a very significant influence on the number and activity of intestinal microorganisms. Intestinal microflora in children is better known than that in the case of the elderly, which is indicated by the fact that the number of described bacteria species coming from the large intestine of children equals 70%, and from the elderly only 8%. The intestinal microflora of an infant is dominated by *Escherichia coli* and enterococci (Rada *et al.*, 2006). The settlement of bacteria in the intestine is influenced by the mode of nutrition. It can be recognized whether a child is breastfed or whether it receives artificial baby milk on the basis of its intestinal microflora. When a child is breastfed by its mother, its intestinal microflora is richer in bacteria producing lactic acid (*Lactobacillus* and *Bifidobacterium*). After about 7–10 days after the delivery the microflora becomes more varied and absolute anaerobes can also be found (*Clostridium* and *Bacteroides*) (Strus *et al.*, 2002). The formation of an adult intestinal ecosystem takes place when children are 7–10 years old. In the case of the elderly, we observe an increase in the number of *Clostridium*, and a decrease in the number of *Bifidobacterium*, as well as an increase of the pH of the intestinal content up to 7–7.5 (Kurokawa *et al.*, 2007). These changes may result in a different amount of enzymes released to the intestinal environment, including the elevated activity of enzymes that are harmful to human health. Therefore, in this work research was undertaken with the intention of determining the activity of β-glucuronidase and β-glucosidase of *Lactobacillus* and *Enterococcus* isolates. The highest activity was discovered in the case of the *Lactobacillus* isolates coming from the 80-year-olds, and the *Enterococcus* isolates were most active in the case of children. It was proven that the activity of both β-glucuronidase and β-glucosidase of the *Lactobacillus* isolates was several times higher than the enzymatic activity of the *Enterococcus* isolates in each age group. This result emphasizes the fact that some endogenic (intestinal) bacteria – *Lactobacillus* or *Bifidobacterium* – to which only positive properties are attributed, may manifest enzymatic activity harmful to human health (*Bifidobacterium longum*) (Russel *et al.*, 2001). The activity of β-glucuronidase of *Lactobacillus* bacteria isolated from the feces of 80-year-olds was the highest among the examined age groups. The reason for this may be a diet rich in fats and poor in probiotic products coming from bacterial species showing properties favorable for the functioning of the digestive tract. A decrease in the activity of the examined enzymes may be achieved by means of the application of probiotic preparations containing controlled strains that can replace the host's own lactic acid bacteria strains manifesting too high activity of the so-called fecal enzymes. The change in the β-glucuronidase and β-glucosidase activity was observed during the application of fermented diary drinks containing *Lactobacillus casei* strain Shirota in the case of healthy adult subjects. The β-glucuronidase activity of the microorganisms present in their large intestine decreased by 30%, and the activity of β-glucosidase by 29% in comparison to the control groups (Edited by Yakult Central Institute for Microbiological Research, 1999). Similarly, a diet supplement in the form of a probiotic strain *Lactobacillus casei* DN-11401 administered to children after liver transplant led to a decrease in the activity of β-glucuronidase by 41% in comparison to children that did not receive such a diet (Pawłowska *et al.*, 2007). High level β-glucuronidase activity of in the case of lactic acid bacteria isolates taken from the group of old people may be related to typical changes in the species composition regarding this group of bacteria, which is also connected with the risk of tumors. The lack of statistically significant differences between the activity of β-glucosidase in the isolates of *Lactobacillus* and *Enterococcus* coming from the two groups of children and the two groups of adults makes it possible to determine the changes in the activity of this enzyme occurring between children and adults. The research results present a decrease in the activity of β-glucosidase in the case of both *Lactobacillus* and *Enterococcus* in adults compared to children. The increased level of the activity of this enzyme in children may be related to the kind of nutrition characteristic for this age group. A diet rich in milk influences shaping of the intestinal ecosystem in a child. A diet containing *Lactobacillus rhamnosus* LC705

and *Propionibacterium freudenreichii* ssp. *shermanii* JS resulted in a decrease of the activity of β-glucosidase in healthy persons by 10% in comparison to the control group (Hatakka *et al.*, 2008). The changes in the number of the intestinal microorganisms that accompany aging may induce an increase or a decrease of the activity level of the so-called fecal enzymes, and thus they can influence the presence of toxic and often carcinogenic substances in an organism.

The conducted research showed that there is a variety as for the activity of β-glucuronidase and β-glucosidase concerning the strains of *Lactobacillus* isolated from subjects of different age and proved that there is an unfavorable increase in the activity of β-glucuronidase accompanying aging. The activity of β-glucosidase of *Lactobacillus* and *Enterococcus* strains was decreased in case of adults in comparison to children, which suggests that the diet of adults ought to be enriched with probiotic products containing bacteria beneficial for human beings.

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ORIGINAL PAPER

Evaluating the Combined Efficacy of Polymers with Fungicides for Protection of Museum Textiles against Fungal Deterioration in Egypt

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Abstract

Fungal deterioration is one of the highest risk factors for damage of historical textile objects in Egypt. This paper represents both a study case about the fungal microflora deteriorating historical textiles in the Egyptian Museum and the Coptic museum in Cairo, and evaluation of the efficacy of several combinations of polymers with fungicides for the reinforcement of textiles and their prevention against fungal deterioration. Both cotton swab technique and biodeteriorated textile part technique were used for isolation of fungi from historical textile objects. The plate method with the manual key was used for identification of fungi. The results show that the most dominant fungi isolated from the tested textile samples belong to *Alternaria, Aspergillus, Chaetomium, Penicillium and Trichoderma* species. Microbiological testing was used for evaluating the usefulness of the suggested conservation materials (polymers combined with fungicides) in prevention of the fungal deterioration of ancient Egyptian textiles. Textile samples were treated with 4 selected polymers combined with two selected fungicides. Untreated and treated textile samples were deteriorated by 3 selected active fungal strains isolated from ancient Egyptian textiles. This study reports that most of the tested polymers combined with the tested fungicides prevented the fungal deterioration of textiles. Treatment of ancient textiles by suggested polymers combined with the suggested fungicides not only reinforces these textiles, but also prevents fungal deterioration and increases the durability of these textiles. The tested polymers without fungicides reduce the fungal deterioration of textiles but do not prevent it completely.

Key words: Egyptian and Coptic Museums in Cairo, fungal deterioration, fungicides with polymers, historical textiles

Introduction

Fungal deterioration seems to be a predominant feature in museums and other culture objects of organic materials such as paper, textiles, wood, *etc.* (Abdel-Kareem *et al.*, 1997; Agrawal, 2001; Bhatnagar and Mani, 2001). The ability of textiles to absorb and retain moisture from the surrounding environment in the museums, coupled with their organic components makes them highly susceptible to fungal deterioration. There are many factors which cause that historical textiles are more liable to fungal deterioration. Textile materials are good nutrient materials for fungi. Progressive changes of the properties of textile materials most commonly happen during natural aging. These changes in the characterization of textile materials cause that historical objects become more susceptible to fungal deterioration (Szostak-Kotowa, 2004).

Fungal deterioration of historical textiles is a serious problem in Egypt (Abdel-Kareem *et al.*, 1997). This is due to the fact that improper environmental conditions in Egypt promote the fungal growth and the nature of the textiles too. Historical textiles in Egypt are more acidic according to the surrounding environments (Abdel-Kareem, 2002), which is considered to create favorable conditions for fungal growth. High humidity accompanied by lack of ventilation in storage rooms in Egyptian museums enhances the fungal growth on textile objects. In some cases contaminated conservation materials such as improper polymers can cause fungal infestation of conserved textile objects (Florian, 1997).

Fungal deterioration causes changes in the properties of textiles such as losses in the strength, their general durability, discoloration, and appearance. In addition, many fungi contain coloured substances that can cause stains and spots on textile objects. Fungal deterioration causes various coloured stains on the surface of a textile object (Mukerji *et al.*, 1995; Abdel-Kareem, 2007). These stains contain chemical substances which can still deteriorate a textile object if the fungus dies or is killed (Montegut *et al.*, 1991; Florian, 2004).

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It is important to think of a way to remove these fungal stains from textile objects. However, it is reported that fungal stains are extremely difficult to remove from historical textile objects as the methods that are known for removing fungal stains are very harmful to textiles (Agrawal, 2001). The chemical changes occurring with the fungal growth result in decreased fabric strength and lead to partial or total destruction of the material (Szostak-Kotowa, 2004). Molds can be dangerous to people working in museums and in some cases can pose a major health hazard (Merritt, 1993). A large number of fungal species are reported to cause deterioration of textiles and culture heritage. There are different methods for prevention of fungal deterioration of textiles with the use of chemicals and nonchemical methods. Chemical treatments include using fungicides and fumigants. Nonchemical methods comprise the use of UV and gamma rays, heat, electron beams and microwaves (Bhatnagar and Mani, 2001). Unfortunately these methods are not evaluated well from conservation perspective. Most of the methods mentioned above may cause damage to ancient textiles such as fading of dyes, dryness of fibers, and breakdown in the strength of the textile fibers and so on.

It is emphasized that the best method to prevent fungal growth on museum textiles, is to protect textile surfaces from contamination, control moisture in materials and relative humidity to be low and avoid the treatments which may activate conidia to start germination (Florian, 1997). In some cases this method could not be applied in all museums (Abdel-Kareem, 2000a). In such a situation some other solutions have to be thought off, such as fumigants and fungicides for preservation of textiles. There are a large number of studies that have been carried out on fungicides used for protection of museum textiles (Agrawal, 1995; Abdel-Kareem, 2000a). Numerous studies for industrial purposes have also been carried out on the microbial deterioration and degradation of polymers and on their protection with biocides (Whitney, 1996; Srivastava, 2001; Lucas *et al.*, 2008). Other papers have also focused on textile conservation methods (Abdel-Kareem, 2000b; 2005). In this study a new approach to prevent fungal growth on consolidated historical linen textiles was evaluated.

Experimental

Material and Methods

Isolation and identification of fungi from historical biodeteriorated textile samples

Biodeteriorated samples. Both cotton swab technique and biodeteriorated textile part technique were used for collecting samples for isolation of fungi from historical textile objects. Although it was confirmed in previous studies that using of parts of the investigated objects is the best method which can be used in identifying fungi from biodeteriorated historical textiles (Abdel-Kareem *et al.*, 1997), in some cases this method is considered destructive. Thus it could not be used with all investigated textile objects in this study. Instead, the cotton swab technique was applied with all investigated textile objects. This method scores highly in most of the criteria required for isolating fungi from ancient objects (Chaisrisook *et al.*, 1995).

Isolation and identification of fungi. Very small biodeteriorated textile parts separated from the original ancient object were washed with sterilized distilled water and were transferred by using sterilized tweezers and were put on 2 modified media in Petri dishes (Abdel-Kareem *et al.*, 1997). The used media are 1 – Medium of Greathous, Klemme and Barker with disk of pure 100% linen fabric with linen textile samples or with disk of pure 100% wool fabric with wool textile samples. 2 – Czapek-Dox agar modified without sugar.

In the case of using the cotton swab technique the fungal species were isolated by using sterile moist cotton buds swabbed onto the surface of textile objects where fungal growth or fungal structures were observed. Cotton swabs were then used to distribute the fungi on media in Petri dishes. The Petri dishes were then incubated for three to four weeks at 28°C (until growth of colonies was observed). For purification and identification, the developed fungi were isolated in pure culture on slants of the appropriate media (Czapek dox agar and malt extract agar) (Booth, 1971). Identification of fungal species was performed according to standardized methods by consulting the appropriate manuals (Domsch *et al.*, 1980; Gilman, 1975; Raper, and Fennell, 1965; Raper and Thom, 1949).

Evaluating the suggested treatment for controlling fungal growth using consolidated polymers

It was confirmed in previous studies that some polymers used in the conservation of historical textiles can accelerate fungal growth on historical textiles (Keyserlingk, 1990); some of them may inhibit fungal growth and others can accelerate it (Abdel-Kareem, 2000b; 2005). However, there is no doubt that all the polymers used in textile conservation cannot prevent the fungal deterioration of textiles. This study introduces a new suggestion by adding some selected fungicides that are commonly used in textile conservation to some selected polymers which are often used in textile conservation. For evaluating the new composed chemical the following processes were carried out.

Polymers. Four selected polymers were used in this study (see Table I). The polymers were selected

Table I
List of polymers used in this study.

	Trade name	Chemical name	Producer
1	Klucel G (SD)	Hydroxypropylcellulose	Lascaux Restauro
2	Lascaux 498 HV (E)	Butyl acrylate / methyl methylacrylate	Lascaux Restauro
3	Mowilith DM5 (E)	Vinyl acetate/acrylic ester copolymer	Hoechst
4	Mowilith DMC2 (E)	Vinyl acetate/dibutyle maleate copolymer	Hoechst
5	Tylose MH300 (SD)	Methyl hydroxy ethyl cellulose	Hoechst

Table II
List of fungicides used in this study.

	Trade name	Chemical name	Producer
1	Preventol O-Na	Sodium o-phenyl-phenol (NaOPP)/2-hydroxybiphenyl sodium salt tetra hydrate	Bayer
2	Neo-Desogen	a water solution of ammonium basic with a strong biocide action	ARTE

according to the relevant references that confirmed that these polymers are suitable, effective and commonly used in the reinforcement of textile artefacts (Abdel-Kareem, 2005; Abdel-Kareem *et al.*, 2008).

Fungicides. Two selected fungicides were used in this study (Table II). The fungicides were selected according to the relevant references that confirmed that these fungicides are suitable and effective in treatment of textile artefacts against fungal deterioration (Abdel-Kareem and Radwan, 2004).

Preparation of samples. Unbleached linen fabric samples were cut into 10×2 cm (length × width) warp test specimens. The warp strips were produced by raveling away yarns on each side forming 1.5 cm wide strips with a 2.5 mm fringe down each side. Five samples were used for each test.

Treatments. Linen textile samples were treated with the selected polymers by using impregnation method (Abdel-Kareem, 2005), with some modification in the technique by adding the tested fungicides to the solution. The Preventol was used in 1% concentration and Neo-Desogen was used in 2% concentration (Table III).

Fungal treatment of samples. Treated and untreated linen textile samples were exposed to attack by pure culture of *Aspergillus niger*, *Chaetomium golobosum* and *Penicillium funiculosum* by using an agar plate test. These fungal species are the most dominant ones isolated from ancient Egyptian textiles textile samples in this study. It was confirmed in previous studies that the selected three fungi are considered to play the greatest role in the decomposition of

Table III
The suggested treatment for controlling the fungal growth.

	Polymer
0	untreated
1	Klucel G 4%
2	Klucel G 4% + Neo-Desogen
3	Klucel G 4% + Preventol
4	Lascaux 498 HV 10%
5	Lascaux 498 HV 10% + Neo-Desogen
6	Lascaux 498 HV 10% + Preventol
7	Mowilith 10%
8	Mowilith 10% + Neo-Desogen
9	Mowilith 10% + Preventol
10	Tylose 4%
11	Tylose 4% + Neo-Desogen
12	Tylose 4% + Preventol

cellulosic materials of all fungi isolated from historical Egyptian textiles (Abdel-Kareem and Szostak-Kotowa, 2005; Garg and Dhawan, 2005). Also, these fungi are commonly used for evaluation of the resistance of polymers to fungal deterioration (Whitney, 1996). Petri dishes containing Czapek-Dox agar medium modified without sugar were used (Abdel-Kareem *et al.*, 1997). The medium was inoculated with spore suspension (14-day old culture) of each one of the tested fungi. The spore suspension of the fungus was spread on the surface of the medium. The textile samples were put on the inoculated surface of medium. The plates were incubated at 28°C. Fourteen days later, linen textile samples were picked out and washed with water to remove mycelium. They were then air dried in room conditions. Before testing, the specimens were conditioned at 20±2°C and 65% 2 RH.

Evaluation methods

All treated and untreated samples before and after fungal deterioration were investigated with tensile tester and colorimeter.

Tensile strength and elongation. Tensile strength and elongation of all samples before and after the fungal treatment were tested using a testing machine, type Zwick 1445. These tests were done according to the ASTM (2000) D 5035-95. The initial distance of the jaws was 50 mm and the testing speed was 25 mm/min, temperature was 23°C, and R.H.65%. Five samples were used for each test and statistical data were calculated for all tested samples.

Colorimetric measurements. The colore values of all textile samples before and after deterioration by different fungi have been carried out with Optimacth 3100 color Spectrophotometer using the CIELab color system. The CIELab color coordinates for L (lightness), a (red/green axis), and b (yellow/blue

axis) values were recorded. Color changes for all samples after the fungal treatment was calculated and expressed as ΔL, Δa, Δb. Calculation of total color change (ΔE) is achieved by the use of the following equations: $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{0.5}$.

Results and Discussion

Isolation and identification of fungi

Fungi isolated from various biodeteriorated textile fabrics from storage area in the Egyptian museum are shown in Table IV. The obtained results show that 207 isolates, representing 31 species of fungi were identified on samples obtained from the Egyptian museum. The most dominant fungi on linen textile fabrics are *Aspergillus* (15 species), *Penicillium* (10 species), *Chaetomium* (4 species), *Alternaria* (1 species), and *Trichoderma* (1 species). The order of the occurrence of fungi on linen textile fabrics is as follows: *Aspergillus* > *Penicillium* > *Chaetomium* > *Alternaria* > *Trichoderma viride*.

Table IV
Isolated fungi from tested bio-deteriorated linen samples from the Egyptian museum in Cairo.

	Fungi	No of isolations
1	*Alternaria alternate* (Fr.) Keissler	8
2	*Aspergillus carbonarius* Bainier	6
3	*Aspergillus chrysellus* Kown & Fennell	3
4	*Aspergillus fischeri* Wehmer	2
5	*Aspergillus flavus* Link	12
6	*Aspergillus flaschentraegeri* Stolk	2
7	*Aspergillus fumigatus* Fresenius	15
8	*Aspergillus nidulans* Eidam	9
9	*Aspergillus niger* Tieghem	13
10	*Aspergillus terrus* Thom	11
11	*Aspergillus ustus* Thom & Church	2
12	*Aspergillus versicolor* (vuill.) Tiraboschi	4
13	*Aspergillus* sp.	5
14	*Aspergillus* sp.	4
15	*Aspergillus* sp.	4
16	*Chaetomium cochlioides* Palliser	11
17	*Chaetomium globosum* Kunze	14
18	*Chaetomium* sp.	7
19	*Chaetomium* sp.	6
20	*Penicillium asperum* (Shear) n. comb.	6
21	*Penicillium citrinum* Thom	8
22	*Penicillium chrysogenum* Thom	4
23	*Aspergillus chrysellus* Kown & Fennell	3
24	*Penicillium cyclopium* Westling	6
25	*Penicillium funiculosum* Thom	10
26	*Penicillium soppi* Zaleski	6
27	*Penicillium wortmanni* Klöcker	7
28	*Penicillium* sp.	4
29	*Penicillium* sp.	4
30	*Penicillium* sp.	4
31	*Trichoderma viride* Pers. Ex Fr.	7

Table V
Isolated fungi from tested bio-deteriorated linen samples from the Coptic Museum Cairo.

	Fungi	No of isolations
1	*Alternaria alternate* (Fr.) Keissler	11
2	*Alternaria tenuissima* Kunze	2
3	*Aspergillus auratus* Warcup	2
4	*Aspergillus carbonarius* Bainier	9
5	*Aspergillus chrysellus* Kown & Fennell	5
6	*Aspergillus fischeri* Wehmer	4
7	*Aspergillus flavus* Link	14
8	*Aspergillus flaschentraegeri* Stolk	4
9	*Aspergillus fumigatus* Fresenius	17
10	*Aspergillus nidulans* Eidam	11
11	*Aspergillus niger* Tieghem	14
12	*Aspergillus proliferans* Smith	3
13	*Aspergillus spinulosus* Warcup	3
14	*Aspergillus terrus* Thom	12
15	*Aspergillus ustus* Thom & Church	4
16	*Aspergillus versicolor* (vuill.) Tiraboschi	5
17	*Aspergillus* sp.	4
18	*Aspergillus* sp.	4
19	*Aspergillus* sp.	4
20	*Chaetomium cochlioides* Palliser	12
21	*Chaetomium globosum* Kunze	13
22	*Chaetomium* sp.	6
23	*Chaetomium* sp.	7
24	*Penicillium asperum* (Shear) n.comb.	8
25	*Penicillium biforme* Thom	2
26	*Penicillium citrinum* Thom	9
27	*Penicillium chrysogenum* Thom	10
28	*Aspergillus chrysellus* Kown & Fennell	12
29	*Penicillium cyclopium* Westling	8
30	*Penicillium funiculosum* Thom	12
31	*Penicillium raistrickii* Smith	2
32	*Penicillium soppi* Zaleski	5
33	*Penicillium wortmanni* Klöcker	9
34	*Penicillium* sp.	6
35	*Penicillium* sp.	5
36	*Penicillium* sp.	3
37	*Trichoderma viride* Pers. Ex Fr.	8

Fungi isolated from various biodeteriorated textile fabrics from a storage area in the Coptic museum are shown in Table V. The obtained results show that 269 isolates, representing 37 species of fungi were identified in samples obtained from the Egyptian museum. The most dominant fungi on linen textile fabrics are *Aspergillus* (18 species), *Penicillium* (12 species), *Chaetomium* (4 species), *Alternaria* (2 species), and *Trichoderma* (1 species). The order of the occurrence

Table VI
Isolated fungi from tested bio-deteriorated wool samples from the Coptic Museum Cairo.

	Fungi	No of isolations
1	*Alternaria alternate*,	5
2	*Aspergillus cervinus* Neill	3
3	*Aspergillus flavus* Link	4
4	*Aspergillus fischeri* Wehmer	3
5	*Aspergillus fumigatus* Fresenius	8
6	*Aspergillus nidulans* Stolk	3
7	*Aspergillus niger* Tieghem	7
8	*Aspergillus raperi* Stolk	2
9	*Aspergillus sparsus* Raper & Thom	2
10	*Aspergillus spinulosus* Warcup	5
11	*Aspergillus wentii* Wehmer	2
12	*Aspergillus* sp.	2
13	*Aspergillus* sp.	2
14	*Penicillium canescens* Sopp	2
15	*Penicillium cyclopium* Westling	2
16	*Penicillium granulatum* Bainier	3
17	*Penicillium lanoso viride* Thom	2
18	*Penicillium paxilli* Bainier	6
19	*Penicillium soppi* Zaleski	6
20	*Penicillium sp.*	2
21	*Penicillium sp.*	2
22	*Chaetomium globosum* Kunze	4

of fungi on linen textile fabrics is as follows: *Aspergillus* > *Penicillium* > *Chaetomium* > *Alternaria* > *Trichoderma viride*.

Fungi isolated from various biodeteriorated wool textile fabrics from storage area in the Coptic museum are presented in Table VI. The obtained results show that 77 isolates, representing 22 species of fungi, were identified on samples obtained from the Egyptian museum. The most dominant fungi on wool textile fabrics are *Aspergillus* (12 species), *Penicillium* (8 species), *Alternaria* (1 species), and *Chaetomium* (1 species). The order of the occurrence of fungi on wool textile fabrics is as follows: *Aspergillus* > *Penicillium* > *Chaetomium* > *Alternaria*.

The results showed that about 37 fungal species were isolated and identified on linen textiles from both investigated museums. Most of the identified fungi in the current study were isolated from other Egyptian textile objects in a previous study by Abdel-Kareem *et al.* (1997) who isolated and identified about 30 fungal species from ancient linen textiles. The results confirm that the textile samples in the current study are more deteriorated by fungi than in a previous study by Abdel-Kareem *et al.* (1997). It is should noticed that about 7 more fungal species were isolated in this study than in the previous one. This may be due to the fact that the examined samples were collected from storage rooms while the investigated samples in the previous study were collected from display showcases and excavations. This result indicates that the textiles in storage rooms in Egyptian museums suffer from fungal deterioration problem more than the textile collections in display areas. Also the results show that linen textiles are more infested by fungi than wool textiles as the number of identified fungi on linen is greater than on wool.

The number of isolated fungi from both investigated museums included in the research shows that their collections suffer from excessive fungal infestation. This is due to the fact that both museums use improper storage methods. The results show that the textile collection in the storage rooms at the Coptic museum are infested by fungi more than the textile collection in the storage rooms at the Egyptian Museum. This may due to that most of the textiles in the Egyptian Museum were excavated from dry tombs, while most of the textiles in the Coptic Museum were collected from churches or tombs in bad condition more than ancient Egyptian tombs. This may also be due to the environmental conditions in the storage area in both the Coptic Museum and the Egyptian Museum. However, the results show that the linen textile fabrics in the Coptic Museum are more liable to fungal deterioration than wool textile fabrics (see Tables V, VI). These results are in agreement with the results obtained by Abdel-Kareem *et al.*, who confirmed that all types of ancient textile fibres are liable to fungal attack; cellulosic fibres are more liable to fungal attack than animal fibres (Abdel-Kareem *et al.*, 1997).

The results show that most of identified fungi belong to the subdivision *Deuteromycetes* class or Fungi Imperfect. These fungi are called conidial fungi because their growth is initiated by conidia (Florian, 2004). These fungi are capable of rapid growth when the environmental conditions are favorable and are also able to survive under unfavorable conditions (Aranyanak, 1995). Most of identified fungal species were reported in previous studies to cause deterioration of textiles. Many authors consider that most of these fungal species are the most active fungi among all fungal genera identified on textiles in the degradation of historical textiles (Montegut *et al.*, 1991; Abdel-Kareem *et al.*, 1997; Agrawal, 2001, Bhatnagar and Mani, 2001; Grag and Dhawan, 2005). Most of identified fungi were reported that they contribute to discolouration of textiles (Aranyanak, 2005; Abdel-Kareem and Szostak-Kotowa, 2005; Abdel-Kareem, 2007). The results showed that the most dominant fungi on the investigated textile samples belong to *Aspergillus* and *Penicillium*. These two genera are very important, since they include species that can grow at relatively much lower conditions of moisture availability than other cellulolytic fungi. Under poor

Table VII
The tensile strength for the samples after treated with fungi.

Polymer	Control		*Aspergillus*		*Chaetomium*		*Penicillium*	
	N/mm^2	S.D.	N/mm^2	S.D.	N/mm^2	S.D.	N/mm^2	S.D.
0	34.35	0.94	4.02	0.70	4.24	1.22	5.98	1.12
1	36.30	1.13	11.30	1.09	13.48	1.27	15.00	1.41
2	36.41	0.94	33.04	1.36	33.48	0.94	34.46	1.22
3	36.74	0.81	28.59	1.05	35.11	1.25	31.63	1.10
4	37.50	1.37	19.02	0.89	19.89	1.22	20.87	1.24
5	37.39	1.30	31.52	0.99	30.87	1.08	35.43	1.07
6	37.28	0.96	31.20	1.43	33.04	0.82	32.50	1.30
7	37.07	1.33	16.63	1.16	17.50	1.34	21.74	1.28
8	37.28	0.85	34.02	1.26	33.26	1.24	34.35	1.35
9	37.50	1.07	33.59	1.25	34.78	1.17	36.09	1.21
10	38.15	0.93	14.59	1.39	15.65	1.17	17.83	1.16
11	38.26	1.02	31.96	1.28	34.02	1.40	32.28	1.41
12	38.37	1.27	26.41	1.14	30.11	1.12	33.15	1.29

Table VIII
The elongation for the samples after treated with fungi.

Polymer	Control		*Aspergillus*		*Chaetomium*		*Penicillium*	
	F max%	S.D.	F max%	S.D.	F max%	S.D.	F max%	S.D.
0	18.00	0.55	6.00	0.89	8.00	0.63	8.00	0.89
1	20.00	0.84	9.00	1.41	10.00	1.10	10.00	1.26
2	20.00	1.26	16.00	1.26	16.00	1.26	16.00	1.10
3	20.00	1.41	16.00	0.63	16.00	0.63	16.00	0.63
4	18.00	1.10	8.00	0.89	9.00	1.41	9.00	1.10
5	18.00	1.41	16.00	1.10	16.00	1.90	16.00	1.26
6	18.00	0.63	16.00	0.63	16.00	0.89	16.00	0.63
7	22.00	0.89	9.00	1.10	10.00	0.89	9.00	1.41
8	22.00	0.89	16.00	1.10	16.00	1.26	16.00	1.26
9	22.00	1.10	16.00	0.63	16.00	0.63	16.00	0.63
10	20.00	0.63	8.00	0.63	9.00	1.41	10.00	1.41
11	20.00	1.10	16.00	1.26	16.00	1.26	16.00	1.41
12	20.00	1.26	16.00	1.10	16.00	1.10	16.00	1.10

storage conditions, the water that such less demanding species produce as a result of their metabolism can accumulate, raising the moisture status of materials to levels at which more highly degradable species may flourish (Szostak-Kotowa, 2004).

For all previous causes there is a need to decontaminate the biodeteriorated textile objects from conidia and mycelium in order to reduce the fungal growth on these textile objects and prevent the contamination of other objects. For that the surface of the biodeteriorated textile objects should be vacuumed cleaned to remove mycelium and conidia. The vacuum cleaning method chosen should be acceptable by conservation standards to protect the integrity of the textile object. The main goal of this process is to reduce the fungal load to the minimal level of the infestation and prevent recontamination (Florian, 2004). A vacuum cleaning method should be applied at low suction power, and through a gauze sheet or fin netting fabric placed over the textile object in order to not disturb loose fibers (Museums & Galleries Commission, 1998). After decontaminating the biodeteriorated textile objects from conidia and mycelium the textile objects should be treated against fungal deterioration.

Evaluating the suggested polymers

Evaluation of the suggested polymers combined with fungicides was carried out based on the obtained results from the changes in the tensile strength and the colour values of treated and untreated linen samples after the fungal treatments.

Tensile strength and elongation. The results of tensile strength and the elongation of the control samples and the biodeteriorated samples are blotted in Table VII and VIII. The loss percent (%) in the

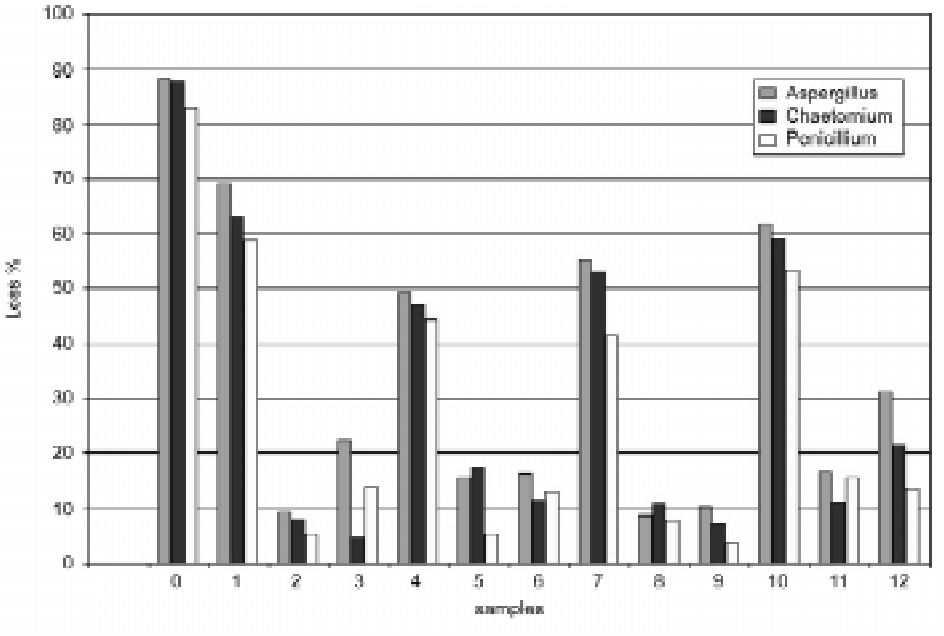

Fig. 1. The loss % in the tensile strength of the sampes after the fungal treatment.

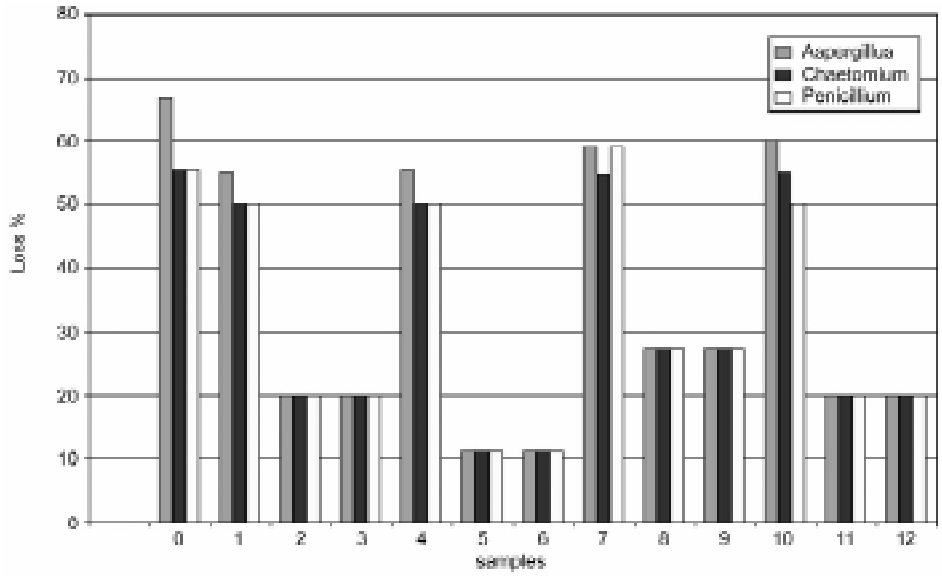

Fig. 2. The loss % in the elongation of the sampes after the fungal treatment.

tensile strength and the elongation of treated and untreated linen samples after fungal incubations are shown in figures 1 and 2. Tensile strength measurements showed that there were considerable differences in tensile strength and elongation between unconsolidated linen samples, linen samples treated with polymers only and linen samples treated with polymers contained different fungicides. The results showed that all tested polymers without fungicides contains have reduce the fungal deterioration of linen samples but not prevent the fungal deterioration completely. These results are in agreement with the results obtained by Abdel-Kareem (2000b). The results in figures 1, 2, show that the least changes in the tensile strength of all tested samples after the fungal treatment were on samples treated with Klucel G + Neo-Desogen. This result confirms that Klucel G + Neo-Desogen is the most effective treatment among all tested treatments that can control the fungal deterioration of linen textiles by all tested fungi. Also the results show that Neo-Desogen is the best fungicides among all tested fungicides can be added to all tested polymers for protection of linen textiles against fungal deterioration.

Colorimetric measurements. The changes in the colour values of treated and untreated linen samples after fungal incubations by *Aspergillus* are shown in Table IX. The changes in the colour values of treated and untreated linen samples after fungal incubations

Table IX
The changes in the color values for the samples after the fungal treatment with *Aspergillus.*

Polymer	dL	da	db	dE
0	–24.57	0.96	2.92	24.76
1	–5.82	0.56	0.78	5.90
2	0.77	0.37	–0.44	0.96
3	–1.86	0.25	–0.29	1.90
4	–9.12	–0.19	–0.31	9.13
5	–1.24	0.05	0.06	1.24
6	–0.75	0.22	–0.55	0.96
7	–9.84	0.4	–1	9.90
8	–2.06	0.94	0.83	2.41
9	–2.02	0.65	0.16	2.13
10	–10.38	0.55	–1.04	10.45
11	–1.69	0.44	–0.78	1.91
12	–1.28	0.51	0.37	1.43

Table X
The changes in the color values for the samples after the fungal treatment with *Chaetomium.*

Polymer	dL	da	db	dE
0	–26.39	1.51	3.13	26.62
1	–5.35	0.12	0.6	5.38
2	–0.55	0.7	0.04	0.89
3	–1.81	0.63	–0.38	1.95
4	–9.39	0.19	4.25	10.31
5	–1.64	0.12	0.62	1.76
6	–1.41	0.6	–0.26	1.55
7	–5.36	1.18	3.19	6.35
8	–1.02	0.5	0.19	1.15
9	–1.49	0.64	0.19	1.63
10	–7.97	0.54	1.35	8.10
11	–0.29	0.35	–0.52	0.69
12	–1.81	0.47	–0.53	1.94

by *Chaetomium* are shown in Table X. Also the changes in the colour values of treated and untreated linen samples after fungal incubations by *Pencillium* are shown in Table XI. Colorimetric measurements showed that there were considerable differences in colour values unconsolidated linen samples, linen samples treated with polymers only and linen samples treated with polymers contained different fungicides. The results showed that all tested polymers without fungicides contains have reduced the fungal deterioration of linen samples but not prevent the fungal deterioration completely. These results are in agreement with the results obtained by Abdel-Kareem (2005), who confirmed that polymers reduce the fungal deterioration of linen samples but not prevent the fungal deterioration completely. The results showed that all tested polymers contained fungicides prevent the fungal deterioration of linen samples completely.

TTable XI
The changes in the color values for the samples after the fungal treatment with *Pencillium.*

Polymer	dL	da	db	dE
0	–22.57	1.22	2.48	22.74
1	–5.07	0.05	4.71	6.92
2	–0.3	0.37	0.19	0.51
3	–1.41	0.64	–0.45	1.61
4	–7.68	–0.09	3.37	8.39
5	–1.03	0.04	0.66	1.22
6	–1.37	0.29	0.05	1.40
7	–10.77	0.13	4.73	11.76
8	–2.02	0.67	0.31	2.15
9	–0.3	0.57	–0.06	0.65
10	–5.3	0.32	3.36	6.28
11	–1.46	0.85	–0.68	1.82
12	–1.54	0.05	0.34	1.58

Suggested guidelines for controlling and prevention of the fungal deterioration on the textile collections in storage rooms of the studied museums

Prevention includes protecting the textile objects from the contamination by fungi and controlling the environment conditions in storage rooms to prevent the development and the growth of fungi (Florian, 1997; Florian, 2004). One of the best methods for protection of textile objects from fungal infestations in museums, can be achieved by controlling the environmental conditions surrounding textile objects (Abdel-Kareem and Morsy, 2004). The following measures should be undertaken: Elimination/prevention of airborne fungi using the considerations mentioned by (Florian, 2004), for example use of protective dust covers for textile objects in storage area. Cleaning dust covers regularly. Performing regular maintenance of storage areas. Environmental conditions should be controllable within the storage storerooms. Temperature should be 18–22°C and relative humidity (RH) 45–55%. This can be achieved by build new storerooms with air conditioning system. Also it is necessary to control in the RH in storerooms using suitable buffer materials such as silica gel. Repairing leaking ceiling in storage area. Placing portable ventilators in the storerooms. Setting a suitable fumigant in storerooms to reduce the chance of microorganisms growing on the collections in the museum.

Conclusion. There are obvious excessive fungal infestations in all tested textile objects in storage areas in both the Coptic Museum and the Egyptian Museum. The textile collection in the storage rooms in the Coptic museum is infested by fungi more than the textile collection in the storage room in the Egyptian Museum. The most dominant fungi isolated from

tested samples belong to *Aspergillus*, *Penicillium*, *Chaetomium*, *Alternaria* and *Trichoderma* species. The order of occurrence of fungi on linen textile fabrics is as follows: *Aspergillus* > *Chaetomium* > *Penicillium* > *Alternaria* > *Trichoderma viride*. The order of occurrence of fungi on wool textile fabrics is as follows: *Aspergillus* > *Penicillium* > *Chaetomium* > *Alternaria*. There is a necessity for using fungicides to be used for disinfection of the biodeteriorated textiles in both the Coptic Museum and the Egyptian Museum. In the cases where it is necessary to use polymers in the conservation of textile objects, the tested polymers containing one of the tested fungicides are very effective in preventing the fungal deterioration of textiles. Klucel G + Neo-Desogen is the most effective treatment among all tested treatments that can control the fungal deterioration of linen textiles by all the tested fungi. Neo-Desogen is the best fungicide among all the tested fungicides and can be added to all tested polymers for protection of linen textiles against fungal deterioration. This study should followed with another study to evaluate the long term effect of the tested polymers supplemented with fungicides on the properties of dyed and not dyed textiles.

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ORIGINAL PAPER

Resistance of Bacterial Biofilms Formed on Stainless Steel Surface to Disinfecting agent

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Abstract

The natural ability of microorganisms for adhesion and biofilm formation on various surfaces is one of the factors causing the inefficiency of a disinfection agent, despite its proven activity *in vitro*. The aim of the study was to determine the effectiveness of disinfecting substances on bacterial biofilms formed on stainless steel surface. A universally applied disinfecting agent was used in the tests. Bacterial strains: *Listeria innocua*, *Pseudomonas putida*, *Micrococcus luteus*, *Staphylococcus hominis* strains, were isolated from food contact surfaces, after a cleaning and disinfection process. The disinfecting agent was a commercially available acid specimen based on hydrogen peroxide and peroxyacetic acid, the substance that was designed for food industry usage. Model tests were carried out on biofilm formed on stainless steel (type 304, no 4 finish). Biofilms were recorded by electron scanning microscope. The disinfecting agent in usable concentration, 0.5% and during 10 minutes was ineffective for biofilms. The reduction of cells in biofilms was only 1–2 logarithmic cycles. The use of the agent in higher concentration – 1% for 30 minutes caused reduction of cell number by around 5 logarithmic cycles only in the case of one microorganism, *M. luteus*. For other types: *L. innocua*, *P. putida*, *S. hominis*, the requirements placed on disinfecting agents were not fulfilled. The results of experiments proved that bacterial biofilms are resistant to the disinfectant applied in its operational parameters. Disinfecting effectiveness was achieved after twofold increase of the agent's concentration.

Key words: adhesion, biofilm, disinfecting agent

Introduction

Settlement of microorganisms on abiotical surfaces is a very common process in various spheres of life. Solid surfaces that are in contact with water environments are the subject of microbial colonization in the first place. The adhesion of single microorganisms to the solid surfaces gives rise to the formation of a specialized cell culture, called a biofilm.

Because of plentiful nutriments and water, the food processing environment is particularly susceptible to biofilm formation. The presence of *Listeria monocytogenes*, *Bacillus cereus* or *Streptococcus thermophilus* cells on food contact surfaces is about 500 to 50 000 times rarer than the biological films forming by these bacteria (Zottola and Sasahara, 1994). This phenomenon can be the direct cause of product organoleptic changes and food deterioration. Moreover, the adhesion of microorganisms to the solid surfaces may cause food contamination by pathogenic and spoilage microorganisms (Pontefract, 1991).

In the food industry, one of the fundamentals of internal control and good manufacturing and hygienic production (GMP/GHP) are regular cleaning and disinfection procedures, since food safety and quality is determined by the efficacy of disinfecting agents. Literature sources report that biofilm formation by some bacteria can take only 2–4 hours (Yuehuei *et al.*, 1997). Our research confirmed this observation as we noted that *Pseudomonas putida* and *Staphylococcus hominis* formed a biofilm after 4 hours of incubation on a stainless steel surface. For practical and

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economical reasons, in manufacturing plants frequent execution of complex hygienic procedures is impossible. Moreover, the time between particular cleaning and disinfection cycles is from several hours to a few days and that promotes the process of biofilm formation. Thus, there is a need for the adaptation of proper procedures and application of agents that enable the efficient eradication of these bacteria.

The effectiveness of hygienic procedures depends mostly on the right choice of cleaning and disinfecting agents. The so far available antibacterial agents may show lower activity towards phenotypically altered sessile bacteria, since they were developed and introduced into production based on determined high activity against planctonic population of microorganisms using classical measurements – minimal ihibitory concentration (MIC) and minimal bactericidal concentration (MBC) (Czaczyk *et al*., 2007).

Several studies results confirm that microorganisms, being part of the biofilm, can be even 1000 times more resistant to the activity of toxic substances than those that remain in suspension. That makes their elimination from utility surfaces difficult (Fett, 2000; Trafny, 2000; Joseph *et al.*, 2001; Pancer *et al.*, 2004; Robbins *et al*., 2005; Pan *et al.*, 2006).

Microbial cells living in clusters form multilevel defense mechanisms against the destructive impact of antimicrobial substances. The nature of the structure of biological films causes slower diffusion of antimicrobial agents through the biofilm matrix composed of polymeric substances such as extracellular polymeric substances (EPS) or proteins and nucleic acids. It also makes it difficult for toxic agents to reach the deeper layers cells in a biofilm (Czaczyk *et al.*, 2007). Resistance is related as well to the specificity of metabolic and genetic changes induced during the growth phase of biofilm co-forming cells. These are mainly growth velocity decreases and biosynthesis of extracellular polysaccharides, enzymatic proteins and formation of proteins known as efflux pumps (Ma *et al.*, 1996; Nikaido, 1996; Berthold, 2007). The chemical communication of cells, called *quorum sensing*, based on the production of extracellular signal molecules in adverse environmental conditions, is also classified to the defense mechanisms of biofilm microorganisms (Golovlev, 2002).

The essence of the research reported herein was to confirm the assumption that the resistance of microorganisms to disinfecting agents is the outcome of their biofilm formation ability. To accomplish this, the bactericidal efficacy of disinfectant, commonly used for disinfection of equipment in the food industry was tested against bacterial biofilms formed on the stainless steel surface.

Microorganisms chosen for experiments dominate among life microflora isolated from food contact surfaces after cleaning and disinfection process. This meant that they exhibit resistance to the disinfectants, and therefore probably have a strong ability to create biofilms. These microorganisms are considered to be non-pathogenic, although some instances of their isolation from opportunistic infections have been reported. They rank as microorganisms responsible for crucial food components changes, related with synthesis of food quality lowering products, and are often defined as SSO (specific spoilage organisms) (Nowak and Piątkiewicz, 2008).

Experimental

Material and Methods

Bacterial strains and growth conditions. The bacterial strains used were *Micrococcus luteus*, *Staphylococcus hominis*, *Pseudomonas putida*, *Listeria innocua*, isolated from food contact surfaces, after a cleaning and disinfection process in a factory without CIP (clean-in-place) system. Stock cultures kept at –25°C in 20% glycerol were spread on tryptic soy agar (TSA) and incubated for 22–24 h at 30±1°C. A single colony of each strain was grown in 100 ml tryptic soy broth (TSB) at 30°C for 48 h to obtain bacterial suspensions of cells at the end of the logarithmic phase, at a density of 10^6–10^7 cfu/ml.

Preparation of stainless steel coupons. Stainless steel type 304 with number 4 finish was used to prepare coupons (5×5×0.1 cm). Coupons were cleaned with acetone to remove grease and were etched by submering in 5N HCl for 15 min and then cleaned in detergent solution The coupons were rinsed with deionized water, allowed to dry at room temperature, and then autoclaved at 121°C for 15 minut (Joseph *et al*., 2001).

Bacterial biofilm analysis. The sterile coupons were placed in sterile Petri dishes containing 2 ml bacterial suspension in TSB and 18 ml low nutrient medium TSB diluted ten times. After incubation at 20°C for 48 h, the samples were aseptically removed, washed in sterile phosphate buffer saline (PBS) to remove unattached cells and placed in Petri dishes with fresh sterile TSB. This procedure was repeated three times every 48 hours to complete the biofilm formation. To enumerate biofilm cells after eight days of incubation, the samples were washed with sterile PBS and the biofilm cells were removed by swabbing with sterile cotton swabs. The swabs were transferred to 100 ml 0.85% physiological saline peptone water, shaken vigorously and enumerated by standard spread plate technique. TSA was used for enumeration and plates were incubated at 30°C for 48–72 h. The bacterial biofilms on stainless steel were also recorded

Table I
Reduction of planctonic cells after 10 minutes exposure to usable concentrations of sanitizer.

Sanitizer concentration (%)	Contact time (min)	Mean population (log cfu/ml) and reduction (log cycles) of bacteria							
		Micrococcus luteus		*Pseudomonas putida*		*Staphylococcus hominis*		*Listeria innocua*	
		Population	Reduction	Population	Reduction	Population	Reduction	Population	Reduction
0,5%	10	8,15±0,19	6,04±0,29	8,00±0,06	7,38±0,35	8,03±0,11	6,34±0,52	8,04±0,13	6,07±0,41
1%			7,36±0,27		ND[a]		7,52±0,49		7,32±0,48

There are means and the standard errors of the means of triplicate in the table.
[a] ND no colonies detected in undiluted samples

by using scanning electron microscope 3000 N Hitachi. The stainless steel coupons (1×1×0.1 cm) with the biofilms before analysis were covered with a thin layer of gold.

Disinfectant agent. The disinfectant agent containing hydrogen peroxide and peroxyacetic acid was designed for use in the food industry. The active ingredients were: 25–30% hydrogen peroxide, 2–5% peroxyacetic acid, and 5–10% octanoid acid. The useable concentration of disinfecting agent was 0.5–1%. The sanitizer was diluted to the required concentrations with sterile deionized water. In the experiment the disinfecting agent was used in concentration 0.5%, 1%, 1.5%, 2% and the contact time for testing the sensitivity of biofilm cells was 10 and 30 min. The effectiveness of sanitizer to planctonic cells was tested in concentration 0.5% or 0.1% for 10 min.

Treatment of planctonic cells with disinfectant agent. Bacterial test suspension at a density from 1.0×10^8 cfu/ml to 5.0×10^8 cfu/ml was added to each tube containing 9 ml of disinfecting agent in concentration 0.5% or 1%. A timer was set and the contents were mixed in a microshaker. After 10 minutes the tested mixture was transferred to a membrane filter apparatus, which contained a membrane filter (∅ 0.45 mm) and 50 ml PBS and the whole was filtered. The filter was washed with 300ml PBS and transfered into Petri dishes with TSA. The number of cfu/ml was calculated after incubation at 37°C for 24–48 h. The decrease of bacteria count was calculated from the formula [log (N/N_a)], where N is the initial count of cfu/ml prior the treatment and N_a is the cfu/ml after treatment with disinfecting agent.

Treatment of biofilms with disinfectant agent. To test the sensitivity of biofilm cells to disinfecting agent, samples with biofilm were dipped in disinfecting agent solutions in the concentration of 0.5%, 1%, 1.5% and 2% for the contact time 10 and 30 min. The samples were then removed and rinsed with sterile PBS. The cells were enumerated after swabbing as described above using 0.85% physiological saline peptone water containing 3% Tween 80 and 0.3% lecitin and plating on TSA. Plates were incubated at 30°C for 48–72 h. The resistance of the biofilm to sanitizer was measured by a decrease in log values [log (N/N_a)].

Results

Bacterial adhesion to stainless steel. Microorganisms used in the examination showed biofilm formation ability on stainless steel (type 304, no 4 finish). After 8 days of incubation 20°C a thick multilayer biological film on the total plate surface was formed by *M. luteus* and *P. putida* (Fig. 1A, 1B), whereas *S. homins* and *L. innocua* formed biofilm, with a tendency to colonize the current irregularities and fissures on the steel surface (Fig 1C, 1D).

Effectiveness of sanitizer against planctonic cells. The test product in usable concentrations, 0.5%, for 10 minutes resulted in a reduction of viable cells of *L. innocua*, *S. hominis* and *M. luteus* by nearly 6 log cycles and *P. putida* by more than 7 log cycles. An increase of agent concentration to 1% for the same contact time caused complete inactivation of *P. putida*, whereas the count of other microbial cells was reduced by 7 log cycles (Table I). At higher concentrations or longer contact time an overall reduction in test cell suspensions of all tested microorganisms was observed (results not shown)

Effectiveness of sanitizer to biofilms. The cell density in the biofilm before and after the application of disinfecting agent is shown in following diagrams (Fig. 2). The average density of biological films formed by the investigated microorganisms reached appropriately 6.68 log cfu/cm^2 – *M. luteus*; 6.56 log cfu/cm^2 – *P. putida*; 6.13 log cfu/cm^2 – *S. hominis*; 5.87 log cfu/cm^2 – *L. innocu,* after 8 days of incubation at 20°C. Application of disinfecting specimen in operational concentration of 0.5% for 10 minutes showed low effectiveness on the biofilms formed by all the bacteria tested and caused the reduction in the number of cells by only about 1–2 log cycles. After 30 min contact time reduction of 3–4 log cycles was observed. Use of the highest concentration recommended by the producer, 1% in 30 minutes, resulted in a reduction in the number of cells by 5 logarithmic cycles for only one organism – *M. luteus*. For other types of bacteria the reduction rate of cells was about 3 to 4 log cycles. When the concentration of applied substance increases, with time of contact 10 minutes, a gradual increase in the reduction of microorganisms

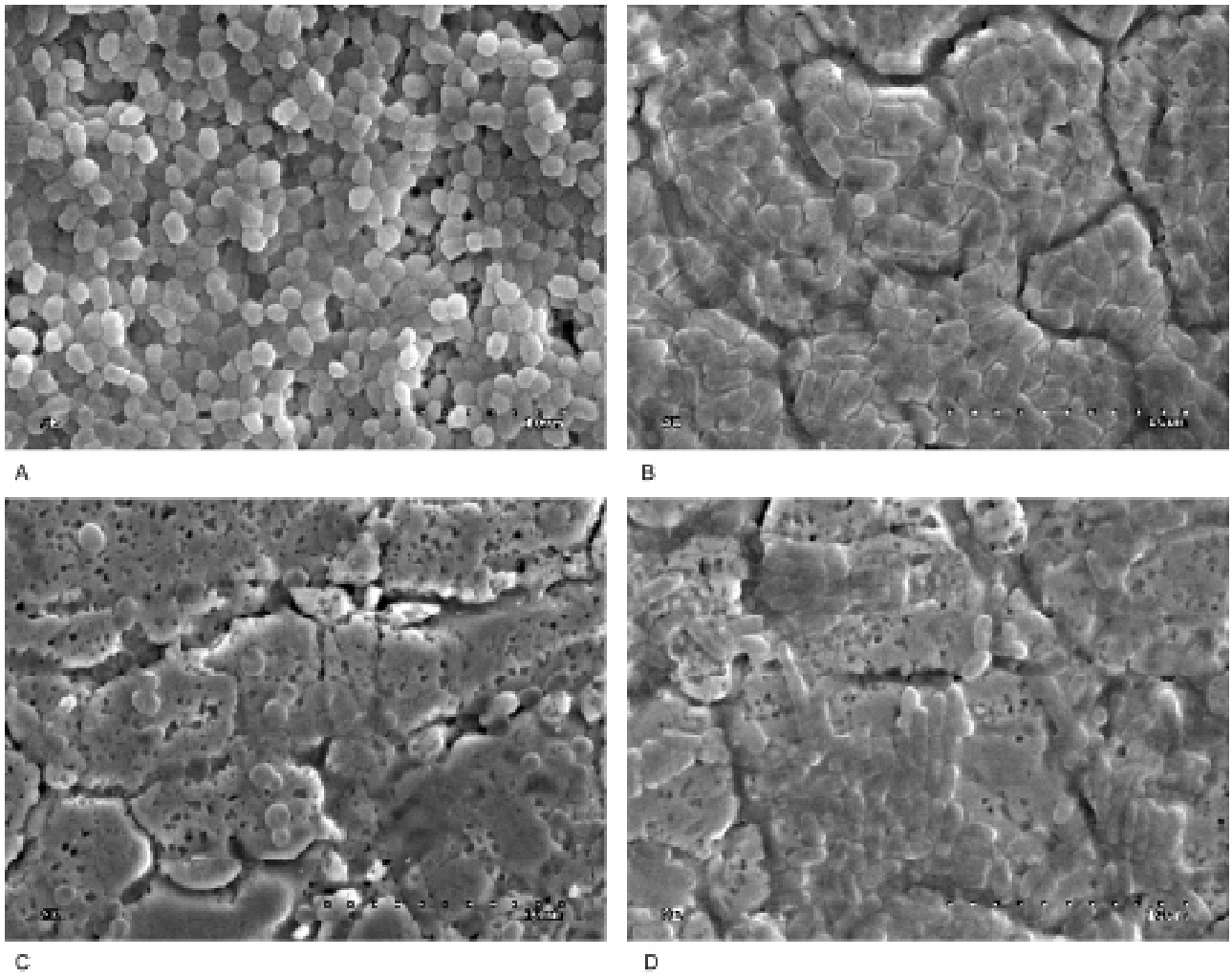

Fig. 1. SEM microphotographs of biofilm: A – *Micrococcus luteus*, B – *Pseudomonas putida*, C– *Staphylococcus hominis*, D – *Listeria innocua* formed on stainless steel for 7 days at 20°C.

in biofilms was observed, although the reduction range was not higher than 5 log. Extending the contact time to 30 minutes resulted in a drop in survival of log cycles. As shown in the figures, only increasing the concentration of the agent twofold and extending the time of contact to 30 minutes brought about a reduction of over 5 log cycles for all the studied microorganisms. The study also indicated that *M. luteus* had a much lower resistance than the other bacteria used in the study – its survival was approximately 1–2 log lower compared to the other species.

Discussion

The purpose of this research was to estimate the antiseptic efficiency of disinfecting agent towards to bacterial biofilms formed on stainless steel. Model studies were carried out on biofilms created in laboratory conditions that simulate the food processing environment. Therefore, the formation of biofilms was carried out in stationary culture for 8 days, oligotrophic conditions of growth were applied and incubation was at 20°C. The objective of this study was to confirm the thesis that the microflora living on production surfaces are resistant to disinfectants, in spite of correctly performed washing and disinfection procedures, and at the base of this resistance is the ability of the microorganisms to form a biofilm.

Research was carried out using microorganisms defined as conditionally pathogenic, which dominated among microflora isolated after cleaning and disinfection. This proves their resistance to applied disinfectants as a possible result of biofilm-forming ability. The experiments carried out by Krogulska (2003) indicate that this group of microorganisms colonizes solid surfaces the most effectively. Moreover, the presence of these microorganisms on the food contact surfaces, determines the deterioration in the quality of food products.

Bacterial biofilm formation on abiotical surfaces is a persistent problem in the food industry. Disadvantageous conditions that prevail after cleaning and disinfection process favor the formation of biological

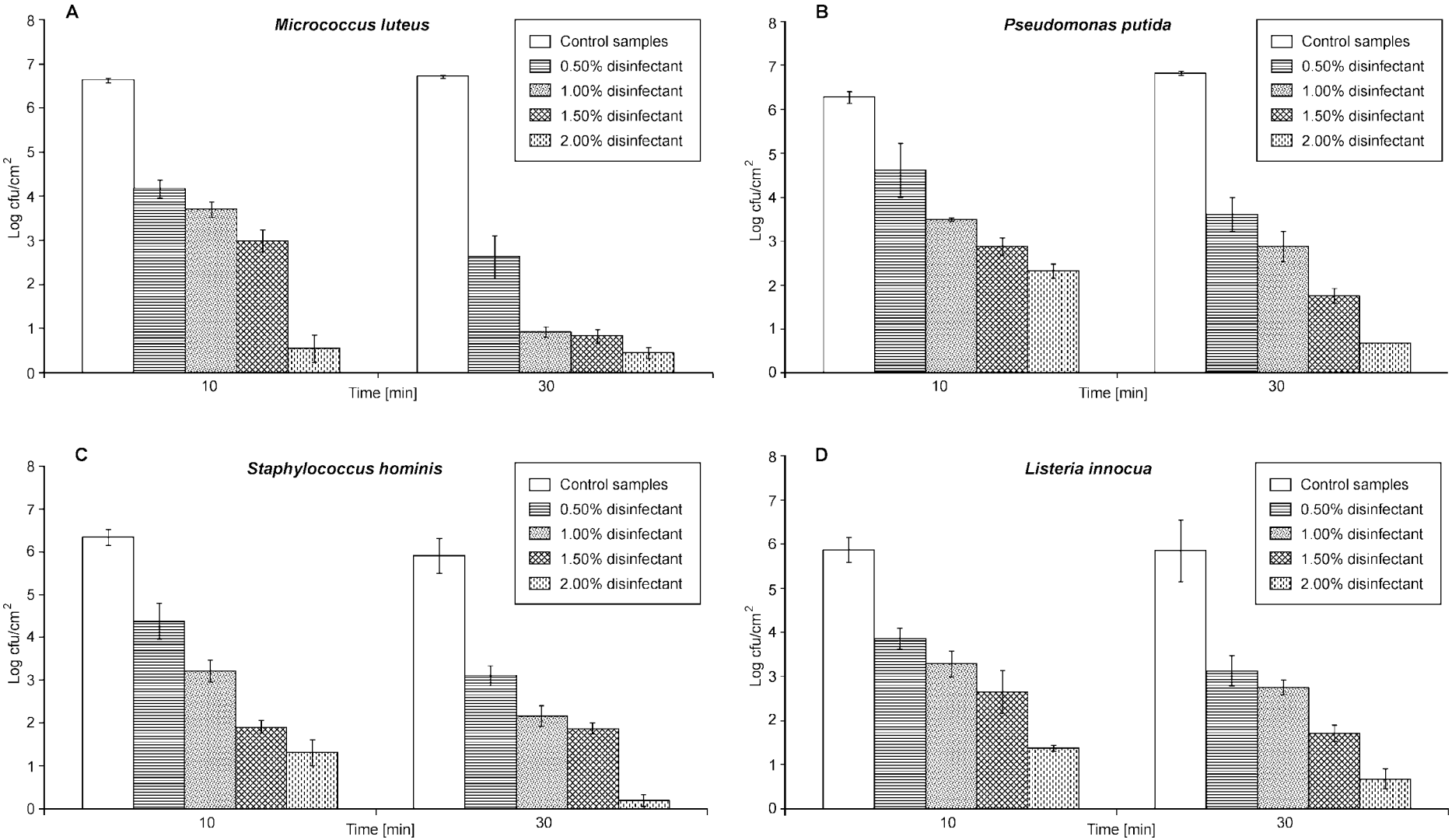

Fig. 2. Effect of tested disinfectant on biofilm formed by *Micrococcus luteus* (A), *Pseudomonas putida* (B), *Staphylococcus hominis* (C) and *Listeria innocua* (D), depending on concentration and contact time.

The white column represents control samples without sanitizer treatment. The colored columns represent samples after sanitizer treatment. The error bars indicate standard deviation.

films. Several studies results prove that the adhesion of bacterial cells to abiotic surfaces is more intensive in hunger conditions. Morphological and physiological changes such as metabolic activity decrease or excessive secretion of extracellular molecules are major determinants of this phenomenon (Fleming and Wingender, 2001; Folsom *et al.,* 2006).

Food production in terms of microbiological safety requires effective eradication of biofilms formed by microorganisms appearing naturally in various ecosystems. The studies conducted as well as several literature sources show that the inactivation of microorganisms in biological films of usable materials is not effective enough (Lee Wong, 1998; Chmielewski and Frank, 2003; Midelet and Carpentier, 2004; Ingham, 2006). Difficulties in elimination of microbiological biofilms from solid surfaces mainly due to ignorance of the characteristics determining the resistance of microorganisms belonging to different taxonomic groups. The result is that the commonly available disinfectants are not suitable for inactivation of biofilm.

Properly carried out disinfection should result in the reduction of microorganisms by at least 5 logarithmic cycles (EN 1040, 2006; EN 1276, 2000; Borycki *et al.*, 2008). The test disinfectant in useable concentration 0.5%, and 10 minutes reduced the number of cells in the biofilm by about 1–2 logarithmic cycles. Only after doubling the concentration to 2%, with a long-time contact of 30 minutes, the disinfectant met the requirements specified in the standard in relation to the cells in the biofilm. Bacteria in suspension were effectively reduced at a concentration of 0.5%, for 10 minutes.

Results of realized experiments showed that microorganisms staying on production surfaces are able to formation of biofilms and they are more resistant to disinfecting agents than planctonic cells. This was also confirmed by other authors. Joseph *et al*. (2001) investigated *Salmonella* spp. biofilms sensitiveness formed on various surfaces. They demonstrated that fivefold active chlorine concentration and twice longer time is needed for *Salmonella* spp. biofilm inactivation of 6 log cfu/ml cells density on stainless steel than for the complete cells reduction in equal density suspension. Similarly, the experiments carried out by Robbins *et al*. (2001), have shown that the effective inactivation of *Listeria monocytogenes* cells in a biofilm requires the use of a twice higher concentration of hydrogen peroxide, than to inactivate planctonic cells.

The high resistance of cells in the biofilm is being explained, among other things, by slower diffusion of antimicrobial agents through the biofilm matrix, which make it difficult to reach the deeper biofilm layers. The rate of diffusion of chemical substances through the biofilm layers can be even 60–80% slower. Moreover, cells that stay in the suspension are exposed to toxic substances on all sides and cells in the biofilm only from one direction (Myszka and Czaczyk, 2007).

To elaborate effective strategy of biofilms removal from food contact surfaces, it is essential to get to know adhesive properties and resistance factors of microorganisms that live in various environment conditions. It is well known that the crucial role in formation of biofilm resistance to antimicrobial specimens is played by the EPS protective layer formed by the colonizing cells. Research carried out by Szumigaj *et al*. (2008) and by Czaczyk *et al.* (2004) showed that excessive secretion of extracellular molecules takes place when the access of nutrients is limited. These extracellular molecules, as a result of solid surface adsorption, mediate in the cohesion and adhesion of microorganisms. Exopolysaccharides are also the main component of a highly hydrated glycocalyx layer that enables to immobilize the microcolony of cells and protects them against biocides and other antimicrobial substances negative effect (Costerton *et al*., 1995).

Three among tested microorganisms (*L. innocua, S. hominis*, *P. putida*) had similar resistance ability to disinfectant used in experiments. Thus, it is advisable to assume that bacteria, adapted to environmental conditions, which have been isolated, have developed similar mechanisms of resistance.

Among all tested microorganisms, *M. luteus* showed the lowest resistance to the specimen compared to other microorganisms. The reason may be due to different properties of the bacterial cells themselves, but also creating the monoculture biofilm during experiments. Biological films that are formed in natural environment conditions, can be composed of one to several species and their functioning is based on particular microorganisms interaction. Metabolites of single species of microorganism can stimulate the growth of other biofilm matrix co-forming cells (Czaczyk, 2004; Dunne, 2002). Studies by Burmolle *et al.* (2006) proved that the synergistic interactions that occur in non-homogeneous biofilms can bring about an increase in their resistance to antimicrobial agents in comparison with homogenous biofilms. It can be assumed, that in the natural environment, the resistance of *M. luteus* could be further enhanced by the presence of other microorganisms, which would explain such frequent isolation of this microorganism from the production surfaces.

The results of our experiments indicate that the tested disinfectant has eradicating activity towards to planctonic cells as well as cells in a biofilm. However, it must be stated that biofilms are characterized by much higher resistance than cells in suspension and they require the application of disinfectants in higher concentrations. Therefore, hygienic procedures performed in the factories, with the use of chemicals should also take biofilms into account.

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ORIGINAL PAPER

Assessment of Microbial Growth on the Surface of Materials in Contact with Water Intended for Human Consumption Using ATP Method

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Abstract

Elaboration of an assessment method for plumbing materials contacting drinking water was the main purpose of this study. The investigation was conducted in 8 week cycles in dynamic conditions using a continuous flow reactor. Microbial growth was measured indirectly by a bioluminescence technique (ATP assay). Every week swabs from the surface of tested materials (polypropylene and different types of polyethylene), from the domestic market were collected and the level of bioluminescence was examined. The results obtained from the surface of tested materials were repeatable and clearly approximated those obtained from the surface of a negative control (stainless steel, low susceptibility for microbial growth). The level of bioluminescence (ATP) on the surface of positive control (paraffin, high susceptibility for microbial growth) was many times higher than that observed on other materials. The presented investigation was the main part of a validation process, which in short time will serve to initiate a complete assessment system for organic materials contacting drinking water.

Key words: ATP, bioluminescence, biofilm, continuous flow reactor, plumbing materials

Introduction

A biofilm is a characteristic living structure growing on every type of surface contacting the water environment. The occurrence of biofilm on the inner surface of plumbing materials like organic ones (PE – polyethylene, PP – polyprophylene, PVC – polyvinyl chloride, PB – polybutylene), steel, cast iron pipes and even copper pipes is well known (Kooj van der *et al.*, 1995; Niquette *et al.*, 2000; Zacheus *et al.*, 2000; Camper *et al.*, 2003; Lehtola *et al.*, 2004; Kim *et al.*, 2006). Microorganisms together with organic and inorganic compounds constitute the main part of this dynamic biological structure which is the prevailing microbial lifestyle in natural microenvironments. The majority of microorganisms have good adhesion properties. The structural development of biofilm formation has been described as a sequence of events including attachment, microcolony development and finally, the establishment of distinct structures as previously described (Martiny *et al*., 2003). At first, the process of the attachment bacteria cells to the surface of a material is reversible. Depending on the size and charge of particle, compensation of ion concentration occurs on both sides of the growing biofilm structure and bacteria forming microcolonies starts to produce organic polymers such as polysaccharides and glycoproteins which is the first stage of the biofilm formation process. Intensive production of polymers allows for permanent attachment to the surface and building of a mature and complicated biofilm structure with diverse microniches. Due to different oxygen, nutrient and metabolite concentration, high level of diversity in microbial communities occurs in every microniche. Depending on specific conditions inside a biofilm structure, microorganisms such as bacteria, fungi, molds, algae, nematodes and protozoans can be found. From among bacteria isolated from cold water biofilms, *Pseudomonas* sp., *Flavobacterium* sp. and *Acinetobacter* sp. are among the most common groups. Bacteria from the genus *Legionella* represent 35 per cent of microorganisms forming biofilms inside hot water tanks and hot water plumbing systems (McBain *et al.*, 2003; Mampel *et al.*, 2006). Biofilms formed in water distribution networks are known to cause public health problems. Elaboration of a method for the assessment of susceptibility of microbial growth on the surface of materials contacting drinking water may provide new tools for improving water quality for the consumer.

According to EU Directive 89/106/EEC concerning construction products and drinking water Directive 98/83/EC, every material contacting with drinking

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water should be examined for its susceptibility for microbial growth. Positive results obtained for examined materials or construction products will allow to issue a CE-EAS certificate on the basis of which admission to use materials and products concerned will be granted. Among the members of European Union there is no single, standardized, unified method and assessment criterion for this evaluation. Every country has its own legal regulations and examination methods regarding materials in contact with drinking water. In some countries, for instance in Germany, Great Britain, the Netherlands, Austria and a few more, different testing and assessment methods for microbial enhancement on materials already exist (van der Kooij *et al.*, 2003). The process of unification of this method is difficult and no rational conclusion has yet been found, In Poland there is no official examination method for evaluation of microbial growth on the surface of materials in contact with drinking water. The National Institute of Public Health – National Institute of Hygiene is a Polish institution authorized to issue hygienic certificates based on the results of chemical testing and assessment of chemical composition of product or material. The hygienic certificate entails admission to use a given material or product in contact with drinking water. The completion of documentation with additional results of microbial growth level will improve Polish regulation according to articles of the EU Directives mentioned above.

The purpose of the present study was to elaborate and implement a Polish method for estimating the susceptibility of materials contacting drinking water on microbial growth.

Experimental

Materials and Methods

Tested materials and products. The assay was conducted on four different pipe materials currently admitted for use in contact with drinking water. Materials included in the investigation were as follows: polypropylene (PP), polyethylene (PE), polyethylene RC (PP-RC) and a multilayer pipe with an aluminum layer (PE-X-Al). As control positive and negative were used glass plates covered with paraffin (SP) and stainless steel plates (S), respectively. All the samples of materials were tested in two or three repetitions.

Continuous flow reactors. Two continuous flow reactors – UPE (polish specific name) working simultaneously and supplied from cold tap water were used in this investigation. The UPE project was elaborated together with employees of Hydro-Engineering and Hydraulics Department from Environmental Engineering Faculty at Warsaw University of Technology. Figure 1. presents the project of continuous flow reactor in detail. The cylindrical body of UPE (inside diameter was 150 mm, high was 550 mm) was made of

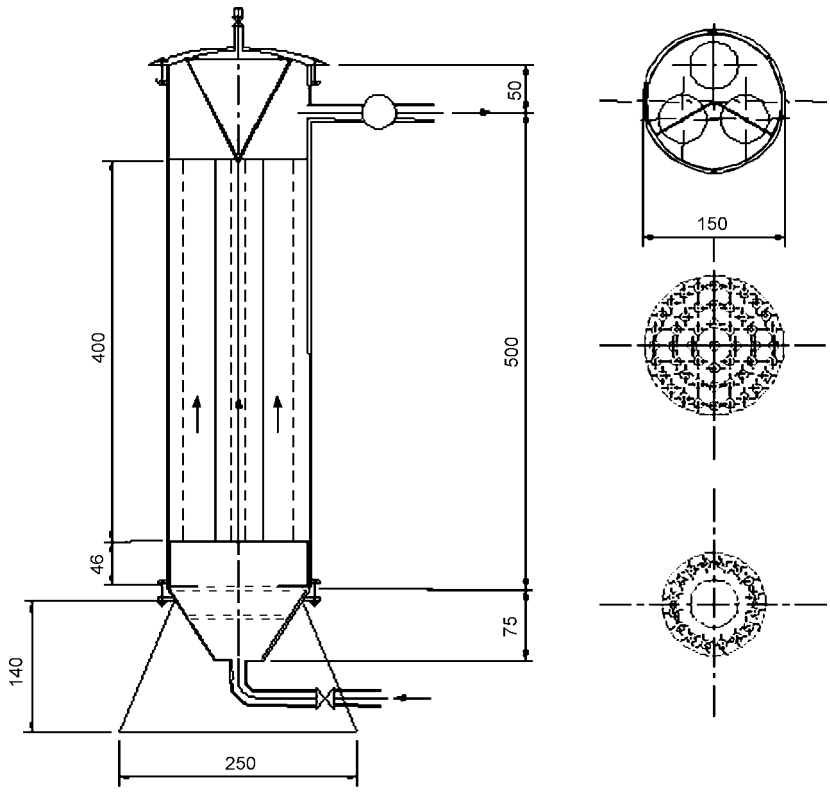

Fig. 1. UPE technical diagram: continuous flow reactor.

high quality stainless steel with teflon seal. On top of the reactor was a removable cover with venting valve.

Water inlet wire was made of teflon and water pressure was regulated by a ball valve. Inside the reactor, at the bottom, a conical diffuser with two partitions was located. A special sample stand made of stainless steel was placed in vertical position inside the cylinder. The water outlet with water meter was located on the side of the UPE about 50 mm below the top. The water flow proceeded from the bottom to the top of reactor so that the reactor could be filled with water evenly and this flow direction protected against the mixing of inlet and outlet water. The average water flow was established at 1 m^3/day.

ATP assay. Luminometer HY-LiTE 2® (MERCK) was applied for the determination of ATP level in swabs taken from the surface of tested materials and controls. A sample measured with this instrument is indicated on the display in relative light units (RLU). The RLU value is directly proportional to the quantity of ATP in the sample tested and therefore also directly proportional to contamination with biological material. The luminometer has a built-in temperature compensation and calibration self check. The test system consists of ready tests, the "Pens" with proper dose of reagents so test results can be obtained in a short time. The linear working range for HY-LiTE 2® is 0–99.000 RLU (0–5.00 log 10 RLU). The tested sensitivity of the used luminometer is 1 RLU.

Sample preparation and testing procedure. Every sample of material (80 cm^2 surface) before examination was mechanically cleaned, treated with 70% ethanol and washed with deionized water. After this pretreatment swabs from the surface were collected for the verification of ATP level on day 0. After the first assay all samples were placed on a stand inside the continuous flow reactor. The examination was conducted during 60 days and every seven days the ATP concentration from the surface of tested pipe materials and controls was determined. The swabs were taken from 2 cm^2 surface of every sample in five repetitions. Temperature of water and flow strength was monitored throughout the whole examination period.

Results and Discussion

The main principles of the presented method are elaborated on the basis of techniques used in food industry which is wide field for bioluminescence assays used to determine microbial contamination in a specific food product, surfaces contacting with food and hygiene monitoring (Hawronskyj and Holah, 1997; Breeuwer and Abee, 2000; Aycicek *et al.*, 2006; Bzducha, 2007). Conventional methods of microbiological analysis, such as plate cultures, swabbing, wash-and-rinse and other are time-consuming and laborious. Time is a very important part of the production cycle. Since the results are obtained 48 or 72 h after sample collection, it is impossible to undertake corrective actions (Cais-Sokolinska and Pikul, 2008). When the cleanliness status of the examined objects is monitored by bioluminescence methods using findings concerning cell biochemistry and the physicochemical properties of microorganisms (Cho and Yoon, 2007), safety assurance is much more facilitated (Larson *et al.*, 2003). An important advantage of this method is short time from collecting a sample to the result, high sensitivity and easy performance (Squirrell *et al*., 2002).

ATP is an energy carrier in all living organisms, linking catabolism and biosynthesis. In the presented method the RLU values, directly proportional to the quantity of Adenosinetriphosphate (ATP), were the main biomass parameters for testing the growth – promoting properties of materials. This type of assay has a number of advantages over the use of other parameters of assessment the presence of microorganisms, as it: includes all active microorganisms, is absent in dead (inactive) microorganisms, enables rapid testing, has low detection limit.

The principle of this assay is based on the firefly luciferine – luciferase reaction, which results in the emission of one quantum of light for each molecule of ATP hydrolyzed.

Results based on bioluminescence assay presented below are a consequence of research on the growt-promoting properties of materials in contact with

Table I
Bioluminescence level on the surface of polypropylene pipe material (PP) placed in UPE1.

Material	Bioluminescence level during successive weeks of conducted assay [RLU/cm^2]								
Weeks	0	I	II	III	IV	V	VI	VII	VIII
PP (1)	10	90	48	234	330	400	324	549	109
PP (2)	12	94	34	90	219	145	70	159	56
PP average	11	92	41	162	274	272	197	354	82
PC*	16	16834	9167	12834	13125	20834	36500	43667	32500
NC*	15	44	62	209	113	49	74	254	75

* PC – Positive Control, NC – Negative Control

Table II
Bioluminescence level on the surface of polyethylene pipe material (PE-80) placed in UPE1.

Material	Bioluminescence level during successive weeks of conducted assay [RLU/cm^2]								
Weeks	0	I	II	III	IV	V	VI	VII	VIII
PE-80 (1)	12	159	39	43	195	188	74	75	82
PE-80 (2)	10	164	38	35	170	69	95	67	42
PE-80 average	11	161	38	39	182	128	84	71	62
PC*	16	16834	9167	12834	13125	20834	36500	43667	32500
NC*	15	44	62	209	113	49	74	254	75

* PC – Positive Control, NC – Negative Control

Table III
Bioluminescence level on the surface of polypropylene (PP-RC) pipe materials placed in UPE2.

Material	Bioluminescence level during successive weeks of conducted assay [RLU/cm^2]								
Weeks	0	I	II	III	IV	V	VI	VII	VIII
PP-RC (1)	12	22	42	50	94	86	60	535	32
PP-RC (2)	15	59	67	49	143	88	58	426	50
PP-RC (3)	11	22	48	67	105	71	58	808	48
PP-RC average	13	34	52	55	114	82	59	590	43
PC*	23	12334	10450	38834	39832	23834	38667	41667	28250
NC*	16	42	115	197	159	187	54	159	97

* PC – Positive Control, NC – Negative Control

drinking water conducted since 2006 (Szczotko *et al*., 2008; Szczotko *et al*., 2009). The applicability of the continuous flow reactor prototype (UPE) was confirmed in 2007. Optimal environmental conditions and duration of this test (8 weeks) were defined and significant differences in the velocity of microbial growth on the surface of few materials contacting drinking water were demonstrated. On the basis of the obtained results optimal time and specific condition of test method were established.

We examined and compared the biofilm formation process on the surface of a few materials contacting drinking water. All the results demonstrated in the tables below were obtained during examination conducted in 2008 and are presented in RLU/cm^2 (Table I).

At the end of the second week of examination of bioluminescence measured on the surface of both PP samples was similar. Beginning from the third week, the results on the surface of PP (1) were over twice as high as on the surface of PP (2). The results of the first assay (week 0) presented an appropriate method of pretreatment applied and amounted 10 RLU/cm^2 and 12 RLU/cm^2 for PP (1) and PP (2), respectively. After the first week of examination, the bioluminescence level increased almost ten times. In successive weeks microbial growth was less rapid. The highest results were obtained after the seventh week when the bioluminescence level reached 549 RLU/cm^2 on the surface of PP (1) and 219 RLU/cm^2 on the surface of PP (2). The average end result for PP samples was 82 RLU/cm^2, this being similar to the result obtained on the surface of stainless steel negative control. Microbial growth on the surface of paraffin negative control was very rapid and bioluminescence measured in the eighth week amounted to 32500 RLU/cm^2 (Table II).

The results obtained in swabs taken from the surface of both PE materials were similar except for those received in the fifth week. The highest microbial growth was detected in the first week of the experiment and in the following weeks the bioluminescence level slightly decreased. The average end result for PE samples was 62 RLU/cm^2. Results obtained during eight weeks of examination clearly showed a similarity between PE pipe materials and stainless steel negative control. Bioluminescence measured on the surface of negative control sample was five hundred times higher (Table III).

Fluctuations of the bioluminescence level measured on the surface of PP-RC pipes samples during eight weeks of examination were insignificant. Only in the seventh week the results from all three samples were high but decreased just one week later. The average level of bioluminescence at the end of the investigation amounted to 43 RLU/cm^2 and was lower with reference to results obtained from the surface of the negative control sample. Microbial growth on positive and negative samples placed inside both continuous flow reactors (UPE1 and UPE2) were very similar (Table IV).

Changes in the bioluminescence level measured from swabs taken from the surface of multilayer pipes

Table IV
Bioluminescence level on the surface of multilayer polyethylene and aluminum pipe material (PE-X-Al) placed in UPE2

Material	Bioluminescence level during successive weeks of conducted assay [RLU/cm^2]								
Weeks	0	I	II	III	IV	V	VI	VII	VIII
PE-X-Al (1)	11	21	70	46	275	134	838	314	162
PE-X-Al (2)	16	21	132	32	198	290	1022	465	214
PE-X-Al (3)	13	50	61	49	300	260	572	273	194
PE-X-Al average	13	31	88	42	258	228	811	351	190
PC*	23	12334	10450	38834	39832	23834	38667	41667	28250
NC*	16	42	115	197	159	187	54	159	97

* PC – Positive Control, NC – Negative Control

(PE-X-Al) were characterized by slow increase during the first six weeks of investigation. In the sixth week bioluminescence values collected from all three pipe samples were the highest and the average value was 811 RLU/cm^2. During the next two weeks all values significantly decreased and the average bioluminescence in swabs taken from the surface of the tested samples was 190 RLU/cm^2 at the end of examination. The result obtained from the surface of the negative control was slightly lower.

The temperature of water flowing through both continuous flow reactors was relatively stable and average values were between 20.6°C and 21.2°C for UPE1 and UPE2, respectively.

On the basis of the obtained results relatively low susceptibility for microbial growth of tested material samples was observed. The presented changes in the bioluminescence level on the surface of each material approximated the values obtained from the surface of negative control (stainless steel). All presented results were low with reference to positive control (paraffin plates) which showed even a few hundred times higher bioluminescence level. The data obtained indicated that applied laboratory equipment and the test method developed were sufficient to assess the growth-promoting properties of materials contacting drinking water. Both positive and negative control materials are suitable for this method (Rosmaninho *et al.*, 2007). Owing to a very sensitive bioluminescence test method applied in this investigation, it was possible to conduct precise monitoring of biofilm growth since the first days of its formation process and afterwards – during the entire 8 week study period. Each assay was relatively simple and results were obtained in a very short time. The presented testing method is currently a proposal for solving the problem of microbiological contamination of drinking water in water distribution systems, particularly in case of different plumbing materials. Several EU countries already have legal regulations concerning the assessment of growt-promoting properties of materials contacting with water and laboratory test methods have been put into practice for many years in national scientific centers and service laboratories.

In the United Kingdom the MDOD (Mean Dissolved Oxygen Difference) according to has become the standard method (BS 6920–2.4). In this test additional oxygen consumption in the presence of the material to be tested is used as parameter for microbiological activity. The method has been used for several decades and a great deal of materials have been tested. Typical MDOD values range from about 0.5 mg/l (glass – negative control) to values of 8 mg/l for paraffin wax (positive control). Materials with a MDOD value above 2.3 mg/l are considered not suitable for use in contact with water intended for human consumption.

The slime production test according to DVGW Technical Standard W 270 test developed in Germany has also been used for nearly 25 years. This method is applied in a dynamic system, with sheets of materials in contact with continuously flowing tap water. The volume of slime on the surface of the tested material is used as a biomass parameter. Typical SP values range from less than 0.1 ml (stainless steel – negative control) to more than 15 ml on solvent – containing bitumen or plasticized PVC. Materials with SP value above 0.1 ml are considered unsuitable for use in contact with drinking water.

In the Netherlands BPP (Biomass Production Potential) Test has been elaborated. In this test adenosinetriphosphate (ATP) is used as parameter for active biomass and test is carried out as a static test without replacement of the water (van der Kooij *et al.*, 2001). ATP method enables the detection of very low concentration of microorganisms and the analysis can be conducted within a few minutes. ATP analysis is also used for determining the biofilm concentration on the walls of distribution system pipes and in the biofilm monitor for determining the Biofilm Formation Rate (BFR) values of drinking water.

An alternative standardized method for testing of pipe materials in contact with drinking water has recently

been published in Austria (Önorm B 5018–1,2002). Pipes are fixed in a vertical position and their bottom ends are closed by mean of foamed PE – stoppers. Next, pipes are filled with tap water and incubated at 22°C with weekly water replacement. The water is aerated from below. Heterotrophic Plate Count (HPC) is determined according to EN ISO 6222 at 22°C after one, two and three months of incubation. At the end of the test biofilm grown on the inner pipe wall is assessed using the ATP – method. The evaluation is performed in relation to a negative (glass pipe) and positive control (PVC – P tube). Pipes are not recommended for use in contact with drinking water if the HPC value of the test water is 10 times higher than in the glass control or when biofilm on the pipe specimen is 5 times higher than in the glass control.

A variety of methods for testing of products impede the uniform assessment procedures and the development of European market. In this situation there is a great need to put into practice a Polish testing method with its own specific requirements.

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ORIGINAL PAPER

Biodeterioration of Optical Glass Induced by Lubricants Used in Optical Instruments Technology

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Abstract

The process of biodeterioration of optical glass was studied after being induced by an auxiliary material (lubricant 4CKP) used in the production of optical instruments. It was determined that the lubricant can initiate growth of conidia of *Aspergillus niger* fungus. Acid spawn metabolites cause deterioration of the glass surface. Measurements of laser light beam transmittance through the glass plate and the AAS chemical analysis method of the post-culture fluid allowed to determine that glass with a high SiO_2 content is most resistant to corrosion caused by the growth of *A*. *niger* fungi spawn

K e y w o r d s: atomic absorption spectrometry (AAS), biodeterioration, lubricant, optical glass, transmitation of laser beam

Introduction

The ubiquitous existence of microorganisms in all natural environments as well as their unusual capability to adapt to various conditions is the cause of biological deterioration and corrosion of most existing technical materials.

It would be difficult to mention a group of materials not affected by microorganisms either in a destructive way or at least with a change of their properties. Microorganisms change the properties of many materials used among others in construction, packing, fabrics, synthetic materials, lubricants, metals and alloys (Zyska, 2001; Cwalina, 2003; Janda, 2005; Szumigaj *et al.*, 2008).

The process of biodeterioration also affects such a stable material as glass. It was determined already in 1943 in Japan that mould grows on optical glass and this was later confirmed in 1958 in the famous Commonwealth Mycological Institute in Kew, Great Britain (Ohtsuki, 1943; Dade, 1958).

Signs of glass microbiological corrosion were more precisely described by Kerner-Gang in 1968. Pits in the optical glass were caused by *Aspergillus versicolor* conidia already after 5 days. This destructive process is slower when *Penicilium funiculosum*, *Alternaria tenuis* and *Aspergillus fischeri* fungi are present.

Gorbuhina and Palinska (1999) studied the role of fungi and cyanide bacteria in the process of destroying glass produced similarly as medieval church windows. On the glass surface they found a film consisting of bacteria such as *Synechococcus* sp., *Merismopedia glauca*, *Chroococcidiopsis* sp. and fungi *Aureobasidium* sp., *Acremonium* sp., and *Trimmatostroma* sp. After removal of the biological material the glass surface also showed pits, slots and figures.

Reports also mention microbiological corrosion of window glass in the Cister church in Hanau (Germany). It is suspected that in the course of earlier restoration works they were covered with a substance rich in carbon. In summer the relative humidity inside the church reached 80% and the average temperature was 18°C. Such conditions and source of carbon enhanced the growth of fungi on the glass surface (Weissaman and Drewello, 1996).

Growth of fungi was also observed on stained glass windows in a medieval monastery in Hanterive (France). Some 20 types of fungi including *Taeniolina*

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deightonii belonging to the *Demiataceae* family (Kaiser *et al.*, 1996) were isolated from infected glass.

Analysis was also made of the deterioration of historical stained glass windows in the 15th century Cartuja de Miraflores monastery in Spain. Biodeterioration of the window glass was found during renovation conducted in 2003. Pits and slots, often filled with white deposits, were observed on the glass surface. *Aspergillus* type are responsible for this phenomena. Together with bacteria they colonized the glass surface (Carmena *et al.*, 2006).

Fungi are the dominant species in microflora on the glass surface and their metabolites are responsible for the irreplaceable changes and degradation of the structure and composition of this inorganic material (Watkins, 2003).

Fungi growth often deteriorate glass surface and cause its darkening and discoloration. On the other hand, deposits are formed on the surface due to the reaction between mould metabolites and glass components (Weismann and Drewello, 1996; Bartosik *et al.*, 2007).

So far studies of microbiologically induced glass corrosion were mainly focused on the analysis of destructive changes occurring on stained glass and windows in old churches. In this area of research, studies of optical glass resistance to various environmental conditions and especially to microorganisms are much less common. Also in Poland such studies have not been undertaken by research centers.

Estimation of the resistance of optical instruments to microbiological corrosion is important and justified since they are used both in civil and military applications and they can be deteriorated both by environmental and microbiological conditions. Knowledge of biodeterioration of optical glass deterioration is badly needed due to marginal and scarce reference in literature to auxiliary materials used in the technology of optical devices. The main aim of our study was to investigate this phenomenon. Earlier studies showed that lubricants used as auxiliary materials in the production of optical devices can be easily assimilated by conidia of fungi.

Experimental

Materials and Methods

Biological materials. The fungus *Aspergillus niger* ŁOCK 0439 from the Microorganisms Pure Culture Collection Institute of Fermentation Technology & Microbiology Technical University of Łódź were used as the biological material in our studies. They were characterized as an acid generating strain which creates citric, malic and oxalic acids when growing on a mineral background with glucose. *Aspergillus* strains are most often mentioned in literature as agents causing glass corrosion.

Technical materials

Optical glass. The chemical composition of the technical material (optically polished smooth glass discs, 3 mm thick and with 30 mm diameter) is shown in Table I. Light kron type glass lens (BK7 and BaK4) have small coefficients of light diffraction n = 1.45–1.6. Heavy flint type glass lens (SF2 i F2 have coefficients of light diffraction n = 1.55 – 1.9 and they contain Pb (*ca.* 50%).

Lubricant 4 CKP. We studied a lubricant of commercial origin used to lubricate spectacle threads and hinges in optical devices exposed to rotations of the lens and eyepiece. The biodeterioration of optical glass was assessed during the growth of fungi in static and submerged conditions.

Components basal medium Mo. glucose 0–0,1%, $(NH_4)_2SO_4$ 0,3%, KH_2PO_4 0,1%, $MgSO_4$ *7H_2O 0,5%, agar 0–0,3%, aqua dest. p H = 6.0

Cultivation of *A. niger* in static conditions. Samples of optical glass were cleansed by immersing them in ethanol (70% conc.) and then they were placed on Petri plates with aqueous agar. The glass surface was covered with 4CKP lubricant layer and then with a small quantity of Mo medium (0.1% glucose and 0.3% agar) to initiate the growth of microorganisms. The culture was grown in a thermostat at 30°C at

Table I
Chemical composition of the tested optical glass

Item	Glass type	Sample mass [g]	Mass composition [%]	
1	BK 7	7.8	SiO_2	68,9
			B_2O_3	10,1
			Na_2O	8,8
			K_2O	8,4
			BaO	2,8
			As_2O_3	1,0
2	BaK 4	6.4	SiO_2	59,6
			B_2O_3	3,0
			Na_2O	3,0
			K_2O	10,0
			ZnO	4,8
			BaO	19,0
			As_2O_3	0,6
			PbO	0,3
3	SF 2	7.8	SiO_2	40,9
			Na_2O	0,5
			K_2O	6,8
			PbO	50,8
			As_2O_3	1,0
4	F 2	7.3	SiO_2	46,96
			K_2O	6,30
			PbO	46,91
			As_2O_3	0,23

controlled relative air humidity of abt. 80% during 3, 6 and 12 months. To prevent drying, the samples were placed in plastic containers. After incubation time, the glass surfaces were cleansed with ethanol and surface changes were observed under of a phase-contrast microscope. Photos of the surfaces were recorded with a high resolution CCD digital camera and then processed with MicroScan graphic programs.

The destruction of the glass surface was also assessed using a helium-neon laser which was emitting a beam with the wavelength of $\lambda = 0.6328$ μm and power of P = 7 mW. The laser beam was directed perpendiculary at the surface of the optical glass. The samples were placed on an optical stand with the possibility of precise movement. The light beam power was measured after it passed through the studied sample. The power measurements for particular glass samples were made in 5 randomly chosen points along the glass diameter. A glass disc that had not been subjected to the influence of microorganisms was the reference sample.

Mean beam power I_{sr} was calculated on the basis of five measurements of transmittance through a parallel wall plate with a perpendicular light using the following formula:

$$I_{sr} = \frac{\sum_{i=1}^{n} I_i}{n} \quad (1)$$

Where: I_i – intensity (power) of the laser beam after passing through the sample; n – number of measurements.

We also estimated the transmittance of the laser light T through the optical glass sample and this parameter was connected to the thickness of the glass plate using formula (2):

$$T = \frac{I_{sr}}{I_0 \times g} \quad (2)$$

Where: I_{sr} – mean power of the laser beam after passing through the sample [mW]; I_0 – intensity (power) of the laser beam in air [mW]; g – thickness of the glass plate [mm]

It must be underlined that in these studies T is the transmittance connected to unitary sample thickness.

Results of measurements of the light transmittance through samples subjected to deteriorating conditions are shown as histograms. Histograms are also shown for laser transmittance through an unaffected clean reference glass sample.

Growth of *A.niger* submerged conditions. Sterile optical glass samples were placed in polypropylene Erlenmeyer bulbs with a liquid medium Mo with pH = 6 with the addition of 5% 4CKP lubricant (no glucose). Such samples were then infected with a suspension of *A. niger* spores with the density of 10^6/ml and at 2% in relation to the medium volume. The culture was grown for 240 h at 30°C on a UNITRONIC OR P SELECTA shaker at 70 rpm. Optical glass samples removed from the bulbs were immersed in ethanol to deactivate the biological material and then it was delicately removed from the surface. Then the culture was pasteurized to stop the activity of the microorganisms. Post-culture liquid was analyzed with quantities of radicals freed from glass with flame atomic absorption spectrometry using a Merck ASA GBC 932 spectrometer.

Changes on the surface of optical glass were assessed on the basis of observations under a phase-contrast microscope and with the use of a laser beam.

The results reported in this paper are an arithmetic mean from three measurements with the exception of laser light transmittance measurements where a mean from five measurements was calculated. Microsoft Excel program was used to analyze the data.

Results

Biodeterioration of optical glass induced by 4CKP lubricant in a static culture of *A. niger*. The macroscopic picture of *A. niger* growth on BaK4 glass covered with 4CKP lubricant is shown in Fig. 1A similar active growth of the spawn was noted for all glass types.

Microscopic observations of glass surface samples after 3 and 6 months of *A. niger* growth did not show any deteriorating changes of glass even if a plentiful growth was noted. Only after 12 months the glass surfaces started to show first signs of structural changes. Observations under a phase-contrast microscope allowed seeing matt areas and spot discoloring (Fig. 2). Similar changes were also observed on other glass surfaces except for BK7 glass.

Measurements of transmittance of light passing through the glass disc confirmed that the culture of

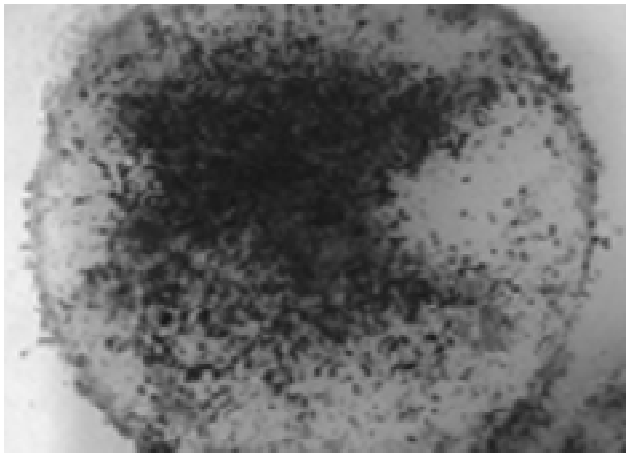

Fig. 1. Surface of glass BaK4 covered with 4CKP lubricant after 12 months of *A. niger* growth.

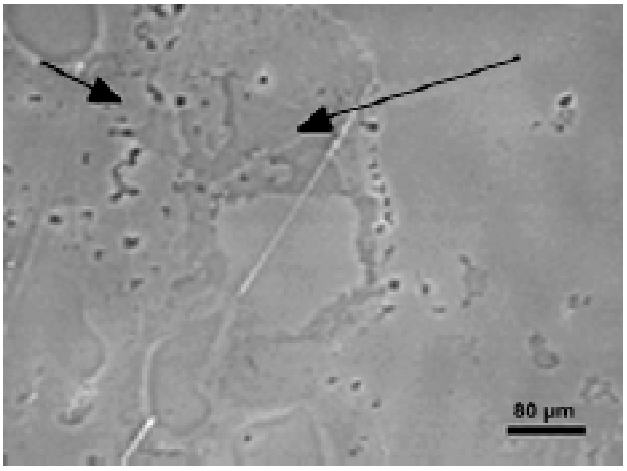

Fig. 2. Surface of BaK4 optical glass covered with 4CKP lubricant after 12 months of *A. niger* growth (arrows-matt areas, discolorations).

A. niger did not show any changes after either 3 or 6 months. After 12 months though, smaller transmittance of laser light through the disc was determined for all types of investigated glass (Fig. 3). The value of transmittance of laser light through BaK4 and F2 glass samples decreased by 1.6% so the surface changes were small. The same value for SF2 glass decreased by 7.8% compared to the initial value. For BK7 glass this transmittance decreased by a statistically insignificant 0.7% indicating that this type of glass is most resistant to deterioration caused by active growth of the *A. niger* spawn on 4CKP lubricant (Fig. 3).

Biodeterioration of optical glass in submerged conditions of *A. niger* growth on a glass base with the addition of 4CKP lubricant. The influence of *A. niger* metabolites on the process of glass deterioration was also observed during immersed culture conditions where, as in the earlier experiment, the 4CKP lubricant was the only source of carbon.

After 240 h of *A. niger* growth, phase-contrast microscopic observations of the glass surface did not confirm any changes in the structure of the investigated glass types. Laser light beam transmittance measurements confirmed earlier determinations that only the BK7 glass structure remained unchanged. Other glass types showed the decrease of this parameter in relation to initial values by 5.2%, 2.3% and 2.8% respectively for BaK4, F2 and SF2 glass samples (Fig. 4).

Atomic absorption spectrometry (AAS) chemical analysis of the post-culture liquid confirmed the destruction of glass caused by *A.niger* metabolites.

Small quantities of Na, Si, K, Zn, Pb cations were found in the medium without glass which was the reference sample (Fig. 5). The % increase of content of particular cations in the solution was assessed in relation to the reference sample. The greatest increase of cation contents in the post-culture liquid was noted for the sample with BaK4 and SF2 glass. The Si and K content increased by 90% while in the solution with SF2 glass the Pb content also increased by 96%. Large quantities of lead were freed from the F2 glass. Its content increased by 97% while for silica and potassium the increase was 59% and 36%, respectively. In the experiment with BK7 glass the quantity of silica in the post-culture liquid increased by only 63% despite the fact that the content of SiO_2 is the greatest in this type of glass. Boron was transferred to the solution in greater quantity, *i.e.* 99% more in relation to the reference sample. This cation can react with organic acids produced by the *A. niger* strain and form soluble salts in the solution. BK7 glass was the only investigated glass type that did not contain any lead cations

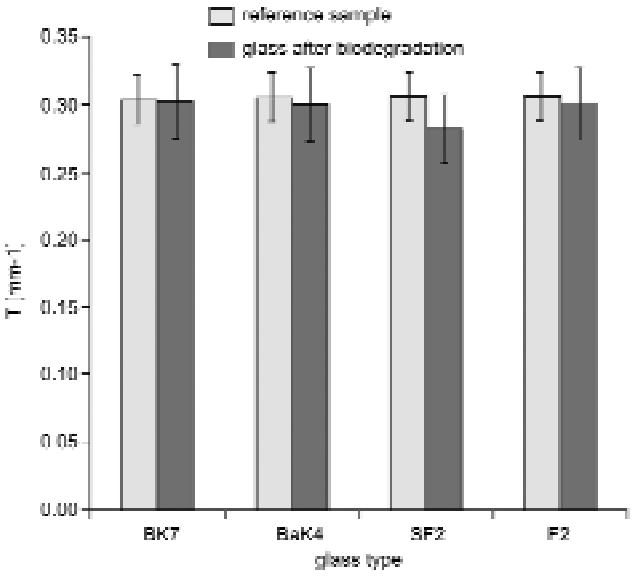

Fig. 3. Transmittance of laser light beam through optical glass after 12 months of cultivation *of A. niger* induced by 4CKP lubricant.

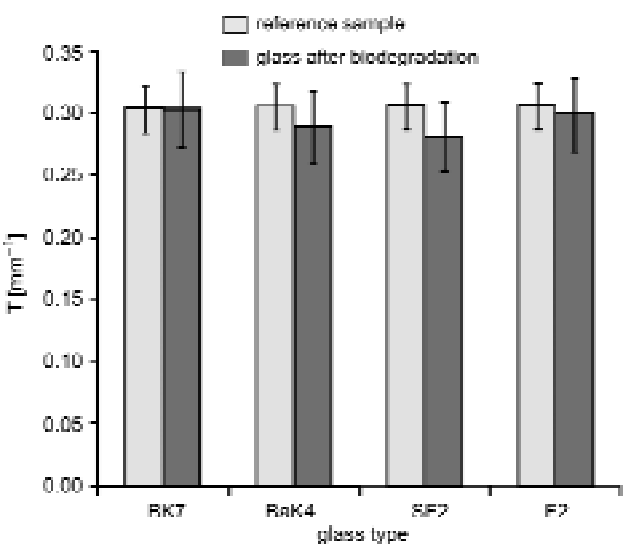

Fig. 4. Transmittance of laser light beam through glass samples deteriorated during 240 h of *A. niger* growth in a Mo base with the addition of 4CKP lubricant.

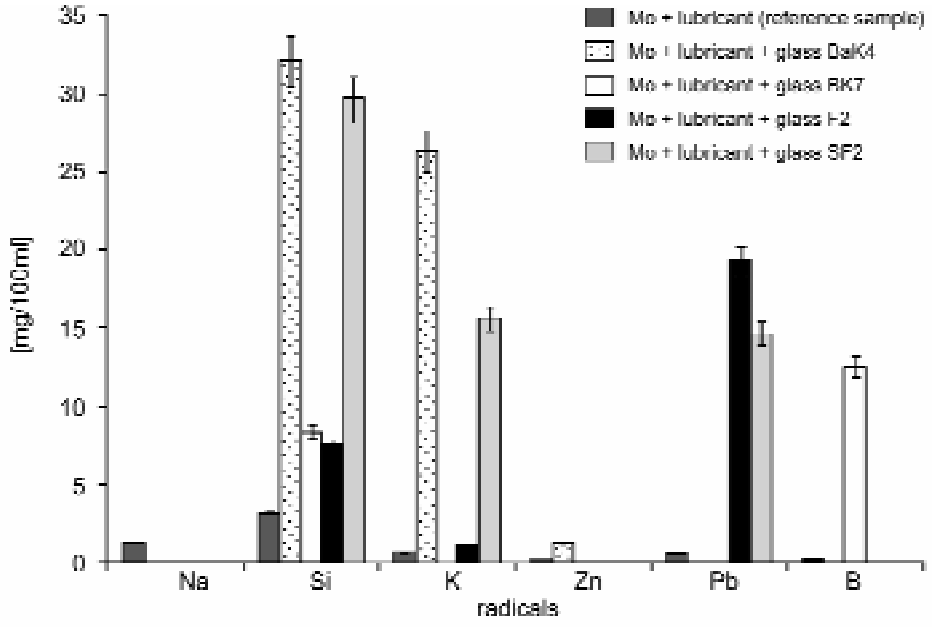

Fig. 5. Chemical radicals in the liquid after 240 h of submerged *A. niger* growth on M_o base with the addition of 4 CKP lubricant and optical glass.

but had the greatest content of SiO_2 (ca 70%). This can explain its greater resistance to destructive influence of biological corrosion caused by *A. niger*. Glass types with low SiO_2 content and with PbO (SF2, F2) have a lower resistance to the influence of acid metabolites. Thus they are more sensitive to surface destruction caused by growth of fungi.

Discussion

Biodeterioration of glass is a long and complex process. Nevertheless, it is known that in an environment of high air humidity, temperature and carbon availability, microorganisms and especially fungi can damage this seemingly durable surface. Surface dirt, putty and lubricant traces or insects can be the source of carbon that initiate the growth of microorganisms. This means that optical glass parts being parts of both civilian and military devices are exposed to the destructive action of microorganisms. Hence the knowledge of the biodeterioration of glass induced by ancillary materials used in the production of optical devices is indispensable to ensure their usefulness and durability. Earlier studies on the resistance of ancillary materials proved that among the studied materials 4CKP lubricant is most susceptible to the growth of *A. niger*. Investigations of glass surface with a thin layer of particularly susceptible ancillary material (4CKP lubricant) and grafted with a suspension of *A. niger* spores showed that changes of the BaK4, SF2 and F2 glass samples take place very slowly. They take the shape of matt areas and spot discolorations and can be seen under the phase-contrast microscope only after 12 months of the culture growth. No such changes were observed on the surface of BK7 glass. A more proper assessment of the surface changes was made when measuring laser light transmittance through the tested material. It was determined that small changes of the SF2 glass structure occurred already after 6 months of the *A. niger* growth. However the BK7 glass resistance was confirmed as high since the transmittance of light was observed only after 12 months (insignificant level of 0.7%).

The content of elements in the post-culture liquid indicate that reactions take place between *A. niger* metabolites and glass components. Silica, lead and potassium were released from SF2 and F2 glass and silica and boron from BK7 glass. The release of boron and potassium from BaK4, SF2 and F2 glass is the result of reactions occurring between glass components and organic acids released to the base by *A. niger*. This strain has been characterized as an acid generating fungus which releases citric, oxalic and malic acids. The investigated glass types can thus be described as not resistant to the influence of the above acidic metabolites. Silica does not react with organic acids so its presence in the post-culture liquid is the result of other mechanisms. It is probable that the wash out of other cations reacting with organic acids could have been the reason for surface changes and some amount of silica could have been transferred to the solution. Richens (1997) and Drewello and Weissmann (1997) state that the reaction of organic acids with cations (components of glass) leads to the formation of soluble complex compounds and thus an

opening and weakening of the silica structure with easier release of the silica.

Results of transmittance of laser light through investigated optical glass types showed that reactions of *A. niger* metabolites (created during the growth process on 4CKP material) decreased the transmittance of light through SF2, BaK4 and F2 glass by 2–8%. They did not, however, cause any changes in the structure of BK7 glass surface which could have been recorded by the light transmittance measurements. Si and B cations found in the post-culture liquid could have been released from the rim of unpolished part of the glass disc without any changes in the transmittance of light through the polished optically smooth surface layer.

The results of these experiments justify the statement that the higher the content of SiO_2 in glass, the higher its resistance to the attack of microorganisms (example-BK7 glass). On the other hand the greater the PbO content (SF2 and F2 glass) and the richer the composition of elements in glass which react with organic acids (BaK4 glass) the greater the susceptibility of the glass surface to microbiological corrosion.

Results obtained from the experiments are in agreement with the analyses of Drewello and Weismann (1997) of the potassium-calcium-silica glass surface (composition: K_2O 12–25%; CaO, 18–25%; SiO_2 47–54%; PbO 0–1.5%) attacked by fungi and of optical F2 glass (composition: PbO 24,1; SiO_2 62.6%; K_2O 8.5%). They determined that the glass surfaces with K_2O, CaO and PbO are more susceptible to the attack of filamentous fungi. The authors also stressed that the boron-silica BK7 glass and the sodium-calcium-silica glass from the year 1900 with a high SiO_2 content (72–74%) have a higher resistance to the effect of organic acids.

Similar results were obtained by Garcia-Vales *et al.* (2003) during studies on the biodeterioration of stained glass windows from XIII–XV centuries. They found most symptoms of microbiological corrosion in stained glass windows with glass of high K and Ca content (*ca.* 18% and 20% respectively) and least in those with high SiO_2 content (*ca.* 60%). References to glass production technology (Jabłkowski, 1962) indicate that the resistance of glass to acids grows with the content of SiO_2 whereas lead type glass is susceptible to the influence of both inorganic and organic acids.

A new element in the state of knowledge on microbiological corrosion of glass is the fact that lubricants (an example here is the investigated 4CKP lubricant) can initiate the growth of conidia of fungi and in consequence cause glass deterioration. Results of investigations of the biodeterioration of glass do not give so far information on the threats of using ancillary materials in inducing microbiological corrosion.

Lubricants used in the production of optical devices should therefore be resistant to biodeterioration and should not contain components assimilable by microorganisms. Additionally, the resistance tests for these materials should be conducted in reference to fungi since organic acids with strong corrosive properties are their main metabolites.

Acknowledgements

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ORIGINAL PAPER

Antimicrobial Activity of Undecan-x-ones (x = 2–4)

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Abstract

As a continuation of our research on the biological activity of undecan-x-ones (x = 2–4), their antimicrobial activity towards bacteria *Escherichia coli* and *Bacillus subtilis*, yeast *Candida mycoderma* and mould *Aspergillus niger,* was investigated. The population viability of the tested microbial strains in the presence of undecan-x-ones was determined by the impedimetric and agar disc diffusion methods. Undecan-x-ones showed low antibacterial activity towards both Gram-positive and Gram-negative bacteria. Undecan-2-one and undecan-3-one exhibited high activity towards *C. mycoderma*. All undecan-x-ones expressed the strongest effect on *A. niger*. The tests have proven that due to high fungistatic activity undecan-x-ones can be used to aid stabilization of food and cosmetic matrices.

Key words: antimicrobial activity, essential oil components, fungistatic activity, ketones, undecan-x-one

Introduction

Essential oils, and specifically their effect on humans, as well as their fungicidal, bactericidal and even virucidal properties, have been the subject of many studies. The results have been documented for a large number of oils (Brud and Chrząszcz, 1998; Chrząszcz, 1998ab). Essential oils are usually mixtures of dozens of chemical compounds and their biological properties depend on the synergy effect of the individual components (Chrząszcz, 1998a; Ochi *et al*., 2005). Large quantities of natural raw materials are required to produce essential oils, and the production process is fairly expensive. When the high prices of essential oils are considered, the following question arises: what is the antimicrobial activity of their individual components? Moreover, it is important to determine if synthetic compounds can replace those from natural sources.

Some oil components can be obtained in a relatively simple and efficient way by organic synthesis and they are commonly recognized as nature-identical. This group also includes aliphatic undecan-x-ones (x = 2–4), the components of essential oils and extracts of exotic plants. Undecan-2-one is the most common undecan-x-one within the plant kingdom. A significant amount of undecan-2-one occurs in the oils obtained from the plants which belong to the *Rutaceae* family (Lawless, 1999). Depending on the species, cultivation conditions and part of the rue plant, essential oils contain from several to dozens percent of this ketone. The essential oil from *Ruta chalepensis* L. growing in Turkey contains 66.5% undecan-2-one (Hüsnü Can Baser *et al.*, 1996), in Iran 66.0–68.0% (Rustaiyan *et al*., 2002), and that from *Ruta montana* L. even 84.2% (Hüsnü Can Baser *et al.*, 1996). Chinese *Ruta graveolens* L. contains 67.0% of this ketone (Lawrence and Reynolds, 1998), the one growing in Cuba 48.7% (Pino *et al*., 1997), and in Malaysia 30.7% (Yaacob and Abdullah, 1989). Large quantities, amounting to 54.3% of undecan-2-one, were isolated from the leaves of *Zanthoxylum pinnatum* (*Rutaceae*) (Brophy *et al.*, 2000), from the fruits and leaves of Brazilian *Siparuna guianensis* (Aubl.) (31.7–32.5%) (Fischer *et al*., 2005), and from the roots of *Philodendron acutatum* Scott. (12.7%) (Viana *et al*., 2002).

Undecan-2-one was also found in the leaves (4.0–7.0%) and fruits (*ca.* 5.0%) of *Pistacia lentiscus* L. growing in Sardinia (Cougiu *et al*., 2002), and in the bark (52–58%) of Indian *Glycosmis pentaphylla* (Ahmed *et al*., 2000). The essential oil from *Cymbopogon schoenanthus* growing in desert areas of Thar contains 14.8% (Shahi and Tava, 1993), and the oil

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produced in Nepal from the fruits of *Cinnamonium glaucescent* contains 3.6% of undecan-2-one (Lawrence and Reynolds, 1997). A small amount, about 1% of undecan-2-one, has been obtained from many other plants, *e.g.* in the essential oils from ginger (Lawrence and Reynolds, 1991a), Chinese rose *Rosa rugosa* (Lawrence and Reynolds, 1991b), Turkish *Salvia blepharochlaena* (Demirci *et al*., 2003), and in the flowers and fruits of *Litsea monopetala* (Roxb.) (Choudhury *et al*., 1997).

Other metameric undecanones are more rarely encountered in nature. Undecan-3-one occurs in the amount of 0.6% in brown sea algae *Dictyopteris membranacea,* which comes from the French Coast of the Mediterranean Sea (Boland and Müller, 1987; Trehan *et al*., 1997). It was found that undecan-3-one is a product of wood-decay processes caused by the fungus *Fomitopsis pinicola* (Rösecke *et al*., 2000). It also is a trail pheromone of African ants *Decophylla longinod* (Boland and Müller, 1987; Trehan *et al*., 1997; Rösecke *et al*., 2000).

Undecan-4-one occurs in concentration 0.25% in the essential oil from the plant *Cymbopogon nardus* (Boland and Müller, 1987).

Undecan-x-ones (x = 2–4) are precious because of their very pleasant, durable floral and fruity odors (Djerassi, 1994; Gibka and Gliński, 2008). Undecan-2-one characterized by a fruity-floral odor with an orange-herbal note is a component of many fragrant compounds and food flavorants (Burdock, 2002). The antimicrobial activity of these ketones has not yet been investigated. They are expected to stabilize microflora and hence act as both an odoriser and preservative in food and cosmetic products.

A simple, ecological and efficient method for the synthesis of undecan-x-ones (x = 2–4) was developed (Gliński and Gibka, 2004). Next, their activity towards Gram-positive bacteria *Bacillus subtilis*, Gram-negative bacteria *Escherichia coli*, yeast *Candida mycoderma* and mould *Aspergillus niger* was established.

Experimental

Materials and Methods

Undecan-x-ones. The experimental materials were undecan-2-one, undecan-3-one and undecan-4-one, obtained in the catalytic ketonization of carboxylic acids according to the procedure previously described (Gibka and Gliński, 2006).

Microorganisms. Bacteria *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 8793; yeast *Candida mycoderma* ŁOCK 0008 and mould *Aspergillus niger* ŁOCK 0436 were used in the experiments. The microorganisms originated from the ATCC Collection and the Pure Culture Collection of the Institute of Fermentation Technology and Microbiology, Łódź Technical University ŁOCK 105. Double passaging activated the microorganisms: bacteria on TSB medium (Trypticase Soy Broth) Oxoid, UK (*B. subtilis* temperature 30°C, 48 h; *E. coli* temperature 37°C, 48 h), yeast and mould on Sabouraud Agar, bioMerieux, Poland (temperature 28°C, 72h).

Determination of antimicrobial activity of undecan-x-ones: The antimicrobial activity of undecan-x-ones was determined by the impedimetric method using a Bactometer M64 System (bioMerieux, Poland). The suspension of tested microbial cells in physiological salt solution (0.85% NaCl) was standardized to the density of about 10^7 CFU/ml. Each well of the impedimeter module was filled with 0.1 ml of the cell suspension, 1, 5, 10, 20 or 30 µl of undecan-x-one and completed to 1 ml volume with GPM medium (bioMerieux, Poland) for bacteria and YMM medium (bioMerieux, Poland) for fungi. A positive control sample was a suspension of microorganisms in the medium without undecan-x-ones. A negative control was the culture of bacteria and fungi with the addition of novobiocin (0.5 µg/ml) and cycloheximide (0.2 µg/ml), respectively. The samples were incubated for 72 h at temperatures optimal for the growth of individual microorganisms, as described in the strain activation procedure. After incubation in the bactometer, the microorganism's viability was controlled by a surface culture on the PCA medium (Plate Count Agar, bioMerieux, Poland). The plates were incubated for 3 days in the case of bacteria and yeasts and for 5 days for mould at temperatures optimal for the growth of the particular microorganisms.

Minimal Inhibitory Concentration (MIC) was assigned as the lowest concentration inhibiting the growth of microorganisms in the bactometer at parallel growth on the PCA plates. Minimal Bactericidal Concentration (MBC) or Minimal Fungicidal Concentration (MFC) was the lowest undecan-x-one concentration at which no microbial growth was observed either in the bactometer wells or on the PCA plates.

To compare the impedimetric method with classical one recommended by CLSI (Clinical Laboratory Standards Institute), the antimicrobial activity of undecan-x-ones was also determined by the agar disc diffusion method. 10, 20 or 30 µl/ml undecan-x-ones were applied on sterile paper discs of 6 mm in diameter (Whatman No 40, Britania). The discs were placed on the surface of the inoculated TSB agar medium and Sabouraud Agar for bacteria and fungi respectively. Cell suspensions of microorganisms for inoculation were prepared as described above and in amount of 0.1 ml of a particular microorganism transferred onto the agar medium. Petri dishes were kept at 4°C for 2 hours, and then incubated at temperatures optimal for

their growth for 72 hours and the zones of inhibition were measured. Novobiocin (0.5 µg/ml) and cycloheximide (0.2 µg/ml) served as controls. The undecan-x-ones activity was classified by the diameter of the inhibition zones as follows: inactive for diameter less then 8 mm, moderately active for diameter 9–14 mm, active for diameter 15–19 mm and highly active for diameter larger than 20 mm (Ponce *et al*., 2003).

Statistical analysis of results. Results were analyzed using a 3-way ANOVA at the confidence level of $p<0.05$. Results of the population viability were presented as an arithmetic mean of three determinations with standard deviation not exceeding 0.2 logarithmic units.

Each assay of the agar disc diffusion method was performed by duplication in two separate experimental runs and the results were presented as a mean with standard deviation.

Table I
Antimicrobial activity of undecan-x-ones presented as MIC (Minimal Inhibitory Concentration) in µl/ml.

Microorganism	undecan-2-one	undecan-3-one	undecan-4-one
B. subtilis	>30	>30	>30
E. coli	>30	>30	>30
C. mycoderma	20	20	>30
A. niger	1	5	5

Table II
Antimicrobial activity of undecan-x-ones presented as MBC/MFC (Minimal Bactericidal/Fungicidal Concentration) in µl/ml.

Microorganism	undecan-2-one	undecan-3-one	undecan-4-one
B. subtilis	>30	>30	>30
E. coli	>30	>30	>30
C. mycoderma	30	30	>30
A. niger	20	10	20

Results

Undecan-3-one and undecan-4-one at the concentration of up to 30 µl/ml had practically no effect on *B. subtilis*. Undecan-2-one appeared to be much more active. However concentrations of 20 and 30 µl/ml of undecan-2-one decreased the population the most, by 2.86 and 5.47 logarithmic units per ml respectively, the MIC and MBC values were not determined (Tables I and II, Fig. 1A). The tested undecan-x-ones showed low activity against *E. coli*. No statistically significant ($p<0.05$) differences in the population in the presence of undecan-2-one and undecan-3-one were observed. The increase in the undecan-4-one concentration from 1 to 10 µl/ml resulted in a gradual reduction of viable *E. coli* cells to 1.90 logarithmic units per ml. A subsequent increase in undecan-4-one doses did not cause further changes in the population viability (Fig. 1B). The results of the impedimetric method were in agreement with those of the agar disc diffusion method and according to the latest (Ponce *et al*., 2003) undecan-3-one and undecan-4-one were classified as inactive against *B. subtilis*. Undecan-4-one at the concentrations of 10, 20 and 30 µl/ml expressed moderate activity against *E. coli* (Table III).

The activities of undecan-x-ones towards yeast *C. mycoderma* were similar (no statistically significant differences, $p<0.05$), when the compounds were added at concentrations of 1 and 5 µl/ml. At the concentration of 20 µl/ml, undecan-2-one and undecan-3-one inhibited the growth of the yeast population completely (MIC), (Fig. 2A, Table I). The concentration of 30 µl/ml of both of these compounds was found to be lethal for *C. mycoderma* (MFC) (Tab. II). Undecan-4-one showed lower activity, and at the

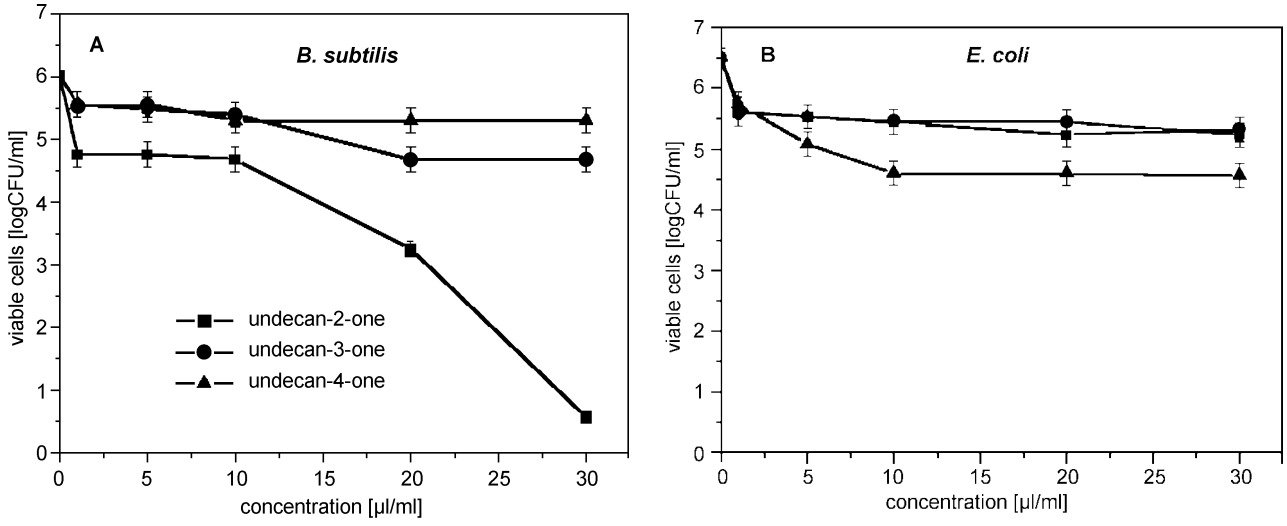

Fig. 1. The effect of undecan-x-ones on bacteria *B. subtilis* (A) and *E. coli* (B).

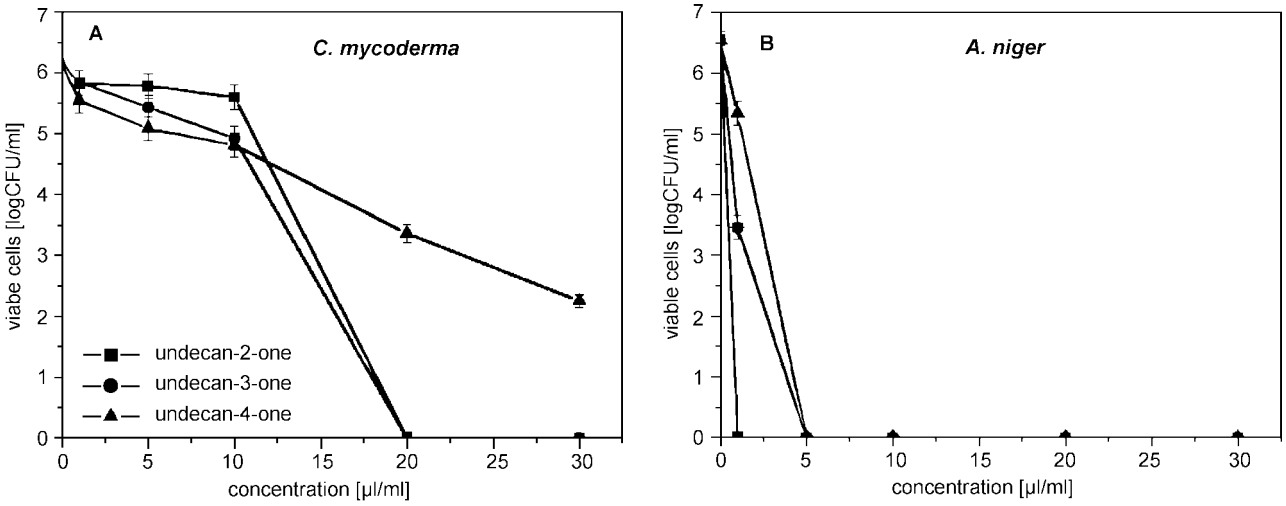

Fig. 2. The effect of undecan-x-ones on yeast *C. mycoderma* (A) and mould *A. niger* (B).

highest tested concentration it caused a reduction of the yeast cell number by 3.89 logarithmic units per ml but on the basis of the results of the agar disc diffusion method (Table III) it was classified as active.

High activity of undecan-x-ones was found in the case of *A. niger* (Fig. 2B), which was also confirmed by the agar disc diffusion method (Table III). Already at the concentration of 1 µl/ml undecan-2-one had an inhibitory effect on this mould (MIC). However, it showed a cidal effect (MFC) only at the 20-fold higher concentration. The Minimal Inhibitory Concentration for undecan-3-one and undecan-4-one was determined to be 5 µl/ml, whereas the concentrations of the cidal effect of these compounds differed, being 10 and 20 µl/ml, respectively (Tables I and II).

Discussion

Undecan-x-ones revealed low antibacterial activity against both tested Gram-positive and Gram-negative bacteria. In the tested range of pure compound concentrations MIC and MBC values were not determined for *B. subtilis* and *E. coli*.

Due to the lack of literature references concerning studies on the antimicrobial activity of undecan-x-ones, their activity can be referred mainly to plant extracts containing these compounds. Although the plant extracts containing significant quantities of undecan-x-ones were selected for comparison, a complex quality of the extracts and synergy or antagonistic interactions of their constituents (Rhiannon, 2002) should be taken into account.

Extracts from the leaves of *Ruta graveolens* did not show activity towards *E. coli* (Valsaraj *et al*., 1997; Ali-Shtayeh *et al*., 1998; Ojala *et al*., 2000; Alzoreky and Nakahara, 2003; Ivanova *et al*., 2005), just like their main component, undecan-2-one. A search for the mechanism of antibacterial action of ketones showed a quick recovery of *E. coli* after 1-hour moderate blocking effect of undecan-2-one (Együd, 1967), which explained its inactivity also in our testing. At the same time, some studies show low activity of the preparations from *R. graveolens* towards *B. subtilis* (Valsaraj *et al*., 1997; Ojala *et al*., 2000; Alzoreky and Nakahara, 2003), which is in agreement with our results for undecan-2-one. A similar effect was found for extracts from the leaves of *R. chalepensis*

Table III
Zones of growth inhibition of tested microorganisms by undecan-x-ones.

Microorganism	Inhibition zone diameter (mm)[a]										
	undecan-2-one			undecan-3-one			undecan-4-one			novobiocin	cyclo-heximide
	10[b]	20[b]	30[b]	10[b]	20[b]	30[b]	10[b]	20[b]	30[b]	0.5[c]	0.2[c]
B. subtilis	11 ± 1	15 ± 1	18 ± 1	8 ± 0	8 ± 1	8 ± 0	7 ± 0	7 ± 0	7 ± 0	20 ± 1	–
E. coli	8 ± 1	8 ± 0	8 ± 0	8 ± 0	8 ± 1	8 ± 1	11 ± 1	12 ± 1	12 ± 1	24 ± 2	–
C. mycoderma	9 ± 0	28 ± 2	32 ± 3	10 ± 1	29 ± 2	35 ± 2	10 ± 0	18 ± 2	19 ± 2	–	19 ± 1
A. niger	32 ± 3	34 ± 2	41 ± 3	29 ± 2	32 ± 3	38 ± 3	18 ± 2	26 ± 2	32 ± 1	–	28 ± 2

Each assay was performed by duplication in two separate experimental runs.
[a] includes diameter of disc (6 mm); [b] compound concentration in µl/ml; [c] compound concentration in µg/ml; – not determined

(Ali-Shtayeh *et al*., 1998; Alzoreky and Nakahara, 2003; Al-Bakri and Afifi, 2007).

Undecan-2-one and undecan-3-one were characterized by high activity towards yeast *C. mycoderma*, as opposed to methanol extracts from the leaves of *R. graveolens* that do not act on members of *Candida* genus, *Candida albicans* (Ojala *et al*., 2000) species. However, the potential activity of undecan-2-one was previously established based on the activity determination of ethanol extracts from the leaves of *R. chalepensis* and *Pistacia lentiscus* towards *C. albicans* (Ali-Shtayeh *et al*., 1998).

Studies on the biological activity of plant extracts and oils refer mainly to bacteria, so there are few literature references concerning moulds. In our studies all tested undecan-x-ones expressed the strongest effect on the mould *A. niger*. Undecan-2-one was noted to be particularly active, which was confirmed in previous studies of *Commiphora rostata* resin components (McDowell *et al*., 1988). Data available in the literature referring to the extract from the leaves of *R. graveolens* (Ojala *et al*., 2000) do not confirm our results, which could be attributed to the lower concentration of undecan-2-one in this extract.

Conclusions. Although all undecan-x-ones are characterized by low antibacterial activity, they act efficiently against fungi. Proven high activity against tested yeast and mould species indicates their potential application as components of a preservative system for the stabilization of food and cosmetic matrices.

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SHORT COMMUNICATION

Toxoplasma gondii: Usefulness of ROP1 Recombinant Antigen in an Immunoglobulin G Avidity Assay for Diagnosis of Acute Toxoplasmosis in Humans

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Abstract

The results present in this study suggest that the *Toxoplasma gondii* recombinant ROP1 antigen in an IgG avidity test can be useful for detection of acute stage of infection. Specific antibodies of low avidity were detected in most of the sera from individuals with acute toxoplasmosis, while the absence or specific antibodies of high avidity were detected in sera from patients with chronic infection.

Key words: *Toxoplasma gondii*, ELISA, recombinant antigen, rhoptry protein 1, serology

Toxoplasma gondii is an obligate intracellular parasite that infects many warm-blooded animals. Although, human toxoplasmosis is generally asymptomatic, a serious disease can occur in the case of a congenital infection and immunocompromised individuals such as AIDS patients and transplant recipients (McAuley *et al.*, 1994; Sàfadi *et al*., 2003). For that reason, it is crucial to estimate the time of infection in pregnant women. Detection of specific IgM and IgG are important but inadequate steps in diagnosis of acute toxoplasmosis (Bertozzi *et al*., 1999; Bessieres *et al*., 1992; Brooks *et al*., 1987; Iqbal and Khalid, 2007; Liesenfeld *et al.*, 1997). The determination of the avidity index of IgG antibodies is considered very important in the diagnosis of an acute infection *vs* a chronic infection (Jenum *et al.*, 1997; Lappalainen *et al.*, 1993; Liesenfeld *et al.*, 2001; Montoya *et al*., 2002). In most cases, the currently available commercial tests are based on antigens obtained from a whole tachyzoites of *Toxoplasma* (TLA). The methods of producing tachyzoites as well as antigen(s) may vary significantly between laboratories and this means that the test is difficult to standardize. Furthermore, it was confirmed that low IgG avidity may be detected for a long time after infection (Ashburn *et al.*, 1998; Villavedra *et al*., 1999). That is why recombinant antigens are considered to replace the antigen obtained from lysed whole parasites. In this case the antigen composition of the test is precisely known and the method can be easily standardized. Several recent studies have reported the use of recombinant antigens (Beghetto *et al.*, 2003; Marcolino *et al*., 2000) or mixture of proteins (Pietkiewicz *et al.*, 2005) in determination of IgG avidity.

The aim of this study was to estimate the diagnostic value of r-ROP1 in IgG ELISA avidity for serodiagnosis of *T. gondii* infection in humans.

A total of 172 serum samples received from a routine toxoplasmosis screening were analyzed and divided into four groups according to the results obtained with the VIDAS Toxo-IgG, VIDAS Toxo-IgG avidity, and VIDAS Toxo-IgM commercial tests (bioMérieux, France): group I – 34 sera from patients suspected of an acute toxoplasmosis (positive IgM, positive IgG with low or borderline avidity); group II – 16 sera from patients with postacute toxoplasmosis (negative IgM, positive IgG with low or borderline avidity); group III – 92 sera from patients with chronic toxoplasmosis (negative IgM, positive IgG with high avidity); IV group – 30 sera from seronegative individuals. Furthermore, we analyzed the avidity maturation of specific antibodies in serum samples received from

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seven pregnant women classified as patients with acute or postacute phase of toxoplasmosis.

In this study, we used in-house IgG ELISA test with r-ROP1 expressed and purified as described in our previous paper (Holec-Gąsior *et al.*, 2009). Each serum sample was used in increasing serial dilutions (1:100–1:800) in separate plates coated with r-ROP1 and there were two different IgG ELISA procedures: normal and avidity ELISA washed with urea solution (PBS, 0.1% Triton X-100 with 6 M urea) three times for 10 min. All groups of human sera were examined by IgG ELISA and three groups (I–III) by IgG ELISA avidity test. Group IV was tested in order to calculate the cutoff which was set as the mean value of the negative serum samples plus two standard deviations.

The results were shown as avidity indexes (AIs) which were calculated as the ratio between optical density for the sample washed with urea solution and optical density for the sample washed with washing buffer (for the dilution which gave OD near 1.0). AI below 0.3 was considered as low avidity, 0.3–0.4 as borderline avidity and values above 0.4 as high avidity. This division was established on the basis of results obtain by Paul (1999) and applied by Pietkiewicz *et al.* (2007).

None of the negative serum samples reacted above cutoff, resulting in a specificity of 100% for ELISAs (Fig. 1). The sensitivity of IgG ELISA for r-ROP1 calculated for three groups of positive sera was at 43%. However, reactivity was different in each group (Fig. 1). Examination of group I sera showed that 29 out of 34 (85%) reacted with rROP1 and in 26 out of 29 cases (90%) reacted at low or borderline avidity. Only three sera (10%) from this group reacted at high avidity. In group II, 6 serum samples out of 16 (37,5%) had specific IgG antibodies against r-ROP1 and 4 of them reacted at high avidity, 2 at low or borderline avidity. Only 23 out of 92 sera (25%) from patients with chronic toxoplasmosis (group III) reacted with r-ROP1 and 22 cases (96%) were with high avidity. Only one serum sample from this group had low avidity (Fig. 2).

In this study we also demonstrated a comparison of avidity maturation of specific IgG antibodies against r-ROP1 and lysed, whole-cell antigen (TLA) in sera from seven pregnant women suspected of acute or postacute toxoplasmosis. Our results showed an increase in time of the avidity indexes (AI) in the IgG ELISA with TLA for all individuals tested (Table I). In the IgG ELISA with r-ROP1 antigen the increase of the avidity indexes was also observed for five individuals, however, there was no reactivity with specific IgG for three patients' serum samples (4 sera) obtained after 2, 8, 10 or 16 weeks from first time

Table I
Comparison of IgG avidity maturation in serum samples obtained from seven pregnant women suspected of acute or postacute toxoplasmosis.

Patient no	Number of weeks after the first sample was taken	IgM (VIDAS TOXO IgM)[a]	Lysed whole cell Assay (VIDAS TOXO IgG AVIDITY)		rROP1 assay	
			Avidity index (AI)	Interpretation[b]	Avidity index (AI)	Interpretation[c]
1	0	+	0.106	Low	0.19	Low
	24	+	0.117	Low	0.24	Low
2	0	+	0.03	Low	0.27	Low
	3	+	0.044	Low	0.37	Borderline
3	0	+	0.231	Borderline	0.66	High
	2	+	0.238	Borderline	–	–
4	0	–	0.121	Low	0.35	Borderline
	8	–	0.114	Low	–	–
	10	–	0.178	Low	–	–
5	0	+	0.108	Low	0.18	Low
	8	+	0.110	Low	0.22	Low
	12	–	0.111	Low	0.25	Low
	16	–	0.125	Low	–	–
6	0	+	0.217	Borderline	0.28	Low
	8	+	0.257	Borderline	0.31	Borderline
7	0	+	0.103	Low	0.22	Low
	4	+	0.12	Low	0.23	Low

[a] + means that patient has IgM, – means absence of IgM.
[b] AI below 0.2 means low avidity index, 0.2–0.3 – borderline avidity index, over 0.3 – high avidity index,
[c] AI below 0.3 means low avidity index, 0.3–0.4 – borderline avidity index, over 0.4 – high avidity index, – means lack of r-ROP1 antigen reactivity with IgG specific antibodies.

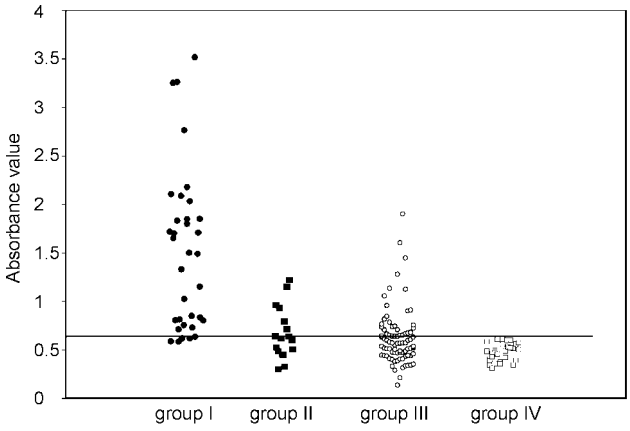

Fig. 1. Immunoreactivity of r-ROP1 protein with four groups of sera (diluted 1:100) from patients with acute (●), postacute (■), and chronic phase of toxoplasmosis (○) and from seronegative individuals (□).

Absorbance was measured at 492nm. Horizontal line represents the cutoff value (0.642).

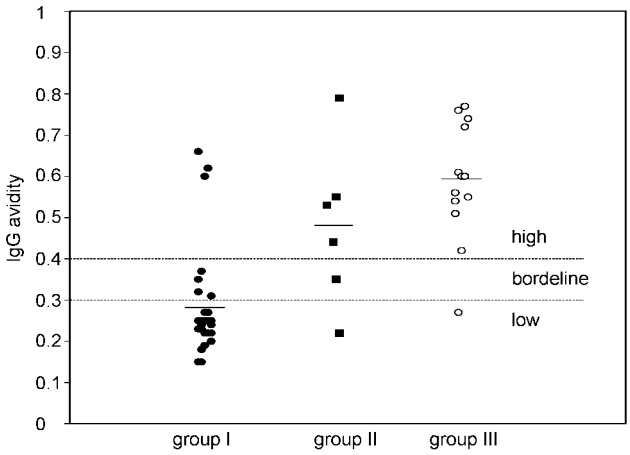

Fig. 2. IgG avidity indexes for r-ROP1 with seropositive sera from patients with an acute (●), postacute (■), and chronic toxoplasmosis (○).

The mean value is indicated for each group by horizontal line (0.282 for group I; 0.480 for group II; 0.594 for group III).

of serum delivery. These results together with those obtained for group III sera, where specific antibodies against r-ROP1 were not detected in 75% of serum samples from patients with chronic toxoplasmosis, confirmed our previously published data (Holec-Gąsior *et al.*, 2009) and Aubert *et al.* (2000) that IgG antibodies against r-ROP1 antigen are produced during the acute stage of toxoplasmosis but are uncommon in the chronic phase of the infection.

In conclusion, when specific IgG antibodies react with r-ROP1 both phases of toxoplasmosis can be suspected. Therefore, IgG avidity assay with r-ROP1 may indicate phase of infection, where low avidity suggests acute toxoplasmosis and high avidity or lack of reactivity may indicate chronic infection. However, further work is needed before an immunoassay with recombinant ROP1 antigen will be useful for clinical purposes.

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SHORT COMMUNICATION

Mechanism of Aniline Degradation by Yeast Strain *Candida methanosorbosa* BP-6

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Abstract

The ability of some bacteria and filamentous fungi to degrade aniline and its derivatives was reported earlier in the literature. However, there was no information about the biodegradation of aniline by yeast strains. The present work is focused on yeast strain *Candida methanosorbosa* BP-6 which was isolated from the wastewater pool of the old dye factory "Boruta" in Zgierz by enrichment technique and identified by standard microbiological methods. We have found that strain *C. methanosorbosa* BP-6 readily grows in the presence of aniline and can degrade this substrate. Relatively good separation of peaks corresponding to aniline and its biodegradation intermediates allowed us their identification and quantification by HPLC methodology. We have found that major intermediates of this degradation are: catechol, *cis,cis*-muconic acid, muconolactone, 3-oxoadipate enol-lactone, 3-oxoadipic acid and succinic acid. Our results provide strong evidence that biodegradation of aniline by the yeast strain *C. methanosorbosa* BP-6 proceeds according to the intradiolic pathway.

Key words: *Candida methanosorbosa* yeast, aniline biodegradation, intradiolic pathway

On the long list of environmentally hazardous chemicals, prominent positions are occupied by organic nitrogen compounds and among them phenylamine, otherwise named aniline. The main source of this xenobiotic, polluting the natural environment, are sewage waters and vapors originating from chemical, tannery, cosmetic or pharmaceutical industry, utilizing aniline as one of raw materials. Due to broad range of toxicity and cancerogenicity, aniline has been subjected to stringent legislative control by US Environmental Protection Agency (1994) as well as by EC Joint Research Centre (2004). The main toxic effects of aniline start with the formation of methemoglobine in blood and are expressed by lowering of oxygen transport, anaemia, jaundice, damage of liver, spleen and lungs, and decreased blood coagulability (Agency for Toxic Substances and Disease Registry, 2009)

Aniline at very low concentrations can be readily degraded in surface waters (rivers, lakes) as discovered by Toräng *et al*. (2002) and Ahtiainen *et al.* (2003). However, the presence of the xenobiotic in wastewaters at concentration exceeding 100 mg/ml can considerably complicate its biodegradation by traditional activated sludge technology (Wang *et al*., 2007). Because of relatively large scale of aniline release into the environment and costly procedure of its chemical remediation (Wang *et al*., 2007), there is an urgent need to find microorganisms able to degrade this xenobiotic. Examples of such organisms were found among various species of bacteria (Surovtseva and Volnova, 1972; Bachofer *et al*., 1975; Helm and Reber, 1979; Aoki *et al*., 1983, 1984; Lyons *et al.*, 1984; Zeyer *et al*., 1985; McClure and Venables, 1986; Fujii *et al*., 1997; Fukumori and Saint, 1997; Zissi *et al*., 1997; Takeo *et al*., 1998; Thomas and Peretti, 1998; Kahng *et al*., 2000; Liu *et al*., 2002; Matsumura *et al*., 2006; Wang *et al*., 2007). The ability to degrade aniline and its derivatives was also found for some strains of filamentous fungi (Arjmand and Sandermann, 1985; Kremer and Sterner, 1996; Emtiazi *et al*., 2001). However, to the best of our knowledge there is no information in the literature about the biodegradation of aniline by yeast strains.

In our search for new microorganisms able to degrade aniline we focused our attention on the surroundings of the old (presently not operating) dye factory

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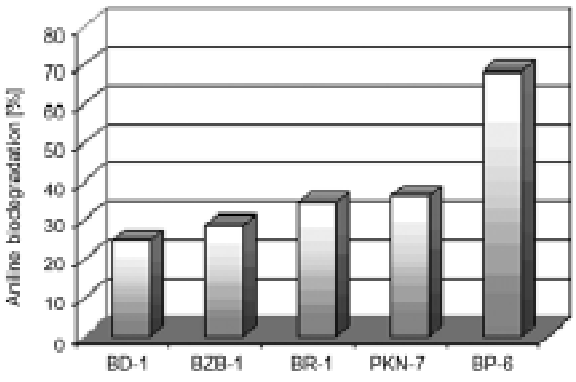

Fig. 1. Biodegradation of aniline by 5 strains of microorganisms isolated from various parts of the area surrounding old dye factory "Boruta" in Zgierz.

"Boruta" in Zgierz. Aniline was one of the basic raw materials utilized in the factory. The material for selection of microorganisms from soil and wastewater was collected in five different places of the factory area. Material from 18 samples, after appropriate dilution, was independently grown at 30°C on Petri dishes filled with solid King medium A (King *et al*., 1954) (glucose 1%, bacto peptone 2%, K_2SO_4 1%, $MgCl_2 \times 6H_2O$ 0.14%, agar 2%, pH 7.5), covered with a thin layer of aniline (20 µl *per* plate). The selected 18 monocultures (single colonies) were transferred into YPG slants (yeast extract 1%, bacto peptone 2%, glucose 2%, agar 2%, pH 7.0) and maintained at 4°C. The selected strains were tentatively identified as either bacteria or yeasts with varying abilities to degrade aniline. For further selection, each YPG slant was washed with isotonic saline to prepare inoculum for submerged shaken cultures. The cultures were run for 9 days at 30°C in liquid AY medium (yeast extract 0.2%, glucose, 0.5%, $NaNO_3$ 0.05%, KH_2PO_4 0.075%, $MgSO_4 \times 7H_2O$ 0.022%, $CaCl_2 \times 6H_2O$ 0.022%, pH 5.5) in dark, in the presence of aniline (0.5%). All strains were found to grow under these conditions with various efficiency as detected by measuring increasing cloudiness of cultures (absorption at 660 nm, data not shown). For the best growing yeast strain isolated from sludge pool near old "Boruta" dye factory (designated by us as BP-6) and 4 best growing bacterial strains isolated from different parts of the post-industrial Zgierz area (designated as BD-1, BZB-1, BR-1 and PKN-7) the level of aniline biodegradadion was estimated by measuring of remaining aniline concentration with a Spectroquant® test at 550 nm (Merck) as azo-dye subsequent to azo-reaction and coupling with N-(1-naphtyl) ethylenediamine dihydrochloride (NEDDC) (Fig. 1). The highest level of aniline biodegradation (68.5%) was observed for yeast strain BP-6. Therefore, this particular yeast strain was a subject of our further investigations.

Standard macro- and microscopic (500-fold magnification) observations allowed us to classify BP-6 as a yeast strain, growing in solid medium (YPG) with cream colored, smooth and glossy colonies, and in liquid medium (AY) as a suspension of oval budding cells 4–6 µm in diameter. Applying diagnostic criteria described by Barnett *et al.*, (1984) and Suye *et al*., (1990) led to a conclusion, that BP-6 yeast should be classified as *Candida methanosorbosa*. The BP-6 yeasts were found to grow readily on D-glucose, trehalose and D-galactose (slowly), whereas no fermentation of sucrose, lactose, maltose, melibiose, raffinose, inulin, melezitose or starch was observed. Also the carbon source and mineral requirements (data not shown) were in full agreement with aforementioned literature data related to *C. methanosorbosa* characterization. Ascospore formation was not observed.

From the technological point of view it was important to check the tolerance of *C. methanosorbosa* BP-6 towards increased concentration of aniline. Thus, several cultures of *C. methanosorbosa* BP-6 were set up in liquid medium, containing all ingredients of AY medium except glucose (30°C, pH 5.5). In addition, the medium was supplemented with either 0.1, 0.3, 0.5, 1.0, 2.0, 3.0 or 4.0% of aniline as a sole carbon source. The experiments showed no decrease of biomass accumulation with increasing aniline concentration in medium within 0.1–4% range during 5 days culture. Microscopic observations, showing the morphology of cells in cultures containing increasing concentration of aniline exhibited no changes for xenobiotic concentrations up to 3%.

The observed high tolerance of *C. methanosorbosa* BP-6 yeasts towards aniline and its efficient biodegradation prompted us to perform a study on the mechanism of this process. The available literature data show that biodegradation of aniline in various bacterial cultures may proceed either according to extradiolic or intradiolic pathway. In both cases the primary intermediate of aniline biodegradation is catechol. The extradiolic pathway, found for example for *Delftia* sp. (Liu *et al*., 2002), involves following sequence of enzymatic transformations: aniline → catechol → *cis,cis*-2-hydroxymuconic-6-semialdehyde → *cis,cis*-2-hydroxymuconic acid → 4-oxalocrotonic acid → 2-oxopent-4-enoic acid → 4-hydroxy-2-oxovaleric acid → pyruvic acid and acetaldehyde. The analogous sequence of transformations for intradiolic pathway involves: aniline → catechol → *cis,cis*-muconic acid → muconolactone → 3-oxoadipate enol-lactone → 3-oxoadipic acid → succinic acid.

For studies of the mechanism of aniline biodegradation, the submerged shaken culture of *C. methanosorbosa* yeasts was run in the dark at 30°C, in liquid AY medium at pH 5.5, in the presence of aniline (0.5%) for 9 days. After each 24 h of fermentation,

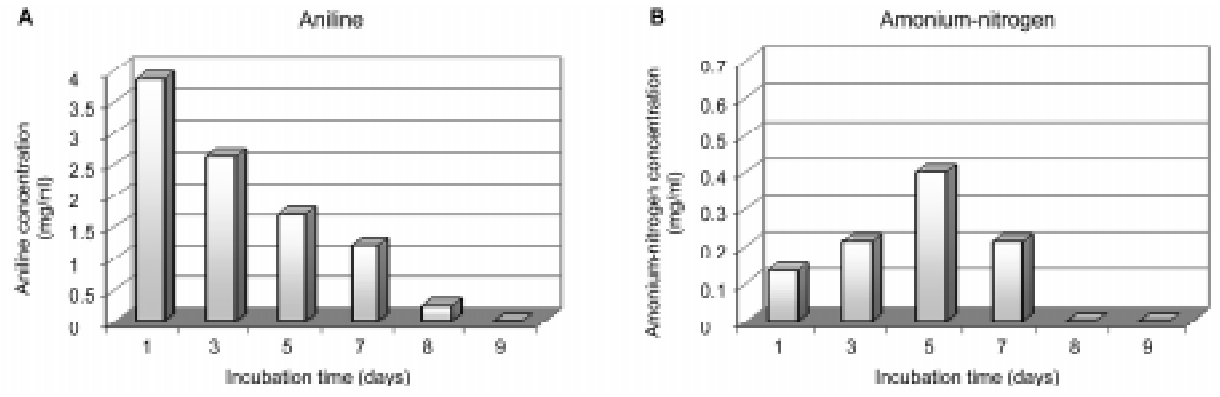

Fig. 2. Spectrophotometric determination of aniline (A) and ammonium nitrogen (B) in cell extracts obtained on consecutive days of *C. methanosorbosa* BP-6 yeast culture in AY medium in the presence of aniline (0.5%).

a portion of the suspension was taken and yeast cells were harvested by centrifugation (30 min at 5 000 g). After washing with 20 mM phosphate buffer (pH 7.0), the yeast cells were sonicated (5 min at 0°C) and the solid parts were removed by ultracentrifugation (20 min at 19 000 g). The supernatants (cell extracts) were then analyzed spectrophotometrically to monitor the concentration of aniline, ammonium-nitrogen,

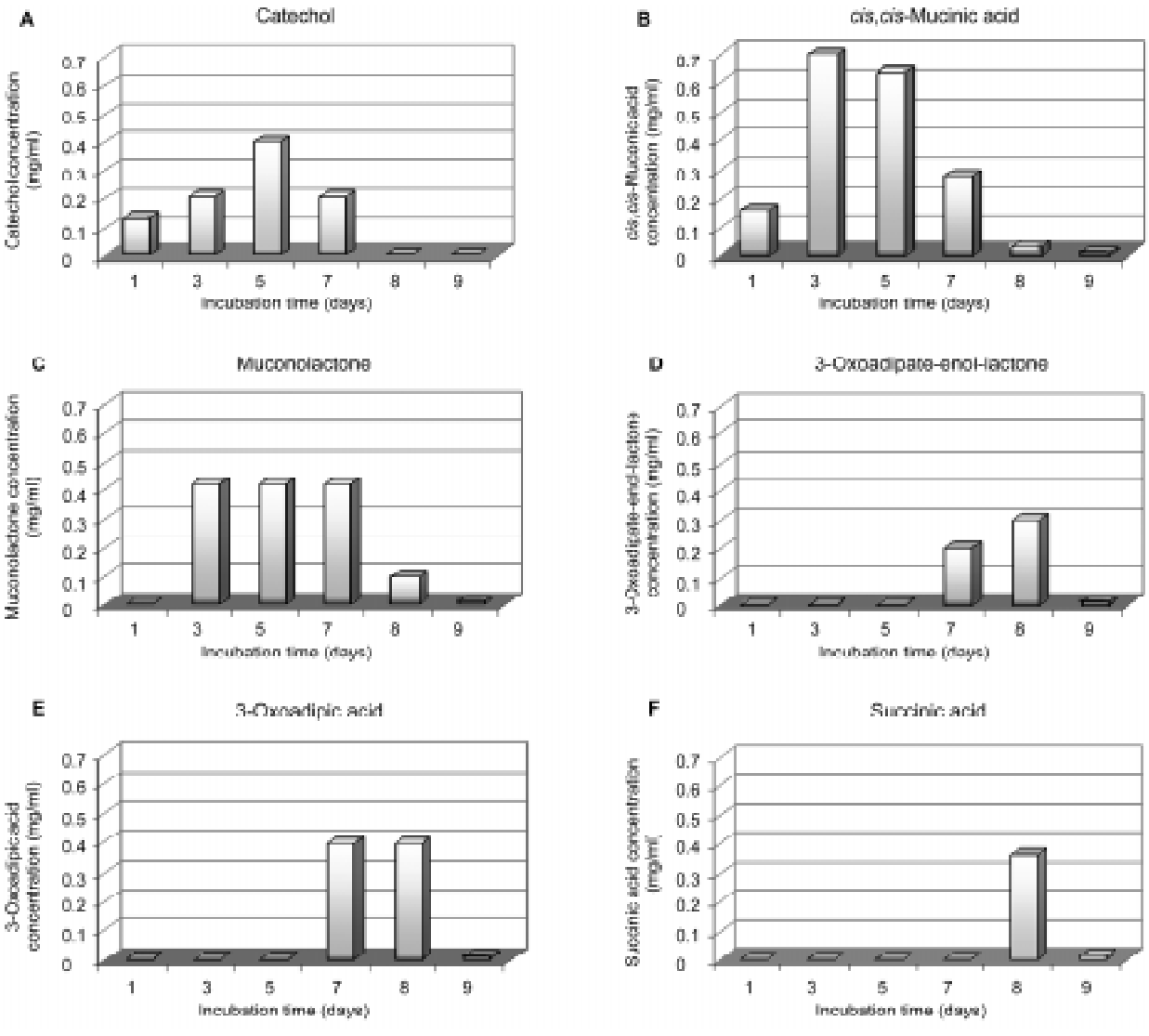

Fig. 3. HPLC determination of intermediate metabolites in cell extracts obtained on consecutive days of *C. methanosorbosa* BP-6 yeast culture in AY medium in the presence of aniline (0.5%).

catechol, *cis,cis*-muconic acid and *cis,cis*-2-hydroxymuconic-6-semialdehyde. In addition, aniline and its metabolites were determined by High Performance Liquid Chromatography (HPLC) method, with authentic samples of each metabolite serving as internal standards.

The analysis performed by photometric Spectroquant® test revealed the stepwise decrease of aniline concentration in *C. methanosorbosa* BP-6 yeast extract prepared from samples taken in consecutive days of fermentation (Fig. 2A). The measurements of ammonium-nitrogen (Hach UniCell™ test) showed the highest concentration of NH_4-N on the day 3 and its subsequent gradual decrease (Fig. 2B). The formation of ammonium ions is clear evidence of aniline dioxygenase activity (Liu *et al*., 2002).

Determination of catechol was performed by spectrophotometric method according to Nair and Vaidyanathan (1964) and showed highest concentration of this metabolite in cell extract (0.50 mg/ml) on the day 5 of fermentation. An important point in discussion on the mechanism of aniline biodegradation is a way of enzymatic transformation of catechol. Its convertion by catechol 1,2-dioxygenase should lead to *cis,cis*-muconic acid (intradiolic pathway) whereas the action of catechol 2,3-dioxygenase should give *cis,cis*-2-hydroxymuconic-6-semialdehyde (extradiolic pathway). The levels of aforementioned metabolites in cell extracts were determined by adaptation of the procedure described by Liu *et al*. (2002). The measurements showed the highest concentration of both metabolites in cell extracts on day 5 of fermentation, amounting to 0.376 mg/ml for *cis,cis*-muconic acid and 0.053 mg/ml for *cis,cis*-2-hydroxymuconic-6-semialdehyde. The clear domination of 1,2-dioxygenase in a cleavage of catechol strongly suggest that biodegradation of aniline by *C. methanosorbosa* BP-6 yeasts under applied conditions proceeds predominantly according to the intradiolic pathway.

In order to confirm this assumption, biodegradation of aniline by *C. methanosorbosa* BP-6 yeasts under conditions as above was followed by HPLC (GYNOTEK instrument, ODS Hypersil RP-18 column, UV-VIS detector, isocratic elution with phosphate buffer, pH 7.5 – methanol, 60:40, v/v). Thus, the yeast cell extracts were prepared as above and, after appropriate dilution, analyzed by HPLC with authentic samples of each metabolite serving as internal standards (see Fig. 3).

The analysis revealed that all intermediates of aniline biodegradation by intradiolic pathway were present in chromatograms. The concentration of catechol was found to be growing up to the day 5 (Fig. 3A) and the level of *cis,cis*-muconic acid was highest between days 3 and 5 of fermentation (Fig. 3B). Muconolactone showed up between days 3 and 7 of aniline biodegradation (Fig. 3C), preceding the formation of 3-oxoadipate enol-lactone (days 7 and 8, Fig. 3D) and 3-oxoadipic acid (days 7 and 8, Fig. 3E). Finally, succinic acid was found in cell extract taken on the day 8 of fermentation (Fig. 3F). Thus, HPLC identification and the sequence of appearance of intermediate metabolites provide strong evidence that biodegradation of aniline by yeast strain *C. methanosorbosa* BP-6 in AY medium proceeds predominantly according to the intradiolic pathway.

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