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New Antibacterial Therapeutics and Strategies

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Abstract
Studies on new antibacterial therapeutics and strategies are currently being conducted in many microbiological, pharmaceutical and biochemical laboratories. The antibacterial activity of plant-derived compounds as well as silver and gold nanoparticles is the subject of this minireview. The application of photodynamic therapy is also discussed.

Key words: nanoparticles, new antimicrobials, phototherapy, plant compounds

Introduction

The growing antibiotic resistance of pathogenic bacterial species is a serious problem for public health. It can be assumed, that although the bulk of traditional antibiotics can still manage drug-resistant bacteria, many commonly used antibiotics are no longer effective (Levy, 1998; Wright, 2010). This situation is aggravated by a decline in the development of new antibiotics, so recently few substances have appeared in the market (Donadio et al., 2010; Högberg et al., 2010). In view of above, the growing interest in the studies aimed at developing new antibacterial therapeutics and strategies is very important not only from medical point of view but also for agriculture and animal breeding (Myles, 2003). The list of such therapeutics is long with stress on the use of bacteriophages as antibacterial agents (Chibani-Chennoufi et al., 2004; Görski et al., 2009). Many papers cover this problem so it will not be the subject of our publication. The present article describes the antibacterial potency and application of plant-derived compounds, nanoparticles and also the very promising photodynamic therapy.

Plant-derived compounds

Plant-derived compounds of therapeutic value are mostly the secondary plant metabolites (Cowan, 1999). Antibacterial phytochemicals are divided in several categories, this article describe two of them – terpenes and phenolics including polyphenols. As a matter of fact, these compounds cannot be considered a new group of antimicrobials because people have applied plants and plant extract for medical purposes for centuries (Rios and Recio, 2005).

Terpenes. Terpenes, also referred to as isoprenoids, are based on an isoprene structure with general chemical formula C_{10}H_{16} and are biosynthesized from the same basic units, isopentenyl diphosphate, IPP, and its isomer dimethylallyl diphosphate, DMAPP (Fig. 1A). Their derivatives containing additional elements, usually oxygen, are called terpenoids. These compounds have a lot of biological functions and are applied as pharmaceuticals, fragrances, colorants. Terpenes constitute a very diverse group of compounds isolated not only from higher plants but also from microorganisms (Sacchettini and Poulter, 1997). Successful approaches in engineering Escherichia coli towards biosynthesis of functional isoprenoids were recently described (Harada and Misawa, 2009).

Of special interest are two pentacyclic triterpenoids oleanolic acid (OA) and ursolic acid (UA) and their derivatives containing sugar moieties – glucosides and glucuronides. The chemical formulas of OA/UA are presented in Figure 1B. The antibacterial activity of these compounds was recently reviewed (Wolska et al., 2010a). The data described in many papers are contrasting and indicate either a strong or weak...
antibacterial effect of OA and UA which likely results from the method of compound purification and the bacterial strain used. A relatively small number of studies has been performed to investigate the basis of OA/UA antibacterial activity. It has been shown that both acids affected peptidoglycan metabolism in *Listeria monocytogenes* (Kurek et al., 2010), oleanolic acid cyclodextrins inhibited insoluble glucan synthesis by *Streptococcus mutans* (Kozai et al., 1999) and oleane-type triterpenoid, glycyrrhizin acted as a potent *E. coli* heat-labile enterotoxin inhibitor (Chen et al., 2009). In turn Ren and coworkers (2005) using microarray techniques demonstrated that UA caused differential gene expression in *E. coli* and inhibited biofilm formation in several bacterial species. Recently Ge and coworkers (2010) proved the synergistic interactions of OA in combination with isoniazid, rifampicin or ethanbutol against *Mycobacterium tuberculosis*. The third pentacyclic triterpenoid with similar chemical structure – betulic acid (BA) is inactive against a large number of Gram-positive and Gram-negative bacteria. This result illustrates the strong structure-function influence of the antibacterial potential of terpenes (Fontanay et al., 2008; Wansi et al., 2010).

Other terpenes or terpenoids, such as monoterpenes and sesquiterpenes and their derivatives, also display antimicrobial activity (Ahmed et al., 1993; Amaral et al., 1998; Habtermariam et al., 1993). Many publications describe the antibacterial potential of diterpenoids. It was demonstrated that six diterpenoids isolated from the bark of *Podocarpus nagi*, of which the most abundant compound was totarol, exhibited potent bactericidal activity against a large number of Gram-positive and Gram-negative bacteria. This result illustrates the strong structure-function influence of the antibacterial potential of terpenes (Fontanay et al., 2008; Wansi et al., 2010).

Phenolics and polyphenols. Phenolics and polyphenols constitute a very large group of chemical compounds. Simple phenols consist of a single substituted phenolic ring, flavones and their derivatives – flavanoids and flavanols are phenolic structures containing one carbonyl group, while quinones contain two carbonyl groups. Tannins are polymeric phenolic substances and coumarins are phenolic compounds made of fused benzene and pyrone rings (Cowan, 1999). The chemical formulas of exemplary phenolic compounds are shown in Figure 2.

The simple phenol, caffeic acid isolated from perennial thorny shrub, *Paliurus spina-christi* was extracted from immature cones of *Pinus nigra* inhibited the growth of multidrug-resistant and methicillin-resistant *Staphylococcus aureus* – MRSA (Smith et al., 2005). Antibacterial activity of diterpenoids isolated from hairy roots of *Salvia sclarea* L. was also studied. The results showed that abietane diterpenoids: salvisipone, aethopinone, 1-oxoaethopinone and ferrugiol were bacteriostatic as well as bacteriocidal for cultures of *S. aureus* and *S. epidermidis* but not for the Gram-negative species, *E. coli* and *Pseudomonas aeruginosa* (Kužma et al., 2007). Salvipisione and aethiopinone expressed staphylococcal anti-biofilm activity, the reduction in the number of live biofilm cells and changes of biofilm morphology were observed. Both diterpenoids showed synergy with several classes of antibiotics, in case of β-lactams this phenomenon was due to the probable alternation of cell surface hydrophobicity and cell envelopes permeability (Walencka et al., 2007). It was also demonstrated that diterpenes inhibited the growth of *M. tuberculosis* (Copp and Pearce, 2007) and recently the Chinese group showed that diterpenes isolated from the genus *Scutellaria* possessed the substantial antimicrobial and antiviral activities (Shang et al., 2010).
effective against Gram-positive bacterial species (Brantner et al., 1996). p-guanidinethyl and its simple parent phenols were active against S. aureus; the simple phenolic species showed lower activity that their calixarene analogues (Mourer et al., 2009). Other groups demonstrated that more highly oxidized phenols were also more active probably due to their ability to oxidize of sulphydryl groups in proteins (Urs and Dunleavy, 1975; Mason and Wasserman, 1987).

Flavonoids and their derivatives, flavonoids and flavonols, due to their extremely large amount of biological properties, including the antibacterial activity, are placed among the most attractive plant derivatives enriching the current therapy options (Cazarolli et al., 2008). These compounds can form complexes with cell wall and also disrupt bacterial envelopes (Tsuchiya et al., 1996; Nakayama et al., 2000). Of special interest are catechins, the subgroup of flavonoids present in the oolong green teas which beneficial effect on human health is well known (Cabrera et al., 2006). These compounds are active against food-borne pathogenic bacteria and therefore exert the beneficial effect in gastrointestinal diseases (Friedman, 2007; Dryden et al., 2006; Koo and Cho, 2004). It was shown that catechins inhibited in vitro the growth of several bacterial species such as Vibrio cholerae, S. mutans and Shigella spp. (Borris, 1996; Sakanaka et al., 1992; Vijaya et al., 1995). Inactivation of specific bacterial enzymes, V. cholerae toxin and glucosyltransferases in S. mutans, was also reported (Borris, 1996; Nakahara et al., 1993). Chrysin, another flavonoid abundant in propolis, also displays substantial antimicrobial activity, preferentially against Gram-positive species, e.g. Streptococcus sobrinus, Enterococcus faecalis and Micrococcus luteus (Uzel et al., 2005). Its activity against certain oral pathogens, such as Peptostreptococcus anaerobius, Peptostreptococcus micros and Lactobacillus acidophilus creates the possibility of propolis application in the treatment of oral cavity diseases (Koru et al., 2007). Recently novel C(7) modified chrysin was synthesized. This modification was deliberately designed in order to enhance the antibacterial effect. The biological assays indicated that this compound is a potent inhibitor of beta-ketoacyl-acyl carrier protein synthetase (FabH) in E. coli (Suresh Babu et al., 2006; Li et al., 2009).

A number of papers are concerned with the antibacterial activity of quinones. This activity is based on their high chemical reactivity inactivating the chemical compounds, mainly proteins. Surface-exposed bacterial adhesins, cell wall polypeptides and membrane bound enzymes are their probable targets (Cowan, 1999; Koyama, 2006). Because the redox-potential of these compounds was so essential to their activity, electrochemical techniques along with biochemical and medical knowledge were successfully combined to design and develop therapeutically efficient derivatives (Hillard et al., 2008). It should be also mentioned that quinones are strong poisons for bacterial type II topoisomerases – gyrase and TopoIV (Pommier et al., 2010). Potent antibacterial activity is attributed to the anthraquinones. It was demonstrated that anthraquinone isolated from Cassia italica is bacteriostatic for Bacillus antracis, Corynebacterium pseudodiphtericum and P. aeruginosa (Kazumi et al., 1994), in turn hypericin and hyperforin isolated from Hypericum perforatum (St. John’s wort) were active against Gram-positive species – S. aureus, S. epidermidis, E. faecalis and Bacillus subtilis (Males et al., 2006; Saddiqe et al., 2010). Recently it was shown that the antibacterial activity of anthraquinone derivatives from Heterophyllaea postulata against S. aureus involved an increase in the level of superoxide anion and singlet molecular oxygen (Comini et al., 2010). The antimycobacterial activity of quinones was also described (Copp and Pearce, 2007).

Tannins are divided in two groups – hydrolysable (derivatives of gallic acid) and condensed, also called proanthocyanidins (derived from flavonoid monomers). Among the various health beneficial activities of tannins their antimicrobial activity is often referred to. These compounds are characterized by strong antiperoxidation properties which may be responsible for the inactivation of microbial adhesions, enzymes and
Silver and gold nanoparticles

Nanoparticles (NPs) are defined as the clusters of atoms of size ranged from 1 to 100 nm. Their network forms are called nanowires. NPs are characterized by a very large surface area to volume ratio (Rai et al., 2009). Copper, zinc, magnesium but especially silver and gold NPs display antibacterial activity and are used for various healthcare, hygiene and personal care purposes and also in water-treatment (Edwards-Jones, 2009). Tannins, especially proanthocyanins, inhibit the growth of uropathogenic E. coli (Cimolai and Cimolai, 2007), S. mutans (de la Iglesia et al., 2010) as well as ruminal bacteria. For the latter it was documented that condensed tannin (sainfoin) inhibited the growth and protease activity of Butyrivibrio fibrisolvens A38 and Streptococcus bovis. The morphological changes of these species implicated the cell wall as a target of tannin toxicity (Jones et al., 1994). In turn, both hydrolysable tannins and proanthocyanidines suppressed the oxacillin-resistance of MRSA (Hatano et al., 2005).

The number of publications dealing with antimicrobial activity of yet another group of phenolics, cumarins, is scarce and generally it can be concluded that this property has not been evaluated systematically (Borges et al., 2005; Cechinel Filho et al., 2009).

The antibacterial effect of AgNPs depends on their size. Smaller-sized particles show stronger antibacterial activity due to their higher surface area to volume ratio (Morones et al., 2005). It was also proved that the truncated triangular AgNPs displayed stronger bacteriocidal action against E. coli compared with spherical and rod-shaped nanoparticles, suggesting shape-dependent interaction at least with Gram-negative bacteria (Pal et al., 2009). Different shapes of silver and gold nanoparticles are shown in Figure 3. Several studies demonstrated that bacterial membranes constituted the main target of AgNPs antibacterial activity, nanoparticles caused their disruption probably due to the production of reactive oxygen species (ROS), including free radicals. Production of ROS is one of the primary mechanisms of nanoparticle toxicity; it may result in oxidative stress, inflammation, and consequent damage not only of membranes but also of DNA and proteins (Singh et al. 2008). The model of bacterium – nanoparticle interactions presented by Neal (2008) is based on contact-mediated membrane lipid peroxidation by arising reactive oxygen species. Contact was facilitated by electrostatic forces between nanoparticles and negatively charged cell envelopes. Scanning and transmission electron microscopy revealed that AgNPs attach to the bacterial cell wall, and that Ag atoms are internalized by the bacteria, probably as Ag ions (Shahverdi et al., 2007; Ingle et al., 2008).

The antibacterial potential of AgNPs over ionic silver because they show reduced toxicity and 1.4–1.9 times higher antibacterial potential (Ingle et al., 2008). Reports pointing to silver toxicity, including aryngia and the deposition of silver in liver are rather scarce (Tomi et al., 2004; Landsdown, 2006).

In this article it should be noted that recently microbial synthesis of selenium, tellurium platinum, thitania, uranitnie and other nanoparticles by bacteria, actinomyces, fungi, yeasts and viruses was also reported (Narayanan and Sakhivel, 2010). Silver in ionic form has been known for centuries to cure venereal diseases, bone and perianal abscesses, eye diseases and burns. It was proved that Ag⁺ was active against various bacterial species e.g. E. coli, S. aureus, Klebsiella sp. and Pseudomonas sp. (Rai et al., 2009; Chopra, 2007). AgNPs have an advantage over ionic silver because they show reduced toxicity and 1.4–1.9 times higher antibacterial potential (Ingle et al., 2008). Reports pointing to silver toxicity, including aryngia and the deposition of silver in liver are rather scarce (Tomi et al., 2004; Landsdown, 2006).

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microscopy images confirmed the formation of pits in the *E. coli* cell wall and the accumulation of silver in bacterial membranes which increases permeability and therefore results in cell death (Sondi and Salopek-Sondi, 2007). AgNPs may target the bacterial membrane, leading to a dissipation of the proton motive force as shown by proteomic data and biochemical studies. Short exposure of *E. coli* cells to AgNPs resulted in alterations in the expression of several envelope proteins (OmpA, OmpC, OmpF, OppA, MetQ) and heat shock proteins, (IbpA, IbpB and 30S ribosomal subunit) (Lok et al., 2006).

AgNPs interaction with membrane sulfur-containing proteins was also proven (Rai et al., 2009). Membrane disruption allowed the passage of AgNPs into cytoplasm causing subsequent damage of DNA and other phosphorus containing compounds and impairing the respiratory chain and cell division. The antibacterial activity of AgNPs is enhanced by their decomposition and the release of Ag ions within bacterial cell (Feng et al., 2000; Morones et al., 2005).

The antibacterial activity of AgNPs was well proven basing on *in vitro* experiments. Activity against MRSA (Panacek et al., 2006), *E. coli* (Sondi and Salopek-Sondi, 2007; Morones et al., 2005; Pal et al., 2007; Yoon et al., 2007), *P. aeruginosa* (Morones et al., 2005), *Vibrio cholera* (Morones et al., 2005) and *B. subtilis* (Yoon et al., 2007) was reported. Kim and coworkers (2007) demonstrated that *E. coli* was inhibited at low concentration of AgNPs whereas the growth-inhibitory effect of *S. aureus* was mild. The efficient antibacterial activity of AgNPs impregnated with bacterial cellulose against *E. coli* and *S. aureus* has also been demonstrated (Castellano et al., 2007). Sucrose and soluble and waxy corn starch can also serve as stabilizers (Valodkar et al., 2010). Synergistic antimicrobial activity of AgNPs with penicillin G, amoxicillin, erythromycin, clindamycin and vancomycin against *S. aureus* and *E. coli* was observed (Shahverdi et al., 2007). It was also shown that AgNPs prevent the formation of bacterial biofilms, e.g. biofilms formed by *P. aeruginosa* and *S. epidermidis* were inhibited in more than 95% (Kalishwaralal et al., 2010). This property made these nanoparticles especially useful in controlling biofilms within the oral cavity (Allaker, 2010). Biofilm formation was inhibited due to the ability of AgNPs to prevent the initial step in biofilm development *i.e.* microbial adhesion to various surfaces (Monteiro et al., 2009).

AgNPs are characterized by so many medical and technological applications that they are considered as new antibacterial agents revolutionizing applied medicine. They are used in wounds and ulcers healing, usually in the form of dressings and creams (Cortivo et al., 2010; Jun et al., 2007). They are also utilized to coat medical devices such as catheters, dressings, and possessing antimicrobial properties (Dastjerdi and Montazer, 2010) and in the production of antimicrobial nanopaints (Kumar et al., 2008).

Gold nanoparticles (AuNPs) also can be used as antimicrobial agents. In this case the majority of papers describes their application as a tool to deliver other antimicrobials or as a factor enhancing photodynamic destruction of bacteria (Pissuwan et al., 2009). AuNPs were very suitable for delivering drugs, including antibiotics; AuNPs conjugates with vancomycin proved to be 50-fold more active that the free antibiotic against *Enterococcus faecium* and *E. faecalis* (Gu, 2003). The AuNPs-ciprofloxacin and aminoglycosidic antibiotics conjugates were also described (Tom et al., 2004; Grace and Pandian, 2007). The role of AuNPs was to facilitate the attachment of conjugated antibiotic to bacterium and therefore penetrating of cell wall. However it should be stressed that there is no consensus concerning the efficacy of AuNPs-antibiotic conjugates compared with the same dosage of free antibiotic, e.g. Rosemary et al. (2006) found that AuNPs enhanced the efficacy of ciprofloxacin against *E. coli* what was not observed for gentamycin (Burygin et al., 2009).

AuNPs were also used as stabilizers for various antimicrobial photosensitzers. Four-fold increase of *S. aureus* elimination was observed when toluidine blue O-AuNPs conjugates and methylene blue-AuNPs conjugates were used in photodynamic therapy compared to dyes alone (Gil-Tomás et al., 2007; Perni et al., 2009). AuNPs enhanced the absorption of light due to their plasmon resonance (Pitsillides et al., 2003) which could cause local hyperthermic effect leading to the efficient destruction of *E. coli* irradiated with X-rays (Simon-Deckers et al., 2009), *P. aeruginosa* exposed to near-infrared laser (Norman et al., 2008) or *S. aureus* irradiated with strong laser light (Zharov et al., 2006).

AuNPs are very useful for detection and diagnosis of bacteria even in complex media like blood (Kaittanis et al., 2010), this being mainly based on the detection of bacterial DNA by change of color (Elghanian et al., 1997). They have been used for fast, accurate and sensitive detection of *Staphylococcus* sp. (Storhoff et al., 2004) and *M. tuberculosis* (Baptista et al., 2006; Veigas et al., 2010). AuNPs were also applied to improve the sensitivity of bacterial detection methods based on flow cytometry and fluorescence (Zharov et al., 2007; Wang et al., 2009).
Natural and synthetic dyes and photodynamic therapy

The antimicrobial effect of natural and synthetic dyes has been known for many decades. Already in the beginning of the last century profavine, acriflavine, crystal violet and brilliant green were used against bacterial infections, mainly to cure infected wounds (Wainwright, 2008; Wainwright, 2010). Pre-operative use of iodine still remains a common practice. Gentian (crystal) violet was shown to be very efficient in the eradication of MRSA from skin lesions (Saji et al., 1995). Recently it was shown that the tiazol dye, thioflavin T, exerted a strong inhibitory effect on S. aureus, the effect on E. coli was less pronounced (Lakatôš, 2010). The list of dyes with antibacterial properties included also pigments synthesized by microorganisms (Wolska et al., 2010b). The pigment produced by P. aeruginosa – pyocyanin, due to its unique redox properties, actively eliminated many bacterial species, especially Gram-positive aerobes (Baron and Rove, 1981). In turn violacein extracted from Chromobacterium violaceum displayed a potent antileishmanial activity (Leon et al., 2001).

Many dyes can serve as photosensitizers, sometimes called photomicrobial agents, and are applied in photodynamic antimicrobial therapy (PACT). Photosensitizers can be activated by visible light to generate cytotoxic radicals, superoxide radicals and singlet oxygen \( \frac{1}{2} \text{O}_2 \) which are the reactive oxygen species (ROS). ROS are highly toxic to various type of cells, including bacteria, causing the damage of the outer membrane, cell wall, ribosomes and nucleic acids and thus impairing many cellular functions (for review see Wainwright, 2010; Ryskova et al., 2010). Photosensitizers are usually dyes such as methylene blue (Gad et al., 2004), porphyrins (Hamblin et al., 2005), crystal violet (Saji et al., 1995), iodocyanine green (Unno et al., 2008) and erytosine (Wood et al., 2006). The latter is of special interest because it can be used for a strong therapeutic effect of the therapy of oral plaque biofilms (Wood et al., 2006). Positively charged (cationic) photosensitizers such as methylene blue and crystal violet act as broad-spectrum antimicrobials, while the negatively charged (anionic) compounds lack efficacy against Gram-negative species (Demidova et al., 2005). This is because anionic photosensitizers are not able to penetrate the lipopolysaccharide outer membrane of Gram-negative bacteria. In practice photosensitizers are usually activated by red light and the preferable source of light is low-power lasers (Ryskova et al., 2010). The stabilization of various photosensitizers by AuNPs was described in the previous chapter. Beside dyes, also fullerenes which are stable chemical molecules composed of 60 carbon atoms arranged in a soccer ball-shaped structure are proved to be the efficient photosensitizers (Krokosz, 2007).

Photodynamic therapy is used mainly in the treatment of local infections. Its efficacy was proved in healing cutaneous infections e.g. poor-healing wounds caused by Mycobacterium marinum (Rallis and Koumantaki-Mathioudaki, 2007) and oral microbial related diseases such as periodontitis (Liu et al., 2009). It was clinically studied for leishmaniasis (Dai et al., 2009). It should be stressed that photodynamic therapy can be applied in the eradication of antibiotic-resistant pathogens e.g. MRSA (Griffiths et al., 1997), VRE (Soncin et al., 2002) and P. aeruginosa (Minnock et al., 1996) which are life-threat danger in hospitals. Recently it was shown that two water soluble photosensitizers: methylene blue and neutral red enclosed in liposomes gave a stronger antibacterial effect than their free forms (Nisnevitch et al., 2010). It should be also noted that photodynamic therapy was applied and found to be efficient in treating lung, stomach and skin tumors (Maisch et al., 2005).

Conclusions

The huge and constantly growing amount of papers describing new antibacterial therapeutics reflects an urgent need to find efficient antimicrobials which can be an alternative to antibiotics. Plants and their extracts have been used even in ancient times for medical purposes. Recently, studies are focused on the activity of purified compounds. Trials aimed at resolving the mechanism of their action are also performed. The majority of studies are held in vitro but the results of preclinical and clinical trial have also been reported. As it was shown that many plant compounds exert a cytotoxic effect (Zhang et al., 2007) efforts have been made to synthesize derivatives with less toxicity and better water solubility (e.g. Farina et al., 1998; Liu, 2005), which can enhance the possibility of their therapeutic application. Studies on the antibacterial activity of silver and gold nanoparticles are more advanced and also comprise their synergism with commonly used antibiotics and the application of AuNPs in stabilizing photosensitizers used in photodynamic therapy, which is a very proficient new antibacterial strategy.

Considering the enormous scientific effort put in elaborating new antibacterial compounds and strategies, alternative possibilities to cope with bacterial diseases can emerge in the near future.

Acknowledgment

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New antibacterial therapeutics and strategies


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Cloning, Expression and Identification by Immunohistochemistry of Humanized Single-Chain Variable Fragment Antibody against Hepatitis C Virus Core Protein

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Abstract

Expression of single-chain variable fragment (scFv) antibodies on the surface of bacteriophage is widely used to prepare antibodies with pre-defined specificities. A phage antibody library containing the gene for scFv antibody against Hepatitis C virus core protein was panned with core protein immobilized on microtiter plate wells. After five rounds of panning 60 phage clones specific to core protein were obtained and one selected clone was sequenced. It was found that the specifically detected antigen consists of 774bp and is capable of encoding 257 amino acids in the patients but not in healthy persons.

Key words: Hepatitis C virus; core protein; phage display; single-chain variable fragment antibody

Abbreviations: HRP, horseradish peroxidase; HCV, Hepatitis C virus; HCV core protein scFv antibody, scFv antibody against HCV core protein; MAb, monoclonal antibody; scFv, single-chain variable fragment.

Introduction

Hepatitis C virus (HCV) was first described in 1989 as the putative viral agent of non-A non-B hepatitis. An estimated 170 million people are infected with HCV worldwide (Armstrong, 2003; Stoll-Keller et al., 2009). HCV is a member of the Flaviviridae family and has been recognized as the major causative agent of chronic liver disease, including chronic active hepatitis, cirrhosis and hepatocellular carcinoma (Di Bisceglie, 2000). It has recently been suggested that HCV core protein suppresses the antiviral cytotoxic T-lymphocyte response by interacting with the C1q complement receptor, thereby playing a key role in the induction and maintenance of chronic HCV infection and liver damage (Kittlesen et al., 2000; Large et al., 1999). The presence of serum HCV core protein is associated with active HCV viremia, and its detection is now used for clinical evaluations (Dickson et al., 1999; Tanaka et al., 2000). In addition, HCV core protein is highly conserved among the various HCV genotypes (Houghton et al., 1991) and elicits a rapid Antibody response after the onset of the disease. Thus, antibody responses to HCV core protein may be considered to confer protection against HCV infection regardless of viral subtypes. The monoclonal antibodies (McAb) of HCV core protein could passively conduce to prevent HCV infection. However, the production periodicity of McAb made by hybridoma technique is relatively long. In addition, clinical testing has shown that murine monoclonal antibodies may evoke human anti-mouse antibody response. Phage display technology offers a means of cloning human anti-HCV antibodies coding gene of a defined specificity that may have potential therapeutic use (Canaan-Haden et al., 1995). We now utilize a semi-synthetic human single-chain Fv antibody library and solid-phase bound hepatitis C virus core protein to screen out the phage display antibody that recognizes the HCV core protein (Zhong et al., 2001), using HCV core protein as the immobilized antigen and proceeding immunohistochemistry.
Experimental

Materials and Methods

Materials. A recombinant HCV core protein from Virostat (USA) was employed. A human scFv antibody phage library in which the genes encoding VL and VH were amplified by PCR with degenerate primers and connected with a glycan linker ([Gly_Ser]), was purchased from Novagen (USA). M13K07 phage was employed as helper (Pharmacia).

Phagemids rescue. To rescue phagemids from the library, 5 ml of 2×TY broth containing 100 mg/ml ampicillin and 1% glucose (2×TY-AMP-GLU) was inoculated with 10 µl of Escherichia coli TG1 taken from the library stock and grown for 3 hrs at 37°C. The bacteria were spun down, resuspended in 50 ml of 2×TY broth containing 100 mg/ml ampicillin (2×TY-AMP), and shaken until A600 reached 0.5. The bacteria were inoculated with M13K07 phage (1×1010 PFU) and incubated for 30 mins at 37°C with shaking.

Materials and Methods

Identification of phage clones. Wells of 96-well-plates were coated overnight with 8 µg HCV core protein per well in the coating buffer. After blocking 100 µl aliquots of diluted (1:50) culture supernatants were added per well for 1 hr at 37°C. The plates were washed and loaded with the secondary antibody as described above. M13K07 phage served as negative control.

DNA sequencing. Plasmid DNA was prepared from the culture of a selected positive clone using Wizard Plus Minipreps DNA Purification System (Promega) and sequenced in an ABI3700 automated DNA sequencer (Perkin Elmer).

Expression of soluble HCV core protein scFv antibody in E. coli. To express the scFv antibody in soluble E-tagged form the selected clone was subcloned into the pCANTAB5E expression vector. Restriction digestion and subsequent 1% agarose gel electrophoresis confirmed the identity of the recombinant pCANTAB5E-scFv vector. Competent E. coli XL1-Blue was transformed with pCANTAB5E-scFv and induced with IPTG for 20 hrs. The culture was centrifuged at 10,000 r.p.m. and the supernatant was subjected to SDS-PAGE and Western blot analysis.

Western blot analysis. The culture supernatant was diluted 1:1 with 2×SDS loading buffer, heated at 100°C for 10 mins, briefly centrifuged again, and 20 µl of the supernatant was used for SDS-PAGE. After the run the gel was blotted onto a PVDF membrane (Millipore). The blot was blocked with 5% non-fat dry milk for 2 hrs, incubated with an anti-E-tag MAb for 1.5 hr and with a secondary HRP-goat anti-mouse IgG antibody for another 1 hr, and stained with DAB and hydrogen peroxide.

Immunohistochemistry. Paraffin-embedded liver tissue slices from patients with positive anti-HCV antibodies and HCV-RNA were examined. After deactivating endogenous peroxidase the slices were submersed in a methanol solution (should be defined) with 0.5% hydrogen peroxide at room temperature for 50 mins, washed with PBS 3 times, and kept in 5%
BSA overnight at 4°C. Then the slices were incubated with the scFv antibody diluted 1:100 for 1 hr at 37°C and overnight at 4°C. A sheep HRP-anti-M13 antibody diluted 1:200 was dropped on tissue preparations and left to react for 40 min at 37°C. The preparations were washed 3 times with PBS, a few drops of a DAB solution (9 mg DAB, 13.5 ml 0.01 M Tris-HCl (pH7.6), 1.5 ml 0.3 % CoCl₂, and 15 ml 30% hydrogen peroxide) were added. After 10 min at room temperature the preparations were washed with PBS 3 times and 1% haematin was used to stain the cell nucleus. After a standard dehydration procedure the preparations were observed under microscope. Negative controls consisted of PBS instead of the scFv antibody and liver tissue preparations from healthy persons.

**Results**

**Identification of HCV core protein scFv-positive clones.** After five rounds of panning (amplification-absorption-elution) 60 clones were picked up and tested for HCV core protein scFv antibody by ELISA. Twenty of these were found positive. Six of these showed a low cross-reaction with BSA (Fig. 1). One positive clone with the highest reaction with HCV core protein and the lowest reaction one with BSA was chosen for a confirmatory test with restriction digestion. The digestion with NcoI and NotI and subsequent gel electrophoresis proved the presence of a 774 bp insert corresponding to the scFv antibody gene (Fig. 2).

<table>
<thead>
<tr>
<th>H chain CDR1</th>
<th>CDR2</th>
<th>CDR3</th>
<th>FR1</th>
<th>FR2</th>
<th>FR3</th>
<th>FR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFTFFSY-AMS</td>
<td>AISQGGSST-YYADSVKG</td>
<td>TRTKRF</td>
<td>EVQLVESGGGLV- RPQGSLRLSAAW</td>
<td>WVQQAPGKG- EWWS</td>
<td>RFTISRNKNTL- YLQMNLSREAEDT -AVYYCAR</td>
<td>WGGQVALTVSR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L chain CDR1</th>
<th>CDR2</th>
<th>CDR3</th>
<th>FR1</th>
<th>FR2</th>
<th>FR3</th>
<th>FR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQGDSL-RSYYAS</td>
<td>GKNRPS</td>
<td>NSPDDS-NHVV</td>
<td>SELTQPAS- VALGQTVRT</td>
<td>WYQQKPGQAP- VLVIY</td>
<td>GIPDRFSGGSS- TASLTITGQAED- EADYYC</td>
<td>FGGGTKLTVLG</td>
</tr>
</tbody>
</table>

**Table I**
The complementarity-determining regions (CDRs) and framework regions (FRs) of deduced amino acids sequence of ScFv against HCV core protein

Fig. 1. Identification of positive clones. The binding activities of the phage antibodies from six clones infected by bound phage particles to coated 8 µg HCV core protein and 20 µg bovine serum albumin.

a-f: positive clones’ culture supernatant with scFv antibody against HCV core protein; g: helper M13K07 instead of phage antibodies, used as negative.

Fig. 2. Restriction map of plasmid DNA carrying HCV core protein scFv gene prepared from a single positive clone by NcoI/NotI digestion.

a, b: DNA fragment with HCV core protein scFv gene; M: DNA Marker DL2000 (Takara Co., Japan)

Fig. 3. Western blot analysis of the supernate protein derived from induced and non-induced E. coli XL1-Blue transformed by expression vector.

Supernate protein from non-induced E. coli XL1-Blue transformed with pCANTAB5E-HCV core protein-scFv (lane a) and supernate protein from induced E. coli XL1-Blue transformed with pCANTAB5E-HCV core protein-scFv (lane b).
Sequencing of HCV core protein ScFv gene. The nucleotide sequence of the selected clone was determined and submitted to GenBank (Acc. No AF271150). The corresponding amino acid sequence was deduced and complementarity-determining regions and framework regions were located according to the Kabat database (Table I).

Expression of HCV core protein scFv in \textit{E. coli}. The HCV core protein scFv antibody was expressed in \textit{E. coli} and confirmed by Western blot analysis (Fig. 3). A negative control consisting of \textit{E. coli} infected with empty vector did not show any specific protein. These results indicated that the soluble form of human HCV core protein scFv antibody was successfully expressed in this system.

Immunostaining of HCV core protein of liver tissue sections. Immunostaining of sections of liver tissues from and 17 patients with chronic hepatitis C and healthy persons gave positive results in the former but not latter group (Fig. 4). HCV core protein was mainly located in the cytomembrane of the hepatocytes.

Discussion

The mature HCV protein is located in the cytoplasm of infected cells, in close vicinity to the perinuclear membranes and the endoplasmic reticulum, where it polymerizes in the presence of genomic RNA to form viral capsids (Reed and Rice, 2000). The HCV core protein is highly antigenic, induces specific cellular and humoral responses, and probably plays a pivotal role in the pathogenesis of HCV infection (Lai and Ware, 2000; Nelson \textit{et al}., 1997). The availability of an anti-HCV core protein humanized scFv antibody allowed the development of an ELISA to detect HCV core antigen in peripheral blood of patients with HCV (Aoyagi \textit{et al}., 1999). The major neutralizing epitope of HCV core protein lies within the first 45 aa of the protein, the major antigenic segment of core recognized both by murine and human antibodies. Noticeable, the recognized epitope (29–37: QIVGGVYLL) has an unusual preponderance of hydrophobic residues, some of which are buried in a small hydrophobic core in the nuclear magnetic resonance structure of the peptide in solution, suggesting that the antibody may induce a structural rearrangement upon recognition (Menez \textit{et al}., 2003).

Phage libraries are a powerful tool for the selection of antibodies of important and useful specificities, particularly for humanized scFv antibodies (Marks \textit{et al}., 1991; Hoogenboom \textit{et al}., 1998; Lamarre and Talbot, 1997; Rondon and Marasco, 1997). It has many advantages. First, it is the only method to get specific antibody by passing the immunization step. It can mimic the maturation process of human antibody in vivo, so that it is possible to obtain a high affinity antibody from this selection. Second, a scFv antibody consisting of antigen-binding domains of heavy and light chain regions of immunoglobin connected by a flexible peptide linker is a small-size molecule compared with the full-length antibody. If an antibody library of human origin is used, the selected antibody is most suitable to human administration and is potentially applicable to clinical diagnosis and treatment of both infectious disease and cancer. Finally, as it contains no Fc fragment, its background in immunohistological study is very low. In contrast, a MAb against the recombinant HCV core protein prepared from hybridomas is of murine origin and hence immunogenic if used systemically in humans (Songsivilai \textit{et al}., 1996). In order to overcome the disadvantages of an intact MAb applied in vivo and to offer an antibody with a stable genetic source, soluble scFv antibodies are currently generated by advanced recombinant phage antibody technique, which may provide novel targeting vehicle for diagnosis and treatment of diseases.
In this study, we succeeded in cloning the HCV core protein scFv gene by means of the phage display library technique. The cloned gene was sequenced and expressed in E. coli. These results illustrate the feasibility of using the antibody-engineering technology that may prove useful in the future for diagnostics and therapy of hepatitis C infection.

Literature


Introduction

Staphylococcus species are considered to be one of the most widespread bacteria found in nature. Bacteria that constitute this genus, colonize a number of domestic animals, including dogs and cats. Usually they belong to the normal skin flora of these animals commonly present on skin and mucosal membranes (Stepanovic et al., 2001; Hauschild and Wojcik, 2007). In 1976 Hajek described a new staphylococcal coagulase-positive species Staphylococcus intermedius which colonized predominantly the skin and the mucosal membrane of the nasal vestibule in dogs (Hajek, 1976). Later studies revealed that S. intermedius was the most frequent species isolated from healthy dogs. It comprised 40.3% of all isolated strains and was identified as a resident of the physiological bacterial flora of healthy dogs (Cox et al., 1988; Król, 1998). Devriese and DePelsmaecker proved in their research that S. intermedius strains were predominantly isolated from nostrils and the rectum region of healthy dogs (Devriese and DePelsmaecker, 1987). These two ecological niches were proposed to be the source for colonization of other areas in healthy dogs by S. intermedius in its carrier-state, inhabits mainly the mucosal membrane of the nasal vestibule. It was also found in the samples taken from the skin, the lumbo-sacralis triangle and perineum, but was rarely isolated from the ears.

Key words: Staphylococcus spp. from dogs, diagnostic, phenotyping, biochemical methods
factors, or systematic diseases (Hendricks et al., 2002; Kizerwetter-Świda et al., 2009). Pyoderma is a type of dermatitis. It occurs fairly often among dogs. In pyoderma, either flesh or deep skin infections can be observed. Commonly in both cases, *S. intermedius* is the main causative agent. Infective skin lesions usually show up in warm, moist and wrinkled skin regions, where there appear to be propitious conditions for bacterial colonization and progression of pyoderma (Allaker et al., 1993; Guardabassi et al., 2004).

Notwithstanding the growing responsibility and microbiological awareness among dog owners and the increasing interest for the *S. intermedius* species among the veterinary society, this species still has not been adequately studied. Bearing this in mind, knowledge of the bacterial flora composition of the dogs skin would be extremely useful in the epidemiological study of a variety of skin infections that occur in dogs.

The main goal of this project was to determine the similarity, disparity and potential relationship between the different colonization patterns of the staphylococcal species characteristic for healthy and diseased dogs, living either in a rescue shelter or households.

Commonly the diagnostics of bacterial strains isolated from dogs are based on phenotypic methods and these type of methods were considered in this study (Miedzobrodzki et al., 2008). Although based on the fact that some staphylococcal species (*S. intermedius* and *S. pseudointermiedius*) can be distinguished almost only by genetic methods, the further introduction of genetic based techniques in routine veterinary diagnostics is essential (Bannoehr et al., 2009; Devriese et al., 2009).

**Experimental**

**Materials and Methods**

**Bacterial strain isolation.** Bacterial samples isolated from 20 healthy and diseased dogs were examined. The examined group of dogs was heterogeneous. It consisted of 20 mongrels, both male and female that weighed from 5 to 45 kg. Among the 20 dogs, 12 had skin lesions and the remaining 8 had none. The dogs living in an animal shelter had been staying there for a few weeks up to 3 years. A similar time range was for the dogs living in household environments. Five out of 7 sheltered dogs, and 7 out of 13 household dogs demonstrated skin lesions. In the 8 remaining dogs, no skin lesions were observed, as shown in Figure 1.

Samples were taken from skin lesions found in sick dogs as well as from healthy dogs without skin changes. The samples were collected by a veterinarian. The material used for research was derived from skin lesions caused by flea allergy dermatitis. These lesions were caused by hypersensitivity, and may have had pus complications. The lesions were not only always exuding with pus or serum-pus, but also painful and strongly pruritic. However, there were no immunological changes, understood as autoagression, such as lupus or pemphigus. There were no spontaneous bacterial infections either.

Swabs were taken from each dog from both the left and right ear, the nasal vestibule, and perineum, as well as from the back, and in the case of the diseased dogs, also from the infection site (Fig. 2). All the isolated samples/strains were described in the following manner: (i) classification of the etiological agent causing from skin infections; (ii) characteristic of the isolated bacterial strain by standard phenotypic methods;
Gram staining was performed (Beveridge, 2001). Identification of all the 104 isolates to Gram-positive cocci, was received from 20 examined dogs. A total number of 104 isolates colonies were isolated and plated on tryptic soy agar. All colonies were screened and staphylococcal-like colonies were isolated and plated on tryptic soy agar for 24 h at 35°C. Pure isolates were then used for future experiments. A total number of 104 isolates was received from 20 examined dogs.

**Microscopic analysis.** To confirm the purity identification of all the 104 isolates to Gram-positive cocci, Gram staining was performed (Beveridge, 2001).

**Biochemical identification.** Throughout the biochemical analysis, two reference strains \( S. aureus \) ATCC25933 and \( S. epidermidis \) ATCC 12228 were tested alongside the canine isolates. All isolates were evaluated for catalase activity and furazolidone resistance. Bacterial identification was performed by a coagulase tube test (Biomed, Poland). Detection of the clumping factor with rabbit plasma was performed by PASTOREXTM STAPH-PLUS test (BIO-RAD). In order to confirm identification of staphylococcal species, ID32 STAPH (BioMerieux, Poland) analysis was done (Sasaki et al., 2007a; Weese et al., 2009).

**Results**

A total of 104 staphylococcal strains were isolated from healthy and diseased dogs living in either animal shelter or households. All strains demonstrated typical colony growth on blood and Baird-Parker selective agar. Coagulase-positive staphylococci produced black, shiny, convex colonies with entire margins and clear zones with or without an opaque zone around the colonies.

**Phenotypic characteristics of isolated strains.** The free coagulase tube test revealed a positive reaction in 61 strains (59%), whereas no coagulation was observed in the remaining 43 (41%) strains. Although the production of the clumping factor was detected in 44 (42%) strains, 57 (55%) did not produce this factor, and for 3 (3%) strains the results were uncertain. The PASTOREXTM STAPH-PLUS test showed that only 39 among all strains (37.5%) have the ability to autoagglutinate. In terms of the ID32 STAPH method, 104 strains were identifiable, Table I. \( S. intermedius \) (46 strains) and \( S. aureus \) (11 strains) were the only two coagulase-positive staphylococcal species isolated among all the tested canine strains. The remaining strains belong to the coagulase-negative group.

**Domicile and Staphylococcus sp. variety.** Table I presents the rate of occurrence of different staphylococcal species in swabs taken from the skin, ears, and mucosal membrane of the nasal vestibule, from dogs living in the animal shelter or households. A total of 17 different species belonging to the \( Staphylococcus \) genus were found. Animals living in the shelter carried 11 different species, whereas 12 species were isolated from dogs living in household conditions. Regardless of living environments, \( S. intermedius \) isolates were the most common. It accounts for 40% and 47.4% of all isolated strains, respectively for the animal shelter and household environment. Based on the obtained results the difference between staphylococcal profiles and the various environments that they colonize was noticeable. Dogs from the animal shelter were carriers for such staphylococcal species, like \( S. gallinarum \), \( S. saprophyticus \), and \( S. simulans \) that were not isolated from any animal living in the household environment. On the other hand, such species as \( S. warneri \), \( S. hominis \), and \( S. chromogenes \), were isolated only from dogs living in the households. A fact worth noticing was that 11 \( S. aureus \) strains (MSSA and MRSA) were isolated only from samples obtained from the shelter.

**Table I** Distribution of 104 strains of the \( Staphylococcus \) species in all examined dogs

<table>
<thead>
<tr>
<th>Staphylococcus spp.</th>
<th>Living environment of examined dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 sheltered dogs-45 strains</td>
</tr>
<tr>
<td>( S. intermedius )</td>
<td>18 (40.0 %)</td>
</tr>
<tr>
<td>( S. aureus ) (MSSA)</td>
<td>3 (6.7%)</td>
</tr>
<tr>
<td>( S. aureus ) (MRSA)</td>
<td>8 (17.8%)</td>
</tr>
<tr>
<td>( S. warneri )</td>
<td>0</td>
</tr>
<tr>
<td>( S. gallinarum )</td>
<td>2 (4.4%)</td>
</tr>
<tr>
<td>( S. hominis )</td>
<td>0</td>
</tr>
<tr>
<td>( S. chromogenes )</td>
<td>0</td>
</tr>
<tr>
<td>( S. epidermidis )</td>
<td>0</td>
</tr>
<tr>
<td>( S. haemolyticus )</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>( S. lentus )</td>
<td>3 (6.7%)</td>
</tr>
<tr>
<td>( S. saprophyticus )</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>( S. equorum )</td>
<td>3 (6.7%)</td>
</tr>
<tr>
<td>( S. xylosus )</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>( S. cohnii )</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>( S. schleiferi )</td>
<td>0</td>
</tr>
<tr>
<td>( S. simulans )</td>
<td>3 (6.7%)</td>
</tr>
<tr>
<td>( S. lugdunensis )</td>
<td>0</td>
</tr>
<tr>
<td>( S. sciuri )</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td><strong>Total number of staphylococcal species</strong></td>
<td><strong>11</strong></td>
</tr>
</tbody>
</table>
shared among household and shelter dogs. The following species were detected in swabs taken from the perineum region of the sheltered animals: *S. aureus*, *S. saprophyticus*, *S. gallinarum*, *S. equorum*, *S. simulans*, and *S. lentus*. Subsequently, domestic dogs carried *S. hominis*, *S. warneri*, *S. chromogenes*, *S. haemolyticus*, and *S. epidermidis* in the material from the same isolation region.

From the lumbo-sacralis triangle region shown in Figure 2, 9 species of *Staphylococcus* were distinguished, Table IV. In comparison to the samples taken from shelter animals, the dogs living at home were characterized by a broad range of staphylococcal species, 2 and 8 species respectively. As mentioned above only two species were isolated from the lumbo-sacralis triangle samples from sheltered dogs: *S. intermedius* (75%) and *S. aureus* (25%).

The above data demonstrated that *S. intermedius* in carrier-state, predominantly colonized nares of tested dogs. Over 50% of all animals, regardless of their living environment, sick or healthy carried strains of this species in the nasal vestibule. The next preferred region was the lumbo-sacralis triangle and perineum. *S. intermedius*, however was isolated more often from the sheltered dogs (75%) then from animals living in households (16.7%), Table IV. In both healthy and diseased dogs, the patterns of colonization were similar, as it is shown in Figure 3A and Figure 3B.

**The etiological factor for skin lesions of canine origin.** The rate of occurrence of different staphylococcal species, isolated from 12 dogs with infection sites are listed in Figure 4. The most common species found in 7 out of 12 examined animals (58.3%) was *S. intermedius*. In 5 out of 7 dogs living in households it was the only staphylococcal species isolated from the skin lesion. Moreover, 7 additional *Staphylococcus* spp. were isolated, including: methicillin-resistant

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### Table II

**Staphylococcal strain distribution in the nasal mucosa**

<table>
<thead>
<tr>
<th><em>Staphylococcus</em> spp.</th>
<th>Living environment of examined dogs total number of bacterial strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 sheltered dogs-19 strains</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>6 (31.6%)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>6 (31.6%)</td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>2 (10.5%)</td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>2 (10.5%)</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>5 (25.6%)</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>1 (5.3%)</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>1 (5.3%)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Staphylococcus</em> warneri*</td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Staphylococcus</em> epidermidis*</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Staphylococcus</em> warneri*</td>
<td>0</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>0</td>
</tr>
<tr>
<td>Total number of staphylococcal species</td>
<td>7</td>
</tr>
</tbody>
</table>

### Table III

**Staphylococcal strain distribution in the perineum region**

<table>
<thead>
<tr>
<th><em>Staphylococcus</em> spp.</th>
<th>Living environment of examined dogs total number of bacterial strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 sheltered dogs-19 strains</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>4 (33.3%)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Staphylococcus</em> warneri*</td>
<td>0</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>0</td>
</tr>
<tr>
<td>Total number of staphylococcal species</td>
<td>7</td>
</tr>
</tbody>
</table>
Staphylococcus spp. isolated from dogs

Staphylococcus spp. isolated from dogs1

Staphylococcus species distribution in diseased dogs was also analyzed based on habitat, Figure 4. Among the 8 species mentioned earlier, 6 originated from animals living in the shelter and three from dogs living at home. Evidently, S. intermedius was the dominant species in each habitat, nevertheless it was isolated more repeatedly from animals living in households (71.4% of all animals). A greater variety of staphylococcal species were found in skin lesion samples taken from animals living in the shelter, where S. intermedius occurred in 40% of these animals. Among these species S. aureus (MRSA), S. simulans, S. equorum, S. xylosus, and S. cohnii were identified. In skin infections of dogs living in household conditions, beside S. intermedius strains, only S. haemolyticus, S. chromogenes were found in skin infections.

Discussion

Normal animal skin is colonized by numerous bacterial species, which contribute to the physiological skin flora. Staphylococcus species are not outnumbered among this flora. The most typical staphylococcal species isolated from canine skins are S. intermedius, S. xylosus, S. sciuri, S. capitis, S. chromogenes and S. lentus, by Nagase et al. (Nagase et al., 2002). At the same time other studies show a slightly different profile of isolated strains from dog skin at carrier state: S. intermedius, S. aureus, S. simulans, S. haemolyticus, and S. saprophyticus (Hauschild and Wójcik, 2007). So far not many studies have been done to determine the staphylococcal colonization pattern based on a specific part of the body or living habitat conditions of the animals from which swabs were taken.

In the recent study we took an effort to characterize the contribution of the different staphylococcal species in the colonization of dogs living in two different environments: the animal shelter and households. Our data confirm the general trend that S. intermedius strains are isolated at a higher rate from dogs that inhabit households, whereas S. aureus is isolated from dogs living at the shelter. These differences could be explained by distinctness in animal exposition to other

![Fig. 3A. Distribution of S. intermedius in the carrier-state at different body sites in sheltered and household dogs](image)

![Fig. 3B. Distribution of S. intermedius in the carrier-state at different body sites in healthy and diseased dogs](image)

![Fig. 4. Distribution of Staphylococcus species found in infected skin lesions of 12 examined dogs](image)
animals and people (Talan et al., 1989; Król, 1998). *S. aureus* strains isolated during our research, seem to be an excellent example. The animals from the shelter living in small closed areas and are in constant contact with other animals and moreover with the personnel, who are one of the main carriers of *S. aureus* (Kloos and Musselwhite, 1975). Humans are colonized by MSSA or MRSA by a carriage rate of 20–40% (Shopsin et al., 2000). These numbers grow even higher in the case of elderly persons or hospital personnel, where they can reach up to 90% (Szewczyk, 2005). Throughout our study, *S. aureus* strains were isolated only from dogs living in the animal shelter and comprise 24.5% of all strains isolated in these animals. Among this group we isolated both MSSA and MRSA strains.

On the other hand our data confirm, that *S. intermedius* is present in carrier-state in dogs. Isolates of this species were the most common, regardless of habitat and dog health conditions. The most favorable place for bacterial colonization was the mucosal membrane of nostrils. It was a reservoir for nearly 55% of all *S. intermedius* isolates, followed by the lumbo-sacralis triangle region (almost 30%).

Dermatitis is the most common type of infectious disease found in dogs (Ackerman, 1994). Dermatoses have a variety of etiological factors, where some of them are caused by particular bacterial strains. 90% of pyoderma in dogs is caused by *S. intermedius* strains infection (Craig, 2003), while *S. aureus* is one of the causative agents in skin lesions of non-pyodermal origin (Biberstein et al., 1984). Our data show that *S. intermedius* species seem to be the only species presented in microbiological samples from infected skin lesions of dogs living in both animal shelter and household environments. It was isolated from almost 60% of all diseased animals. Five out of seven of these dogs had only *S. intermedius* strains presented in the infected skin lesion. In the remaining two cases, *S. intermedius* strains were accompanied by other coagulase-negative strains.

The achieved data enriches our knowledge of the colonization of dogs by not only staphylococcal coagulase-positive strains like *S. aureus* or *S. intermedius* but also by coagulase-negative strains. The presented results are also important for understanding the epidemiology of infectious animal diseases, in which bacteria from the staphylococcal genus play a crucial role. In the last few years, a new *Staphylococcus* species – *S. pseudintermedius* was separated from the group previously considered to be *S. intermedius* species (Devriese et al., 2005; Wladyka et al., 2008). *S. pseudintermedius* can be easily misclassified as *S. intermedius* by routine biochemical methods used in standard diagnostics. Furthermore, it seems that all the isolates classified in our and other projects by routine biochemical identification tests seem to be *S. pseudintermedius*. Therefore, the final microbiological identification must require the use of genetic methods (Sasaki et al., 2007a; Bannoehr et al., 2009).

The number of dogs used in the project was twenty which was not very high. However they were used to show whether the similarity in lesions is because of same etiological factors in diseased dogs (twelve) and to recognize the staphylococcus species in healthy dogs at carrier state (eight). The staphylococcal species isolated from healthy and diseased dogs were compared to understand the colonization although disease was not studied. The main question was, do the carriage or the life conditions (house/shelter) effect the occurrence of disease?

The less number of dogs taken in the project resulted from trouble in selection of animals. There were often abrasions or wounds present on the skin. Only the dogs with skin lesions were taken for the project. The idea was that the similarity in skin lesions is caused by staphylococci, the carriage in same area and the localization of dogs in environment. Thus the work was to be done with high precision. Dogs were taken from one acute shelter hostel and the skin lesions were clinically evaluated. All the samples were taken by same veterinary doctor.

Analysis shown in the paper is an introduction on the general elaboration of carriage phenomenon in dogs living under different environments (house/shelter) and of isolated strains from skin lesions in part of dogs in the population. Thus showing the relation between diseased to healthy dogs and house to shelter conditions.

A total of one hundred four bacterial strains were isolated from twenty dogs. Higher species heterogeneity was observed among bacteria in dogs from shelter and that this difference did not affect pathology, which is mainly caused by *S. intermedius*, independent of the living environment of dogs. So the etiological factor can be easily observed among the tested dogs and the number of dogs used were enough to give the phenomenon. Authors were interested neither in the numbers of dogs nor in possessed isolates, but in observation of differences in colonization and etiological factors according to dogs’ life environment.

In conclusion, we described the isolation of various staphylococcal strains which were constituents of the normal biocenosis of the skin of dogs. We also reported staphylococci as etiological factors of wound infections in household or rescue shelter dogs, healthy or diseased. Our observations enriched by recent reports by other authors expand the knowledge to be more precise which brings focus to ecological phenomena and epidemiological pathways. A carriage phenomenon and transient colonization by such strains in dogs and/or their owners facilitate the transmission of staphylococci between humans and domestic animals.
(Simoons-Smit et al., 2000; Nagase et al., 2002) with a particular share of staphylococci from poultry (Wieliczko et al., 2002). As it is reported the genomic similarity of strains isolated in veterinary pathology is particularly specific (Cuteri et al., 2004, Jakubczak et al., 2007). The dissemination of methicillin-resistant \emph{S. pseudintermedius} in various animals and the confirmed presence of their resistance genes places them as potentially serious pathogens (Ruscher et al., 2009). They have to be precisely analysed by a number of validated and recommended methods (Cuteri et al., 2004; Ma³achowa et al., 2005). Recently elaborated molecular methods should be introduced to restriction analysis of the chromosomal DNA with the final aim of increasing the effectiveness in discriminating closely related strains (Krawczyk et al., 2007; Black et al., 2009). A need of such an investigation is additionally enhanced by single reports on human colonization by \emph{S. intermedius} (Talan et al., 1989; Mahoudeau et al., 1997; Kikuchi et al., 2004) as well as recently reported infections caused by \emph{S. pseudintermedius} (Van Hoovels et al., 2006; Sasaki et al., 2007b). Thus the elaboration of new procedures and diagnostic schemes for both veterinary and human bacteriology are an emerging challenge.

Acknowledgements

The authors thank Miss Ma³gorzata Wójcik from the Jagiellonian University for helping prepare this manuscript.

Literature


**Introduction**

Brucellosis is a major zoonotic disease that causes a serious health and economic problem worldwide (Elfaki et al., 2005). In spite of the growing number of countries declared Brucella-free, the disease remains one of the main zoonotic infections throughout many parts of the world with major economical and public health implications. About 500,000 new cases occur annually worldwide, with predominance in the Middle East, Mediterranean countries, South America and Central Asia (Godfroid, 2002; Sauret and Villissova, 2002). The causative organisms of brucellosis are Gram-negative facultative intracellular pathogens that may affect a range of different mammals including man, cattle, sheep, goats, swine, rodents and marine mammals and in most host species, the disease primarily affects the reproductive system with concomitant loss in productivity of animals affected (Cutler et al., 2005). In man, infection is associated with protean manifestations and characteristically recurrent febrile episodes that led to the description of this disease as undulant fever (Abdoel et al., 2008). Currently, the diagnosis of brucellosis is based on microbiological and serological laboratory tests. However the diagnostic value of serological tests is unsatisfactory in the early stages of the disease due to low sensitivity, serological cross-reactions, and the inability to distinguish between active and inactive infection due to antibody persistence after therapy (Diaz and Moriyo, 1989; Navarro et al., 2002). Furthermore in patients with persistent or relapsing brucellosis, dependence on blood culture analysis is usually impeded by the low yield of microorganisms as a result of dormancy of brucellae in the mononuclear phagocytic cells (Elfaki et al., 2005). Blood cultures (which represent the ‘gold standard’ of laboratory diagnosis) are among the most important tests used for the diagnosis of infectious...
diseases including brucellosis. However, contamination with skin type flora like coagulase negative staphylococcus could over grow the slow growing organisms like \textit{Brucella} in addition to a serious threat to laboratory personnel (Yagupsky, 1999; 2004). Therefore, other diagnostic methods are needed to overcome such limitations of conventional approaches for the diagnosis of brucellosis. DNA-based methods such as gene probes and polymerase chain reaction (PCR) are attractive means for the confirmation of brucellosis. Because of the prevalence of brucellosis in Saudi Arabia, a precise diagnostic method should be established for the control of brucellae in this population. Different target genes, primer pairs, PCR techniques and extraction procedures have been previously investigated for detection, however, most of these assays have used \textit{Brucella} DNA of pure cultures and only a few of these primers have been used in clinical animal, and human samples (Zervou et al., 2001; Abdoel et al., 2008; Bogdanovich et al., 2008; Hinić et al., 2008) and there is no enough report about comparison of these assays. Therefore the aims of the current work were to compare different DNA extraction method for DNA purification from \textit{Brucella} cells, compare different targets and PCR methods for detection of \textit{Brucella} and apply it to clinical human samples.

\textbf{Experimental}

\textbf{Material and Methods}

\textbf{Clinical samples and bacterial strains.} A total of 200 clinical blood specimens were collected from the Armed Forces Hospitals (Riyadh, Saudi Arabia) including 160 blood samples obtained from patients with clinically proven or suspected systemic brucellosis infection and 40 control samples from healthy subjects without any clinical evidence or history of brucellosis. The diagnosis of brucellosis was confirmed by isolation and identification of \textit{Brucella} spp. from blood culture. Blood (8 to 10 ml) was inoculated into BACTEC Plus aerobic/F blood culture bottle (enriched soybean-casein digest broth) and incubated for 28 days or until the bottles were positive. All blood cultures were evaluated in the BACTEC 9600 blood culture system (Becton Dickinson Diagnostic Instrument Systems), which detect microbial growth by continuous monitoring. One ml aliquots from bottles shown to contain Gram-negative coccobacilli bacteria were removed and stored at \textdegree C until use. All isolated strains were identified in the lab. The reference strain used in this study was \textit{Brucella melitensis} 16 M, which was obtained from the Central Veterinary laboratory (Weybridge, UK). It was propagated on chocolate agar (Oxoid) medium and incubated at 37°C in a humidified atmosphere supplemented with 5% CO\textsubscript{2}. Brain heart infusion broth (Oxoid) with 20% glycerol (Sigma) was used for the storage of bacterial strains at \textdegree C.

\textbf{Preparation of bacterial cells suspensions.} Freshly cultured \textit{Brucella melitensis} was killed by the addition of 70% methanol in sterile saline (0.9% NaCl) and recovered by centrifugation at 5000 rpm for 5 min, washed twice with 5 ml of sterile distilled water then recovered by centrifugation at 5000 rpm for 5 min. The cells were serially diluted with sterile distilled water and adjusted to a 0.5 McFarland standard (which is approximately 1.5×10\textsuperscript{8} CFU/ml). Different cell dilutions were prepared and suspended in either sterile distilled water or whole blood collected in EDTA Vacutainer from healthy individual with no evidence or history of brucellosis infection, to give a final cell count in the range of 25 to 10\textsuperscript{3} CFU/ml. The inoculated whole blood samples and cells suspended in water were subsequently used for DNA extraction. Sterile water inoculated blood samples served as a negative control.

\textbf{Bacterial DNA extraction methods.} Four different DNA extraction kits were used to extract the genomic DNA of \textit{Brucella melitensis} according to the manufacturer instructions including QIAamp kit (Qiagen), GenomicPrep DNA Isolation Kit (Amersham Biosciences), Automated Nucleic Acid Purification system (MagNA Pure LC Systems), in addition to 10% Chelex-100 resin suspension (Bio-Rad Laboratories) where 0.2 ml cell suspension was mixed with 0.1 ml of a 10% Chelex-100 resin suspension (Bio-Rad Laboratories), and the mixture was boiled for 10 min. After centrifugation at 10000 rpm for 5 min, about 0.1 ml of supernatant was removed and used for PCR.

\textbf{Detection of \textit{Brucella melitensis} by conventional PCR.} The sensitivity of conventional PCR was investigated for detection of \textit{Brucella melitensis} using a modification of previously reported methods (Navarro et al., 2002; Baddour and Alkhailfa, 2008) using four different primers pairs (TIB MOLBIOL, Berlin, Germany), specific to four different targets in \textit{Brucella} spp. (Table I). The PCR reaction contained (25 µl): reaction buffer (50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 10 mM Tris HCl, pH 9.0), 200 µM of each of dATP, dCTP, dGTP, and dTTP and 2.5 U of puReTaq DNA polymerase (Amersham-Pharmacia). For optimization of PCR conditions different concentrations of primers (5–25 pmol) and MgCl\textsubscript{2} (1.5–4 mM), amplification at different temperature settings and cycling programs were used (Table I). Following PCR reaction, 10 µl of the reaction mixture was mixed with 2 µl of loading buffer ReddyRun (ABgene) and was run in 2 % agarose gel electrophoresed in Tris-borate-EDTA buffer (TBE) at 120 V for about 50 min and the amplified DNA bands were visualized in ethidium bromide staining and
Detection of *Brucella* spp. by different PCR methods

Photographed under UV light. 100 bp Superladder (ABgene) was used as DNA Marker. Sterile water instead of DNA was used as a negative control.

Detection of *Brucella melitensis* by Real-time PCR. Detection of *Brucella* using Real-time PCR was investigated using modified of previously reported method (Redkar et al., 2001). The reaction mixture for real time contained 2 µl of 10x LightCycler-FastStart DNA master hybridization probes (Roche Diagnostics), 2.4 µl MgCl₂ (final concentration of 4 mM), 4 µl Reagent mix (Brucella-specific primers and hybridization probes), and 6.6 µl nucleases free water. Thermocycling conditions were as follows: one cycle of initial denaturation at 95°C for 10 min, followed by 55 amplification cycles (temperature transition rate of 20°C/s), each including denaturation (95°C for 10 s), annealing (54°C for 45 s), and extension (72°C for 90 s). Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of the LightCycler Red 640-labeled sensor probe at the F2 channel.

Results

Sensitivity of the DNA extraction methods. Four different DNA extraction methods were evaluated for whole DNA purification from *Brucella melitensis*. Serial dilution of the *Brucella melitensis* cells was prepared in one ml blood. Total DNA was extracted using different methods and the purified DNA was used as template in PCR reaction. The sensitivity and efficacy was measured as the minimum number of CFU required to produce DNA showing a positive PCR. The results for the approximate sensitivity of each DNA extraction method are shown in Table II. It was found that the MagNA Pure LC method was the most efficient and sensitive method as it showed positive PCR reaction with DNA extracted from as low as 25 and 100 CFU suspended in one ml blood and whole blood, respectively.

### Table I

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>B4-B5</th>
<th>ISP1-ISP2</th>
<th>F4-R2</th>
<th>JPF-JPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR target</td>
<td>gene encoding a 31 kDa <em>Brucella abortus</em> antigen</td>
<td>IS6501</td>
<td>sequence 16S rRNA of <em>B. abortus</em></td>
<td>gene encoding an outer membrane protein (omp-2)</td>
</tr>
<tr>
<td>Product size (bp)</td>
<td>223</td>
<td>600</td>
<td>905</td>
<td>193</td>
</tr>
<tr>
<td>Primer sequence</td>
<td>F-5-TGCTCGGCATTATACAAA-3 R-5-GGATACGGTTACCTTACGTTAGTCGGA-3</td>
<td>F-5-TCAGAGCGCGGGAAGGGG-3 R-5-AATGTTAGTTTCACCCACTA-3</td>
<td>F-5-GGCCTCAAGGCAGCCAGCACA-3 R-5-ACGACATGTTGCGGTCGGA-3</td>
<td></td>
</tr>
<tr>
<td>PCR conditions: MgCl₂ conc. (mM)</td>
<td>1.5, 2.5, 4</td>
<td>1.5</td>
<td>1.5</td>
<td>3</td>
</tr>
<tr>
<td>Taq polymerase (IU)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Annealing temp</td>
<td>60</td>
<td>56</td>
<td>54</td>
<td>60</td>
</tr>
<tr>
<td>Extension time (s)</td>
<td>60</td>
<td>45</td>
<td>90</td>
<td>60</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivities of different DNA extraction methods.</th>
<th>DNA extraction Count of <em>Brucella</em> cells (CFU/ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MagNA Pure LC</td>
<td>+</td>
<td>25000 600 80 40 20 10 5 25</td>
</tr>
<tr>
<td>QIAmp silica column</td>
<td>+</td>
<td>25000 600 80 40 20 10 5 25</td>
</tr>
<tr>
<td>GenomicPrep Blood</td>
<td>+</td>
<td>25000 600 80 40 20 10 5 25</td>
</tr>
<tr>
<td>Chelex resin</td>
<td>–</td>
<td>25000 600 80 40 20 10 5 25</td>
</tr>
</tbody>
</table>

The results shown in Tables I and II were as follows: one cycle of initial denaturation at 95°C for 10 min, followed by 55 amplification cycles (temperature transition rate of 20°C/s), each including denaturation (95°C for 10 s), annealing (54°C for 45 s), and extension (72°C for 90 s). Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of the LightCycler Red 640-labeled sensor probe at the F2 channel. Water was used instead of DNA as a negative control.

† Positive PCR – Negative PC
one ml water respectively, followed by GenomicPrep Blood method and QIAamp silica column purification method respectively (Table II and III). However none of the extracted DNA using Chelex resin was able to give positive PCR reaction. Based on these results the MagNA Pure LC method was selected for further analysis.

Detection of Brucella by conventional and real time PCR. Detection of Brucella melitensis by conventional PCR was investigated using four different targets. The results presented in Table IV and V and Figure 1 indicated that the B4-B5 amplification method was the most sensitive as it could amplify DNA extracted from as low as 25 and 100 CFU/ml suspended in one ml water and blood, respectively, followed by ISP1-ISP2 and F4-R2, respectively. However, none of the bacterial DNA from whole blood or water gave a positive PCR using the JPR-JPF method. Based on these results the B4-B5 method was used in analysis of the clinical samples. The sensitivity of the real-time PCR was determined using Brucella specific probes. The reaction was carried out using DNA extracted from serial dilution of bacterial cells suspended in blood and water. Real-time PCR was able to detect Brucella using DNA extracted from as low as 50 and 15 CFU suspended in one ml blood and water respectively (Fig. 2).

Clinical samples. During the study period, 200 clinical blood specimens (160 patients and 40 controls) were tested for brucellosis by blood culture, optimum conventional PCR and real-time PCR. Among the 160 clinical samples tested, 89 specimens were...
Detection of *Brucella* spp. by different PCR methods

Blood culture positive for *Brucella* and 71 were negative but 9 of them were positive for other bacteria (six coagulase negative staphylococci, one *Staphylococcus aureus*, one *Klebsiella* spp. and one *Acinatobacter* spp). One of the blood culture positive for coagulase negative *staphylococcus* (detection after 33 h) was positive for *Brucella* by conventional PCR and light cycler PCR in blood, negative in serum and negative by blood culture. DNA was extracted from the 89 blood samples (which were found to be positive for *Brucella* by blood culture) and detection was carried out.

**Fig. 2. Detection of* Brucella* by real time PCR**

Fluorescence is plotted against number of PCR cycles to monitor amplification of different cells counts *Brucella* suspended in blood (A) and water (B) 1: 800 CFU/ml, 2: 200 CFU/ml, 3:100 CFU/ml, 4: 50 CFU/ml, 5–25 CFU/ml, 6:15 CFU/ml, 7&8: Negative control.

**Table III**

<table>
<thead>
<tr>
<th>DNA extraction</th>
<th>Count of <em>Brucella</em> cells (CFU/ml water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA extraction</td>
<td>25000 6000 800 400 200 100 50 25</td>
</tr>
<tr>
<td>MagNA Pure LC</td>
<td>+   +   +   +   +   +   +</td>
</tr>
<tr>
<td>QIAmp silica column</td>
<td>+   +   +   +   +   +   –</td>
</tr>
<tr>
<td>GenomicPrep Blood</td>
<td>+   +   +   +   +   +   –</td>
</tr>
<tr>
<td>Chelex</td>
<td>+   +   +   –   –   –   –</td>
</tr>
</tbody>
</table>

+: Positive PCR, –: Negative PCR

**Table IV**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Count of <em>Brucella</em> cells (CFU/ml blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA extraction</td>
<td>25000 6000 800 400 200 100 50 25</td>
</tr>
<tr>
<td>B4-B5</td>
<td>+   +   +   +   +   +   –</td>
</tr>
<tr>
<td>ISP1-ISP2</td>
<td>+   +   +   +   +   +   –</td>
</tr>
<tr>
<td>F4-R2</td>
<td>+   +   +   –   –   –   –</td>
</tr>
<tr>
<td>IPF-JPR</td>
<td>–   –   –   –   –   –   –</td>
</tr>
</tbody>
</table>

+: Positive PCR, –: Negative PCR
Table V
Sensitivities of four PCR methods for detection of Brucella suspended in water specimens determined by amplifying the DNA extracted from the dilution series

<table>
<thead>
<tr>
<th>Methods</th>
<th>Count of Brucella cells (CFU/ml water)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25000</td>
</tr>
<tr>
<td>B4-B5</td>
<td>+</td>
</tr>
<tr>
<td>ISP1-ISP2</td>
<td>+</td>
</tr>
<tr>
<td>F4-R2</td>
<td>+</td>
</tr>
<tr>
<td>JPF-JPR</td>
<td>-</td>
</tr>
</tbody>
</table>

+: Positive PCR, -: Negative PCR

Table VI
Detection of Brucella in blood and serum samples by conventional PCR in comparison to blood culture method

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Positive</th>
<th>False pos.</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>89 (89)</td>
<td>0 (40)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Blood Culture</td>
<td>89 (89)</td>
<td>0 (40)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Blood</td>
<td>64 (89)</td>
<td>2 (40)</td>
<td>72%</td>
<td>95%</td>
</tr>
<tr>
<td>Serum</td>
<td>48 (89)</td>
<td>0 (40)</td>
<td>54%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table VII
Detection of Brucella spp. in blood and serum samples by real-time PCR in comparison to blood culture method

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Positive</th>
<th>False pos.</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture</td>
<td>89 (89)</td>
<td>0 (40)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Blood</td>
<td>69 (89)</td>
<td>0 (40)</td>
<td>77.5%</td>
<td>100%</td>
</tr>
<tr>
<td>Serum</td>
<td>54 (89)</td>
<td>0 (40)</td>
<td>60%</td>
<td>100%</td>
</tr>
</tbody>
</table>

An accurate diagnosis of brucellosis is very important for treatment, control and eradication of brucelae and due to the prevalence of brucellosis in Saudi Arabia, an efficient and sensitive diagnostic method should be established for the control of brucelae in this population (Elfaki et al., 2005). PCR offers an alternative choice over the conventionally available methods for an accurate diagnosis of brucellosis. However, sufficient nucleic with removal of inhibitory substances is essential for optimal detection of the microbial pathogens by PCR. The aim of this study was to optimize the DNA extraction and PCR conditions for detection of Brucella spp. and apply the optimum conditions in the clinical samples and compare it with blood culture approach. Therefore four different DNA extraction methods were evaluated to purify total DNA from Brucella melitensis. Although blood is known to possess substances inhibitory to PCR, the DNA purification methods used in this study (except chelex resin method) were successful in eliminating these inhibitors, the most sensitive and efficient one being the MagNA Pure LC method showing positive PCR reaction with DNA extracted from as low as 25 and 100 CFU suspended in one ml blood and one ml water respectively. The detection of bacterial DNA in blood specimens by PCR usually requires sensitive DNA amplifying method with sensitive primers and optimized PCR conditions because of the presence of human DNA and inhibitors in blood (Bricker, 2002; Bogdanovich et al., 2004). With the aim of finding the most efficient and sensitive methods for detection of Brucella DNA in blood specimens, four DNA amplifying methods were evaluated using four primers pairs including B4-B5, ISP1-ISP2, F4-R2 and JPF-JPR. The results indicated that the detection limit varied between 25 to 800 CFU/ml, depending on the amplifying method (except JPF-JPR method). The B4-B5 amplification method was the most sensitive one as it could amplify DNA extracted from as a low as 25 and 100 CFU/ml suspended in one ml water and blood respectively, followed by ISP1-ISP2 and F4-R2, respectively. This result is consistent with that previously reported by Elfeki et al. (2005) where PCR using primers B4-B5 was the most sensitive one for detection of Brucella spp. However in another study by Navarro et al. (2002) for comparison of three PCR methods for detection of Brucella, F4/R2 was the most sensitive primers. Furthermore the specificity of the real-time PCR was determined using Brucella specific probes. The reaction was carried out using DNA extracted from serial dilution of bacterial cells suspended in blood and water. Real-time PCR was even more sensitive than conventional PCR as it was able to detect Brucella spp. using DNA extracted from as low as 50 CFU /ml blood 15 CFU/ml water.

The best DNA extraction method was used to extract DNA from the clinical samples and optimum conventional PCR conditions, RT-PCR and blood culture were compared for detection of Brucella spp. in the clinical blood samples. It was found that 72%...
and 54% of the positive blood culture was detected by PCR with specificity of 95% and 54% in blood and serum, respectively. The use of PCR for the detection of Brucella DNA in blood samples of certain groups of patients with brucellosis has been previously studied with sensitivity in the range of 50% to 100% (Mattar et al., 1996; Navarro et al., 1999; Zerva et al., 2001). Several systems of real-time PCR have been developed. They are user-friendly, rapid, and free of contamination. Moreover, these PCRs overcome the conventional PCR by allowing quantification of the targeted copies in the specimen (Newby et al., 2003; Probert et al., 2004). Evaluation of the real-time PCR for detection of Brucella spp. in the clinical blood samples showed excellent specificity and good sensitivity. The real-time PCR confirmed 77.5% of the results obtained with the blood culture assays with specificity of 100%. In this study it appears that the real-time PCR has greater sensitivity and specificity than conventional PCR.

Conclusions. In conclusion comparison of blood culture, conventional PCR conditions and RT-PCR for detection for detection of Brucella spp. in the clinical blood samples indicated that PCR amplification technology is promising method for the detection of Brucella in clinical samples with high sensitivity and specificity close to that reported by conventional blood culture. Although the PCR detection of Brucella spp. using peripheral blood is not without difficulties, it presents considerable advantages. Compared to standard bacteriological methods, the PCR assays are safer and more rapid to perform. Therefore, these assays may be important diagnostic tools to detect Brucella spp.

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Literature
**Antibiofilm Activity of Selected Plant Essential Oils and their Major Components**

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**Abstract**

The aim of the study was to examine the antibiofilm activity of selected essential oils (EO): *Lavandula angustifolia* (LEO), *Melaleuca alternifolia* (TTO), *Melissa officinalis* (MEO) and some of their major constituents: linalool, linalyl acetate, α-terpineol, terpinen-4-ol. Biofilms were formed by *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* NCTC 8196 on the surface of medical biomaterials (urinary catheter, infusion tube and surgical mesh). TTC reduction assay was used for the evaluation of mature biofilm eradication from these surfaces. Moreover, time-dependent eradication of biofilms preformed in polystyrene 96-well culture microplates was examined and expressed as minimal biofilm eradication concentration (evaluated by MTT reduction assay). TTO, α-terpineol and terpinen-4-ol as well as MEO, showed stronger anti-biofilm activity than LEO and linalool or linalyl acetate. Among the biomaterials tested, surgical mesh was the surface most prone to persistent colonization since biofilms formed on it, both by *S. aureus* and *E. coli*, were difficult to destroy. The killing rate studies of *S. aureus* biofilm treated with TTO, LEO, MEO and some of their constituents revealed that partial (50%) destruction of 24-h-old biofilms (MBEC₅₀) was achieved by the concentration 4–8×MIC after 1 h, whereas 2–4×MIC was enough to obtain 90% reduction in biomass metabolic activity (MBEC₉₀) after just 4 h of treatment. A similar dose-dependent effect was observed for *E. coli* biofilm which, however, was more susceptible to the action of phytochemicals than the biofilms of *S. aureus*. It is noteworthy that an evident decrease in biofilm cells metabolic activity does not always lead to their total destruction and eradication.

**Key words:** biofilm eradication, biomaterial-associated infections, essential oils

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**Introduction**

A frequent complication while using artificial devices in medical practice (BAI, biomaterial-associated infections) are infections dependent on microbial adhesion and biofilm formation. They should be considered a serious health problem requiring a rapid solution. Very often BAI are a consequence of direct biomaterial contamination during implantation but they can also be caused by haematogenous spread of bacteria from an infection site anywhere within the human body. A similar medical problem is posed by wound-associated chronic infections, which are also often characterized as having a biofilm nature. The biofilm mode of growth results in an increased bacterial resistance against classical antimicrobial treatment and host immune factors (Bryers, 2008; James *et al.*, 2008; Dai *et al.*, 2010; Hoiby *et al.*, 2010).

Thus, the search for alternative therapies is a necessity and using, for example, natural plant/animal products and/or their combinations with antibiotics or synthetic counterparts seems to be one of the promising solutions (Dürig *et al.*, 2010).

Higher plants evolved through developing mechanisms for avoiding the effects of microbial attack based on the production of protective substances, usually their secondary metabolites. Compounds with strong bacteriostatic or bactericidal activity belong mostly to phytalexins and, within this group, essential oils are the most important members (Gibbons, 2008). Essential oils are complex mixtures of chemical substances, at least one of which shows antimicrobial activity. They consist mainly of monoterpenes, sesquiterpenes, diterpenes and other aromatic or aliphatic compounds (Kalemba and Kunicka, 2003; Bakkali *et al.*, 2008; Reichling *et al.*, 2009). Essential oils of several plants...
are widely used in ethnomedicine for their antimicrobial and anti-inflammatory properties but their anti-biofilm activity has not been so far studied extensively (Carson et al., 2006; Fabian et al., 2006; Cavanagh and Wilkinson, 2002; Karpanen et al., 2008; Warnke et al., 2006; Chao et al., 2008; Gursoy et al., 2009; Hossainzadegan and Delfan, 2009).

Essential oils derived from *Melaleuca alternifolia*, *Lavandula angustifolia*, *Melissa officinalis* and their major constituents were selected for the present study. Tea tree oil (TTO) is an essential oil from the leaves of the Australian *M. alternifolia* tree, a member of the botanical family Myrtaceae. The oil from the leaves is used medicinally, being effective against bacterial, viral and fungal organisms as well as having immunostimulatory activity. It is also known that it can alleviate inflammation and may help wound healing (Carson et al., 2006). Essential oils distilled from members of the genus *Lavandula* have been applied both cosmetically and therapeutically for centuries with the most commonly used species being *L. angustifolia*, *L. latifolia*, *L. stoechas* and *L. intermedia*. The claims made for lavender oil include its antibacterial, antifungal, smooth muscle relaxing, sedative, antidepressive properties (Cavanagh and Wilkinson, 2002; Roller et al., 2009). *Melissa* is commonly used in Europe as a tea, liquid extract, and topical preparation. Melissa essential oil (lemon balm) is also a common antibiotic and antifungal agent (Mimica-Dukic et al., 2004).

The attention of our study was focused on the possibility of using essential oils in the fight against biofilms of two members of the “alert” pathogens group. These were Gram-positive staphylococci, *Staphylococcus aureus* and Gram-negative enteric bacteria – *Escherichia coli*, since the epidemiological data on the incidence of infection with their participation sound serious. Moreover, these strains were chosen because the known differences in the cell wall structures between Gram-positive and Gram-negative bacteria imply the extent of their susceptibility to various antimicrobial agents (Gibbons, 2008; Fabian et al., 2006; Chao et al., 2008).

**Experimental**

**Materials and Methods**

**Biomaterials, bacteria, phytochemicals.** The following biomaterials/culture surfaces were used: short term urine drainage catheter of Nelaton type (Bicakcil, Turkey), infusion tube (Pollfa Lublin, Poland) (both 0.5 cm in length); surgical mesh support for muscle/fascia (Tricomed, Łódź, Poland) (0.5 × 0.5 cm); 96-well polystyrene tissue culture microplates (Nunc, Denmark). Eradication of *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* NCTC 8196 biofilms by essential oils (EO) and some of their main components was evaluated. There were oils of *Melaleuca alternifolia* (tea tree oil, TTO), *Lavandula angustifolia* (lavender essential oil, LEO), *Melissa officinalis* (Melissa essential oil, MEO or lemon balm) and α-terpineol, terpinen-4-ol, linalool, linalyl acetate. All essential oils were purchased from Pollena Aroma, Poland and the isolated compounds of EO were purchased from SAFC, USA, via Sigma, USA.

**Evaluation of bacterial susceptibility to essential oils and their major constituents.** MIC (minimal inhibitory concentration) of phytochemicals was determined by the standard CLSI (Clinical Laboratory Standards Institute) microdilution method, with minor modifications (Budzyńska et al., 2009). Essential oils/components were initially diluted in 96% ethanol (1:1 v/v), and later in Mueller-Hinton Broth (MHB, Sigma) with 0.5% Tween 20 (Sigma). The tested concentrations of phytochemicals (range 6.75–0.024%) were deposited in triplicate, in a volume of 100 µl in the wells of flat-bottomed polystyrene 96-well microplates (Nunc, Denmark). Then, 100 µl of bacterial suspension with a density of 10^6/ml in MHB/0.5% Tween 20 was added to each well. The positive control was a suspension of bacteria in 200 µl of MHB/0.5% Tween 20, and a negative control was the medium without bacteria. After 24 h incubation at 37°C, the absorbance at A600 (Victor 2, Wallac, Finland) was determined by the standard CLSI (Clinical Laboratory Standards Institute) microdilution method, with minor modifications (Budzyńska et al., 2009). Antimicrobial substances with a concentration of 10^4/ml in MHB/0.5% Tween 20 were added to each well. The positive control was a suspension of bacteria in 200 µl of MHB/0.5% Tween 20, and a negative control was the medium without bacteria. After 24 h incubation at 37°C, the absorbance at A_600 (Victor 2, Wallac, Finland) was determined. The concentration at which the A_600 of the wells did not exceed the value of absorbance of the control medium was accepted as the MIC. The lowest concentration of essential oils/components, bactericidal to ≥99.9% of the original inoculum (MBC, minimal bactericidal concentration) was determined from broth dilution MIC tests, by subculturing 100 µl (from the wells of MIC, 2×MIC, 4×MIC, 8×MIC) to the broth medium without any antimicrobial agents. No visible growth after subsequent 24 h incubation means MBC on condition that the concentration is not higher than the 4-fold value of MIC. Antimicrobial substances – oxacillin and ofloxacin (Sigma, USA) were accepted as a reference.

**Surface colonization and biofilm eradication.** Fragments of biomaterials (three pieces of each type) were incubated with bacterial suspension in tryptic soy broth (TSB) supplemented with glucose (TSB/0.25% Glc) for 24 h at 37°C. Colonized surfaces, after their washing with phosphate-buffered saline (PBS), were transferred to the wells of a 96-well tissue culture microplate containing dilutions of essential oils/components and were incubated for the next 24 h at 37°C. Then, biomaterial fragments were rinsed with PBS and transferred to a new 96-well microplate containing TSB with 1% TTC (2,3,5-triphenyltetrazolium chloride) for 24 h incubation at 37°C, as previously
described (Różalska et al., 1998). TTC is a redox indicator used to differentiate between metabolically active and inactive cells; the colorless compound is enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living cells due to the activity of various dehydrogenases. The presence/reduction of biofilm biomass was evaluated by comparing the color intensity of biofilm deposit on biomaterials treated and untreated with phytochemicals, according to arbitrarily fixed scale (4+, 3+, 2+, 1+, –). The experiments were performed twice to confirm reproducibility of the results.

The results of the semi-quantitative method were compared with those of a standard biomass quantification method by CFU determination. The fragments of colonized biomaterials treated with phytochemicals, which were scored by TTC assay as (+) and (–), were removed from the wells and rinsed in PBS. Then, they were placed in 1 ml of sterile broth medium and the remaining bacterial deposit was removed by ultrasonic disruption for 5 min. One hundred µl of the sonicate was spread onto two agar plates for overnight incubation and CFU counting.

**Time-dependent biofilm eradication.** Time-dependent inhibitory concentration of phytochemicals for biofilms preformed in a polystyrene 96-well culture microplate, used at concentrations from MIC to 8×MIC was determined. Mature (24-h old) biofilms of *S. aureus* ATCC 29213 and *E. coli* NCTC 8196 were exposed to the action of essential oils/components for 1, 4, 6 and 24 h co-incubation at 37°C. The essential oils/components concentrations causing 50% or at least 90% reduction in biomass metabolic activity (MBEC$_{50}$ and MBEC$_{90}$) at each time point were determined using MTT-reduction assay, as described earlier (Walencka et al., 2005; 2007). MBEC$_{50}$ means that A$_{550}$ of the sample dropped below the half value of the positive control (A$_{550}$ = 1.5) and MBEC$_{90}$ means that A$_{550}$ of the sample was close to the value of the negative control (A$_{550}$ = 0.2). To avoid interference between the phytochemical’s red-ox potency and MTT, before its application, the fluid above the biofilm was aspirated and removed. In this assay, pale-yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by living, respiratory active cells. A solubilizer was added to dissolve the formed formazan product into a colored solution whose absorbance was quantified (550 nm (Victor 2, Wallac, Finland). The experiments were performed in duplicate to confirm reproducibility of the results. Mean A$_{550}$ ± S.D. values were presented.

Evaluation of viability of bacterial cells treated with essential oils. The potential effect of essential oil on bacterial cell membranes was assessed using EO-mediated SYTO 9 and propidium iodide uptake (LIVE/DEAD BacLight Bacterial Viability kit, Molecular Probes, Invitrogen, Poland). Cell suspensions of *S. aureus* ATCC 29213 and *E. coli* NCTC 8196 in PBS (1.0×10$^6$ CFU/ml) were incubated with 4×MIC, 2×MIC or MIC of EO at 37°C for 4 h, with shaking. After the incubation, the bacteria were washed with 5 ml sterile PBS and stained using the LIVE/DEAD kit, according to the manufacturer’s recommendations. The cells with LIVE/DEAD and no EO served as negative controls. After the incubation all the samples were washed and resuspended in PBS, and a drop of each suspension was examined with a confocal scanning laser microscope for green/red fluorescence to visualize SYTO 9 and propidium iodide, respectively. Six areas of each of the triplicate samples were photographed with an integrated Hamamatsu digital camera (C4742–95; Nikon UK).

**Results**

The minimal inhibitory concentration (MIC) of selected essential oils and their major constituents was determined using the broth microdilution method and defined as the lowest concentration able to inhibit visible microbial growth. The essential oil of *Melaleuca alternifolia* (TTO, α-terpineol and terpinen-4-ol, as well as the essential oil of *Melissa officinalis* (MEO), showed stronger antibacterial activity than the essential oil of *Lavandula angustifolia* (LEO) and its major components, such as linalool and linalyl acetate. Their MICs values against *Staphylococcus aureus* and *Escherichia coli* are presented in Table I. The experiments performed to check whether the tested phytochemicals are bactericidal showed that MBC values did not exceed the obligatory 4×MIC, since more than 99% of the bacterial inoculum was killed even by MIC or 2×MIC. One exception was the *S. aureus* culture which was killed by 8×MIC of linalool – one of the components from the lavender and melissa essential oils. The data on this set of experiments are presented in Table I.

The answer to the question whether TTO, LEO, MEO are bacteriostatic or bactericidal was also provided by the experiments for EO-mediated SYTO 9/PI uptake, evaluated by fluorescent confocal microscopy. As assessed by the emission of green/red fluorescence, non-treated *S. aureus* or *E. coli* cells, showed only green fluorescence (live with undamaged cell wall), except for very few red cells. However, EO-treated cells exhibited increased uptake of propidium iodide (leaking cell wall and membrane), proportional to the increase in the compound concentration. All bacterial cells from planktonic cultures treated with EO at the concentration of 2–4 MIC, exhibited red fluorescence (data not shown).

In the further experiments, essential oils/compounds were shown to possess the bactericidal activity against
mature *S. aureus* and *E. coli* biofilms formed earlier on the surface of polystyrene culture microplate wells. The time-dependent inhibitory concentration of phytochemicals for biofilms preformed in the polystyrene 96-well culture microplate was determined by MTT reduction assay. All biocides were used for this purpose at MIC up to 8×MIC. There was only a small difference between planktonic and biofilm biocidal concentrations, usually not exceeding 2–4-fold higher values. Killing rate studies of *S. aureus* biofilm eradication by TTO and its constituents revealed that partial (50%) destruction of 24-h-old biofilms (MBEC<sub>50</sub>)

![Fig. 1. Time- and concentration-dependent effect of essential oils on biofilm viability, determined by MTT-reduction assay. A1, A2, A3 (*S. aureus*), B1, B2, B3 (*E. coli*) biofilms exposed to tea tree oil (A1, B1), lavender oil (A2, B2), or lemon balm (A3, B3). A<sub>550</sub> mean ± S.D.](image_url)
Antibiofilm activity of plant essential oils was achieved by the concentration of 4–8×MIC just after 1 h, whereas 2×MIC was enough to obtain TTO MBEC90 after 4 h of treatment. Effective MBEC90 of LEO and its constituents was as high as 4–8×MIC. The time-dependent activity of MEO was comparable to TTO. With few exceptions, the tested phytochemicals reduced the metabolic activity of bacterial cells in biofilms after 24 h exposure at concentrations close to MICs. A similar dose- and time-dependent effect was observed for \textit{E. coli} biofilm, however, surprisingly, it was more susceptible to the action of phytochemicals than the \textit{S. aureus} biofilms, similarly to what was observed for planktonic cultures. (Table I, Fig. 1).

Among the medical biomaterials tested, surgical mesh was the surface most prone to persistent colonization, since biofilms formed on it by both bacterial strains were more difficult to eradicate by the tested compounds. Modified Richard’s method was used to detect the presence or eradication of biofilm, as described earlier (Różalska \textit{et al.}, 1998). The obtained visual results scored by TTC assay as (+) and (–), were compared with the data from the standard biomass quantification method by bacterial dislodgement and CFU determination. Only TTO and terpinen-4-ol used at MIC – 2×MIC caused visible biofilm eradication (TTC reduction) from the surface of urological catheter and infusion tube, evaluated later as greater than 90% decrease in the number of live bacteria (CFU). However, the use of higher concentrations (4–8×MIC) of these and other compounds (MEO, LEO, α-terpineol, linalool, linalyl acetate) was necessary to achieve such an effect on the surface of surgical mesh. In all cases it was proved that indeed TTC score (–) means that the lack of metabolically active cells is equal to the lack of viable bacteria, since the CFU counting method used for the measurement of biofilm survival showed its total eradication (data not shown).

Our study leads to the conclusion that essential oils and some of their major constituents are able to efficiently reduce biofilms of both \textit{S. aureus} and \textit{E. coli} on biomaterial surfaces. However, a satisfactory effect is strongly dependent on the surface structure and properties. The example of TTO antibiofilm activity is presented in Fig. 2.

![Fig. 2. Image of infusion tube colonized by \textit{S. aureus} biofilm: A, B – treated for 24 h with TTO, respectively at MIC or \(\frac{1}{2}\) MIC. C(+) – not treated positive control, C(–) – not colonized negative control. TTC-reduction assay – scored according to an arbitrarily fixed scale as described in Material and Methods.](image-url)
Discussion

In the present study, the in vitro strong effects of Melaleuca alternifolia (TTO), Lavandula angustifolia (LEO), Melissa officinalis (MEO) essential oils and some of their main constituents (α-terpineol, terpinen-4-ol, linalool, linalyl acetate) on the biofilms of Gram-positive S. aureus and Gram-negative E. coli were shown. Remarkably, the in vitro activity of the oils against the biofilm of both strains tested was equal to or only slightly lower than that against bacterial planktonic culture (Table I). This is worth emphasizing because the biofilm resistance to classical chemotherapeutics is typically 100–1000 times higher (Bryers, 2008, Hoiby et al., 2010). It is a promising observation since plant-derived compounds have gained a general interest in the search to identify alternatives for the control of infections, especially those connected with the use of artificial medical devices. Research on this topic is focused on natural or synthetic substances, which have potent broad-spectrum microbicidal and antibiofilm activities and pose a low risk for the development of resistance. It is accepted that there are two main reasons why essential oils do not create resistant strains of bacteria: they are complex and comprise numerous compounds in variable proportions depending on the plant chemotype. Hence, even if bacteria did figure out an oil’s effective components, they would have to start all over with the next set (Bakkali et al., 2008; Reichling et al., 2009).

In the present study we demonstrated that essential oils, not only of M. alternifolia well known in this respect from other studies (Brady et al., 2006; Kwieciński et al., 2009; Karpanen et al., 2008), but also of M. officinalis and L. angustifolia have antibiofilm potency. According to our knowledge, this is the first report concerning such activity of MEO and LEO. Both S. aureus and E. coli biofilms were eradicated efficiently by MEO. However, this effect was more time-dependent than the activity of TTO and LEO. The antibacterial, antifungal and antioxidiant properties of M. officinalis essential oil, but not antibiofilm activity, have been earlier described. The main constituents of MEO are citrals (geranial + neral, 39.9%), citronellal (13.7%), limonene (2.2%), geraniol (3.4%), β-caryophyllene (4.6%), β-caryophyllene oxide (1.7%), and germacrene D (2.4%) (Mimica-Dukic et al., 2004). Our further research will be devoted to examining the activity of individual components. However, it is also possible that they can be more active when they are in their natural proportions in the native oil. The main chemical components of lavender oil – the second active in our experiments are α-pinene, limonene, 1,8-cineole, cis-ocimene, trans-ocimene, 3-octanone, camphor, linalool, linalyl acetate, caryophyllene, terpinen-4-ol and lavendulyl acetate (Roller et al., 2009; Cavanagh and Wilkinson, 2002). Three of these components linalool, linalyl acetate, terpinen-4-ol were tested for antibiofilm activity since they are also major constituents of active TTO. Among them, terpinen-4-ol seems to have the most potent activity, causing more than 90% reduction in biofilm metabolic activity (MBEC90) after just 4 h of exposure.

Our results on TTO antibiofilm activity are slightly different from those published by Brady et al. (2006). The authors showed that biofilms formed by MSSA and MRSA isolates were completely eradicated following an exposure to 5% TTO for 1 h. In our study, S. aureus biofilm was also eradicated in such a short time but only by 66%. However, the concentration of TTO used was much lower – 3.1% (8 × MIC). On the other hand, total eradication of S. aureus biofilm was achieved not earlier than after 4 h but the concentration of TTO needed for that was only 0.78% (2 × MIC). Similarly to our results, TTO activity against S. aureus biofilms was reported by Kwieciński et al. (2009).

Usually plant-derived compounds show considerable activity against Gram-positive bacteria but not against Gram-negative species or yeast, which have evolved significant permeability barriers (Bakkali et al., 2008; Gibbons, 2008; Reichling et al., 2009; Amalradjou et al., 2010). Thus, it is worth emphasizing that in our experiments the Gram-negative bacteria were very susceptible to damage by all the essential oils used. For example, the potent and very fast antibiofilm activity of TTO was noticed for E. coli which was eradicated within 1 h exposure to concentration 0.78%.

It is noteworthy that the evident decrease in biofilm cells metabolic activity measured by MTT-reduction assay (biofilms in microplate wells), is not equal to their total destruction and eradication from the surface of the real medical biomaterials. We, like some other authors, have defined a drug concentration resulting in ≥90 biomass reduction measured by MTT staining as the MBEC (minimal biofilm eradication concentration). This method indeed has shown excellent applicability as it is able to detect dose-dependent and time-dependent differences in the effect of antimicrobials. But, contrary to other investigators, we have not found a strong correlation between the amount of biofilm mass exposed to essential oils, quantified with TTC staining on medical biomaterials and the metabolic activity quantified with MTT in the wells of microplate. A great advantage of the TTC reduction method is the fact that the metabolic reduction of colorless TTC into red-colored formazan by bacteria adhering to the surface of a synthetic implant occurs regardless of the type, shape or color of the biomaterial. However, this method is only semi-quantitative and requires confirmation using a more objective evaluation method, which was done in our study. This
suggests that research on given compound’s antibiofilm activity should be conducted using simultaneously at least two independent methods. It is well known that the growth conditions and the type of surface may affect the architecture of a biofilm which can influence the cells sensitivity to antimicrobial agents (Bryers, 2008; Flemming et al., 2009; Hoiby et al., 2010). However, our results encourage the exploration of the other essential oils and their constituents showing the most potent antibiofilm activity since the oils used in this study have superior antimicrobial activity in *S. aureus* and *E. coli* biofilms, compared with conventional antibiotics.

**Literature**


Competitiveness of *Rhizobium leguminosarum* bv. *trifolii* Strains in Mixed Inoculation of Clover (*Trifolium pratense*)

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**Abstract**

*Rhizobium leguminosarum* bv. *trifolii* (*Rlt*) establishes beneficial root nodule symbiosis with clover. Twenty *Rlt* strains differentially marked with antibiotic-resistance markers were investigated in terms of their competitiveness and plant growth promotion in mixed inoculation of clover in laboratory experiments. The results showed that the studied strains essentially differed in competition ability. These differences seem not to be dependent on bacterial multiplication in the vicinity of roots, but rather on complex physiological traits that affect competitiveness. The most remarkable result of this study is that almost half of the total number of the sampled nodules was colonized by more than one strain. The data suggest that multi-strain model of nodule colonization is common in *Rhizobium*-legume symbiosis and reflects the diversity of rhizobial population living in the rhizosphere.

**Key words:** clover growth promotion, competitiveness, mixed inoculation, rhizobia

**Introduction**

Biological nitrogen fixation (BNF) is a process in which atmospheric dinitrogen is reduced to forms available to plants and is provided by free-living bacteria or bacteria symbiotically associated with leguminous plants, commonly known as rhizobia. Rhizobia belong to different taxonomic families of α-proteobacteria such as *Rhizobiaceae*, *Phyllobacteriaceae* and *Bradyrhizobiaceae*, and to β-proteobacteria such as the recently described *Burkholderia* spp. (reviewed by Perret *et al.*, 2000; Masson-Boivin *et al.*, 2009).

Significance of rhizobial symbioses in agriculture stems from providing an extra source of nitrogen for plant growth, supplementing low nitrogen level in the soil and replacing nitrogenous fertilizer (Peoples *et al.*, 1995; Ramos *et al.*, 2001). *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*) induces and colonizes nodules elicited on roots of clover (*Trifolium* spp.). The symbiosis between rhizobia and legumes is a multi-step process including the exchange of molecular signals between the microsymbiont and its host (reviewed by, Perret *et al.*, 2000; Spaink, 2000; Jones *et al.*, 2007). Initially, flavonoids released by plants attract bacteria to the roots and induce synthesis of bacterial Nod factors that are lipochitooligosaccharide signals that trigger several plant responses, such as root hair curling and differentiation of plant meristems leading to the formation of root nodules. Nodules are colonized by rhizobia via tubular structures known as infection threads in which rhizobia multiply. After releasing from infection threads bacteria surrounded by peribacteroid membranes colonize nodule tissue forming symbiosomes in which bacteria are converted into bacteroids. Bacteroids localized in the symbiotic zone of mature nodules reduce atmospheric dinitrogen into forms, which are easily assimilated by plants (Vasse *et al.*, 1990; Timmers *et al.*, 2000).

Competition for nodulation is a quantitative phenotype, which determines the ability of *Rhizobium* strains to dominate in the nodules of a given legume host in competition with other strains present in the root rhizosphere (Dowling and Broughton, 1986). Soil populations of *Rhizobium* usually consist of numerous strains,
which compete with each other during the phase of vegetative growth in the soil, as well as during several stages of plant colonization and nodule formation (Wilson et al., 1998; Stuurman et al., 2000; Duodu et al., 2009). Rhizobial strains are genetically and metabolically diverse, reveal significant differentiation in competitive fitness to infect and occupy nodule of the host plant and differ in the symbiotic activities (Maier and Triplett, 1996; Wielbo et al., 2007; Depret and Laguerre, 2008). To investigate the competition between rhizobial strains, numerous molecular markers were developed and employed. Spontaneous or acquired antibiotic resistance markers are commonly used due to feasibility of detection and a broad differentiation allowing simultaneous identification a number of strains (Sharma et al., 1991; Cresswell et al., 1994; Sessitsch et al., 1998; Wilson et al., 1998; Stuurman et al., 2000).

In this work, we investigated the competition ability of 20 Rlt strains originating from clover root nodules under laboratory conditions. The results showed that in most cases simultaneous inoculation of plants with a mixture of rhizobial strains carrying different antibiotic resistance markers resulted in nodule colonization by more than one strain. The commonly observed nodule colonization by multiple strains that differ in respect to the efficiency of plant growth promotion may have significant impact on overall symbiosis in the soil.

**Experimental**

**Materials and Methods**

**Rhizobium leguminosarum bv. trifolii strains.** 20 Rlt strains used in this study were obtained from nodules of clover (Trifolium pratense) cultivated in arable sandy loam soil in the region of Lublin, Poland. Rifampicin, streptomycin and trimethoprim-resistant spontaneous mutants were obtained as follow: from overnight cultures of Rlt isolates grown in liquid TY medium (Sambrook et al., 1989) with constant shaking, about $5 \times 10^9$ cfu/ml of the given culture was plated on TY agar medium supplemented with rifampicin, streptomycin or trimethoprim (40 µg/ml, 200 µg/ml, 200 µg/ml, respectively). Single colonies resistant to appropriate antibiotic were isolated. Nalidixic acid for selection of naturally resistant strains were obtained by introducing pJBA21Tc plasmid. Nal R – natural nalidixic acid resistance. Tc R – tetracycline resistant strains were obtained by introducing pJBA21Tc plasmid. Nal R – natural nalidixic acid resistance.

**Plant tests.** Red clover seeds (Trifolium pratense L. cv. Dajana) were surface-sterilized, germinated on TY agar medium, and then transferred onto agar slants with nitrogen-free Fåhraeus medium (Vincent, 1970). Clover seedlings were inoculated with 200 µl single strain suspension in water, which was scratched from TY agar medium plate, or with 200 µl mixture of four strains differently tagged with antibiotic-resistance marker (1:1:1:1 v/v). In both cases the bacterial suspensions contained about $1.0 \times 10^9$ cfu/ml. Plants were grown in a greenhouse under natural light supplemented with artificial light (14 h day/10 h night, at 24/19°C). After 5 weeks, clover plants were harvested and nodules were counted. The efficiency of symbiotic nitrogen fixation was estimated by weighing fresh mass of shoots and roots. For each experimental group 20 plants were used and two independent experiments were conducted.

**Estimation of rhizobial competitiveness.** Ten clover plants were randomly chosen from each experimental group inoculated with a mixture of four strains and wet masses of shoots and roots were estimated. Root nodules were surface-sterilized and the content of individual nodules was plated on TY agar medium. Colonies derived from individual nodules were used for preparation of water suspension an $OD_{550}$ of 0.05 ($5 \times 10^7$ cfu/ml). 20 µl of each suspension was spotted on a set of TY agar medium plates containing one of four antibiotics, depending on antibiotic resistance markers carried by strains in a particular mixture. Bacteria were grown for three days at 28°C. Detectable growth on only one plate from a set was interpreted as the presence of pure culture in the nodule, whereas the growth on more than one plate from a set was interpreted as mixed colonization of nodule.

**Rhizobial growth assay.** 5 ml Fåhraeus medium supplemented with appropriate amount of TY medium was inoculated with 50 µl of overnight culture of Rlt strains growing in TY medium, and incubated for 2 days at 28°C. Bacterial growth was measured by monitoring $OD_{550}$. For all tested strains the experiment was conducted in triplicate.

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The relevant characteristics of Rhizobium leguminosarum bv. trifolii strains used in this study

<table>
<thead>
<tr>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>K416 TmR</td>
<td>K417 RfR</td>
<td>K38 RfR</td>
<td>K29 TeR</td>
<td>K313 TeR</td>
</tr>
<tr>
<td>K411 RfR</td>
<td>K108 TmR</td>
<td>K413 NaR</td>
<td>K87 SrR</td>
<td>K312 SrR</td>
</tr>
<tr>
<td>K36 SrR</td>
<td>KO17 TeR</td>
<td>KO18 TeR</td>
<td>K322 NaR</td>
<td>K415 RfR</td>
</tr>
<tr>
<td>KO27 TeR</td>
<td>K71 SrR</td>
<td>K91 SrR</td>
<td>K810 RfR</td>
<td>K107 TmR</td>
</tr>
</tbody>
</table>

The relevant characteristics of Rhizobium leguminosarum bv. trifolii strains used in this study
Results

To study nodulation competitiveness, twenty *Rlt* strains were divided into five groups (I–V). Strains were assigned to groups randomly, and four strains belonging to the same group differed from each other with respect to the antibiotic resistance markers they were carrying (Table I). Clover seedlings were inoculated with individual strains or with a mixture of four strains belonging to a given group. After 5 weeks, samples of nodules were taken and rhizobia were recovered from nodules by selection on TY medium supplemented with an appropriate antibiotic. In the case of groups II, III and V, all four strains used for clover inoculation were recovered from the nodules, whereas in the case of groups I and IV, three of four strains used for inoculation were found in the sampled nodules. For *Rlt* strains assigned to a given group the “relative competition ratio” (RCR) was calculated as follow: (a) the number of nodules occupied by the least frequently found strain was normalized to 1; (b) RCR for the three other strains were calculated by dividing the number of nodules colonized by a particular strain by the number of nodules occupied by the least frequent strain; (c) if a strain was absent in all tested nodules, the RCR was 0 (Fig. 1A, B, C, D, E). As regards their high “relative competition value”, K411 (group I), K417, K71 (group II), K38, K413 (group III), K87 (group IV) and K415 (group V) strains can be considered competitive. On the other hand, KO27 and K810 strains were not recovered from the sampled nodules and they were considered uncompetitive.

Based on the antibiotic resistance selection of *Rlt* strains recovered from clover nodules, it has been found that nodules occupied by only one strain comprised at average 56 ± 7% (Fig. 2 A, B, C, D, E). The others nodules were occupied by two or three strains that were distinguished by different antibiotic markers. Two-strain nodule occupancy ranged from 28% to
40%, and three-strain nodule occupancy from 3% to 16% of nodules, depending on the group of strains (I–V) used for clover inoculation (Fig. 2 A, B, C, D, E). Nodules simultaneously colonized by all four strains were not detected. The obtained results showed that nodule colonization by more than one strain was prevalent, at least in competition experiments conducted under laboratory conditions.

*Rlt* strains used in competition experiments were characterized with regard to the symbiotic properties such as nodulation ability and plant growth promotion (Table II). The tested strains varied both in the number of nodules elicited and in the impact on fresh mass of clovers. In the case of some strains the differences in nodule number and shoot and root mass were statistically significant. It is worth noting that plants inoculated with a mixture of four strains showed intermediary values of symbiotic parameters, which can reflect averaged effects of individual strains on symbiosis. In almost all cases, fresh mass of total plant per nodule was lower in the mixed inoculation in comparison to the same parameter in clover inoculation by individual strains (Table II).

The differences observed in the nodule colonization ability of *Rlt* strains might result from some physiological differences between the strains, for instance, in the growth rate. In the competition experiment, the mixtures of strains used as inocula were prepared by mixing equal volumes of four bacterial water suspensions with the same OD$_{550}$, which should minimize the effect of possible differences in the initial cell density. In addition, growth of rhizobia was

<table>
<thead>
<tr>
<th><em>Rlt</em> strain number</th>
<th>No of nodules/plant</th>
<th>Fresh mass of shoots/plant (mg)</th>
<th>Fresh mass of roots/plant (mg)</th>
<th>Fresh mass of plant/nodule (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I of <em>Rlt</em> strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K 416</td>
<td>5.4 ± 1.5 a</td>
<td>26.3 ± 3.5 a</td>
<td>22.7 ± 1.3 a</td>
<td>9.0</td>
</tr>
<tr>
<td>K 411</td>
<td>6.8 ± 0.7 a</td>
<td>31.3 ± 9.4 a</td>
<td>29.7 ± 10.2 a</td>
<td>8.9</td>
</tr>
<tr>
<td>K 36</td>
<td>4.3 ± 1.0 a</td>
<td>36.8 ± 2.1 a</td>
<td>18.5 ± 3.6 a</td>
<td>12.8</td>
</tr>
<tr>
<td>KO27</td>
<td>6.6 ± 2.3 a</td>
<td>28.6 ± 6.6 a</td>
<td>32.3 ± 5.3 a</td>
<td>9.2</td>
</tr>
<tr>
<td>Mixed inoculation (I)</td>
<td>7.4 ± 2.2 a</td>
<td>36.2 ± 8.3 a</td>
<td>23.7 ± 7.4 a</td>
<td>8.1</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K 417</td>
<td>7.0 ± 0.7 b</td>
<td>45.8 ± 9.3 ab</td>
<td>25.6 ± 11.6 a</td>
<td>10.2</td>
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<tr>
<td>K 108</td>
<td>5.4 ± 0.7 ab</td>
<td>50.9 ± 8.5 b</td>
<td>26.5 ± 10.0 a</td>
<td>14.3</td>
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<tr>
<td>KO17</td>
<td>3.6 ± 0.4 a</td>
<td>33.4 ± 1.9 a</td>
<td>12.6 ± 4.2 a</td>
<td>12.7</td>
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<tr>
<td>K 71</td>
<td>3.7 ± 0.6 a</td>
<td>34.2 ± 8.6 a</td>
<td>13.7 ± 3.8 a</td>
<td>13.0</td>
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<tr>
<td>Mixed inoculation (II)</td>
<td>4.7 ± 1.9 ab</td>
<td>30.5 ± 10.0 a</td>
<td>10.5 ± 6.3 a</td>
<td>8.8</td>
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<td><strong>Group III of <em>Rlt</em> strains</strong></td>
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<td>K 38</td>
<td>5.2 ± 2.0 a</td>
<td>36.6 ± 4.1 a</td>
<td>16.0 ± 0.9 a</td>
<td>10.1</td>
</tr>
<tr>
<td>K 413</td>
<td>8.6 ± 2.1 a</td>
<td>44.3 ± 2.5 a</td>
<td>39.4 ± 7.0 b</td>
<td>9.8</td>
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<td>KO18</td>
<td>10.2 ± 0.9 a</td>
<td>68.5 ± 7.3 b</td>
<td>43.3 ± 7.1 b</td>
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<td>K 91</td>
<td>5.8 ± 2.7 a</td>
<td>56.6 ± 3.0 c</td>
<td>40.0 ± 12.5 b</td>
<td>16.6</td>
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<tr>
<td>Mixed inoculation (III)</td>
<td>9.0 ± 2.8 a</td>
<td>33.9 ± 3.4 a</td>
<td>24.9 ± 6.3 ab</td>
<td>6.5</td>
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<tr>
<td><strong>Group IV of <em>Rlt</em> strains</strong></td>
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<tr>
<td>K 29</td>
<td>3.6 ± 1.2 a</td>
<td>31.2 ± 7.0 a</td>
<td>12.1 ± 2.0 a</td>
<td>12.2</td>
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<td>K 87</td>
<td>2.8 ± 0.7 a</td>
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<td>12.4 ± 4.1 a</td>
<td>13.4</td>
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<tr>
<td>K 322</td>
<td>4.2 ± 1.8 a</td>
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<td>18.8 ± 8.2 ab</td>
<td>12.7</td>
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<tr>
<td>K 810</td>
<td>6.8 ± 1.6 b</td>
<td>29.5 ± 4.5 a</td>
<td>21.8 ± 5.5 b</td>
<td>7.5</td>
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<tr>
<td>Mixed inoculation (IV)</td>
<td>3.3 ± 0.9 a</td>
<td>25.2 ± 7.1 a</td>
<td>10.4 ± 2.5 a</td>
<td>10.9</td>
</tr>
<tr>
<td><strong>Group V of <em>Rlt</em> strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K 313</td>
<td>3.8 ± 1.5 a</td>
<td>44.1 ± 12.9 a</td>
<td>23.7 ± 10.5 ab</td>
<td>17.7</td>
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<tr>
<td>K 312</td>
<td>3.4 ± 1.0 a</td>
<td>35.0 ± 7.0 a</td>
<td>13.3 ± 2.1 a</td>
<td>14.4</td>
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<tr>
<td>K 415</td>
<td>6.2 ± 1.2 ab</td>
<td>41.3 ± 0.9 a</td>
<td>22.8 ± 5.0 b</td>
<td>10.4</td>
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<tr>
<td>K 107</td>
<td>5.1 ± 1.6 ab</td>
<td>55.3 ± 10.4 a</td>
<td>27.4 ± 8.1 b</td>
<td>16.4</td>
</tr>
<tr>
<td>Mixed inoculation (V)</td>
<td>6.8 ± 0.4 b</td>
<td>44.7 ± 7.0 a</td>
<td>25.7 ± 6.1 b</td>
<td>10.3</td>
</tr>
<tr>
<td><strong>Average values</strong></td>
<td></td>
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<tr>
<td>Inoculation by individual strains</td>
<td>6.2 ± 2.3 a</td>
<td>34.1 ± 7.2 a</td>
<td>19.0 ± 7.9 a</td>
<td>12.1 ± 2.8 a</td>
</tr>
<tr>
<td>Inoculation by a mixture of strains</td>
<td>5.4 ± 1.9 a</td>
<td>39.4 ± 11.4 a</td>
<td>23 ± 9.6 a</td>
<td>8.9 ± 1.8 b</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation of 20 clover plants in experiment. Means within the same column and followed by the same letter are not significantly different ($P>0.05$).
assayed in the medium composed of nitrogen-free Fähraeus medium used in plant tests supplemented with 1%, 2%, 5%, 10% or 20% TY medium. It was assumed that addition of some amount of TY to the plant medium allows for the growth of bacteria while simultaneously resembling the plant growth medium. Rhizobia were grown in these media for 2 days and $OD_{550}$ was measured. The results showed that $Rlt$ strains used in the competition experiments grow weakly and only in Fähraeus medium supplemented with 10% or 20% of TY, reaching $OD_{550}$ values from 0.13 to 0.2 (data not shown). Differences in growth of strains on Fähraeus medium supplemented with 10% TY, as well as on medium supplemented with 20% TY were observed, however, the analysis of variance indicated that these differences were statistically insignificant (data not shown). In conclusion, the possible differences in the initial growth of $Rlt$ strains on plant medium at the time of inoculation could not essentially affect their competitiveness.

**Discussion**

Local population of *R. leguminosarum* specific for a given legume host is composed of numerous strains characterized by great variability in genetic and metabolic traits (Lakzian et al., 2002; Wielbo et al., 2010). Rhizobial competitiveness is a complex process dependent on genetic and overall metabolic status of bacteria (Wielbo et al., 2007), susceptibility to plant molecular signals (Mabood et al., 2008; Maj et al., 2010), motility of bacteria (Mellor et al., 1987), production of resistance to bacteriocins (Wilson et al., 1998) or even distribution of bacteria in the soil (Lopez-Garcia et al., 2002). Several studies indicate that both the legume hosts and microsymbionts affect the outcome of rhizobial competition (Kiers et al., 2006; Depret and Laguerre, 2008; Rangin et al., 2008).

All these and other variables characterizing individual strains influence their overall competition abilities. As a result, appreciable variability in rhizobial competitiveness is observed, both in highly controlled experiments under laboratory conditions, and in experiments carried out in the soil (Maier and Triplett, 1996; Svenning et al., 2001; Wielbo et al., 2007; Duodu et al., 2009; Sachs et al., 2009).

The strains compete with each other on the level of root adsorption, root hair infection, growth inside the infection threads, nodule tissue colonization and survival after release into the soil, where they constitute part of population capable of symbiotic interactions (Duodu et al., 2005; Silva et al., 2007; Rangin et al., 2008; Sachs et al., 2009). Less competitive rhizobia comprising another part of the population may be eliminated from nodule infection (Streeter, 1994). Finally, nonsymbiotic rhizobia constitute the fraction that lost the nodulation genes but is still able to colonize root surfaces and can play an important role in competition (Sachs et al., 2009).

In several studies, the competition between rhizobia was commonly investigated in a two-strain competition assay under controlled conditions. In this assay both strains were tagged with different markers such as antibiotic resistance genes (Bromfield, 1984), *lux* (Cresswell et al., 1994) or *gfp* reporter genes (Stuurman et al., 2000) and occupancy of both strains was easily determined in the nodule. The drawback of this method is the extreme simplification of the model, which does not reflect native conditions. The second most frequently used method for rhizobial competition analysis was to use a single marked strain vs. the entire unmarked soil population composed of numerous strains. Major drawback of this approach is lack of any information about the unmarked strains possibly present in the nodules. Recently, the competitiveness of unmarked rhizobial strains was studied by molecular identification of particular genotypes occupying the nodules (Svenning et al., 2001).

In the present work, the two methods have been combined, and clover plants were inoculated by four differently marked strains. It allowed determining the differences in competitive ability of the individual strains, and the predominance of nodules occupied by more than one strain, in the case of a mixed inoculation. It is worth noting that infection by several strains negatively affected plant growth promotion when compared to single-strain infection.

Past studies showed mixed colonization of infection threads and nodules (Bromfield, 1984) in a two-strain competition assay (Stuurman et al., 2000; Gage, 2004). Expanding the competition test to clover inoculation by four strains, we demonstrated the possibility of single clover nodule colonization by three different strains. The percent of nodules colonized by three strains was relatively low and ranged from 3 to 16% of the sampled nodules. Nodules colonized by four strains were not found. It is possible that the number of the sampled nodules should have been greater to allow detection of nodules occupied by all four strains. On the other hand, the limited space available in the infection threads and/or in young nodules might restrict the growth of rhizobia, and this might also explain why four strain nodule colonization was not observed. The most important finding from the presented study is that almost half of the total number of the sampled nodules was colonized by more than one strain. It suggests that multi-strain model of nodule colonization is predominant also in the soil environment, and reflects the complexity and diversity of rhizobial population in the rhizosphere.
The comparison of symbiotic efficiencies of clover inoculation by individual strains vs. a mixture of strains was not conclusive. Whereas, significant differences between the averaged data of shoots and roots masses in single strain inoculation and in the mixed inoculation have not been found, significant difference in ratios of fresh mass of the entire plant to nodule number was evident when these two types of inoculations were compared (Table II). A possible explanation for this result might be that the concentration of several Nod factors produced by particular strains in the mixed inoculation is substantially greater than in a single strain inoculation. Nod factors stimulate nodule organogenesis (Perret et al., 2000; Spank, 2000) and that nodule number increase was not accompanied by increased plant growth probably due to the relatively short timeframe of the experiment. This observation is consistent with our previous studies, in which treatment of clover seeds with Nod factors under controlled conditions enhanced the growth of clover only when a Nod factor was applied in appropriate concentration (Maj et al., 2009).

In summary, our data revealed that *R. leguminosarum* strains investigated in the experimental competition model were greatly differentiated with respect to competitiveness for nodule occupancy. In the mixed inoculation of clover plants, nodule coinfection by two or three strains was commonly observed. The prevalence of nodule coinfection by native soil population is yet to be determined.

Acknowledgements

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**Literature**


Introduction

Bio-ethanol production by yeast is a growing industry due to energy and environmental demands (Schubert, 2006). *Saccharomyces cerevisiae* and related yeast species have been extensively used in fermentation, wine-making, sake-making and brewing processes. Successful performance of alcoholic fermentations, however, depends on the ability of the yeast strains used to cope with a number of stress factors occurring during the process (Van Uden, 1985; Viegas *et al.*, 1989; Hirasawa *et al.*, 2007), including osmotic pressure imposed by the initial high sugar concentration and stress induced by fermentation end-products such as ethanol or acetate. Among these, the stress induced by increasing amounts of ethanol, accumulating to toxic concentrations during ethanol fermentation, is the major factor responsible for reduced ethanol production yields and, ultimately, for stuck fermentations (Gibson *et al.*, 2007). Thus, yeast strains that can endure stress imposed by high ethanol concentrations are highly desirable. Throughout the years many efforts have been made to characterize the mechanisms underlying ethanol stress tolerance, aiming to increase ethanol productivity (Van Uden, 1985; You *et al.*, 2003; Alper *et al.*, 2006; Hirasawa *et al.*, 2007). To overcome fermentation problems, sophisticated refinements of fermentation processes, involving extractive fermentation (Jones *et al.*, 1993; Da Silva *et al.*, 1999), cell immobilization (de Vasconcelos *et al.*, 2004; Verbelen *et al.*, 2006), and recycling or retention by membranes (Nishiwaki and Dunn, 1998; Wang and Lin, 2010), were employed with a view to obtaining a large quantity of fermenting biomass as well as removing the inhibitory ethanol product. Successful engineering of yeast transcription machinery for this purpose was also reported (Alper *et al.*, 2006).

The present study was conducted to select the best ethanol-producing yeast strains from our collection, to improve yeast fermentation performance by adaptation, and to optimize the conditions for alcohol production from sucrose. There are, to our knowledge, only a few studies that describe the creation of ethanol-tolerant *S. cerevisiae* mutants using adaptation and ethanol stress as the selection pressure (Brown *et al.*, 1982; Remize *et al.*, 1999; Stanley *et al.*, 2010).

**Selection and Adaptation of *Saccharomyces cerevisiae* to Increased Ethanol Tolerance and Production**

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**Abstract**

A total of 24 yeast strains were tested for their capacity to produce ethanol, and of these, 8 were characterized by the best ethanol yields (73.11–81.78%). The most active mutant *Saccharomyces cerevisiae* ER-A, resistant to ethanol stress, was characterized by high resistance to acidic (pH 1.0 and 2.0), oxidative (1 and 2% of H₂O₂), and high temperature (45 and 52°C) stresses. During cultivation under all stress conditions, the mutants showed a considerably increased viability ranging widely from about 1.04 to 3.94-fold in comparison with the parent strain *S. cerevisiae* ER. At an initial sucrose concentration of 150 g/l in basal medium A containing yeast extract and mineral salts, at 30°C and within 72 h, the most active strain, *S. cerevisiae* ER-A, reached an ethanol concentration of 80 g/l, ethanol productivity of 1.1 g/l/h, and an ethanol yield (% of theoretical) of 99.13. Those values were significantly higher in comparison with parent strain (ethanol concentration 71 g/l and productivity of 0.99 g/l/h). The present study seems to confirm the high effectiveness of selection of ethanol-resistant yeast strains by adaptation to high ethanol concentrations, for increased ethanol production.

**Key words:** adaptation to high ethanol concentration, ethanol tolerance, fermentation, yeast
Experimental

Material and Methods

Microorganisms and media. The strains of the yeast listed in Table I were maintained at 4°C on malt agar slants. For inoculum preparation selected strains were cultivated on a growth medium A containing glucose, 2%; bactopepton, 2% and yeast extract, 1%. Fermentations were performed using a basal medium A containing: sucrose, 15.0%; yeast extract, 1.0%; \((\text{NH}_4\text{)}_2\text{SO}_4\), 0.3% and \(\text{KH}_2\text{PO}_4\), 0.1%.

Adaptation for increased ethanol production. Enrichments for increased ethanol production were carried out according to the method of Dinh et al., (2008) with some modifications. The cultivation of the \(S.\text{ cerevisiae}\) ER strain was carried out in malt medium containing ethanol and then the culture was transferred to a fresh medium containing the same ethanol concentration. Adapted cultures were grown in culture tubes (18 by 150 mm) containing 10 ml of that medium and were incubated at 30°C without agitation. After that, the culture was transferred to a medium containing a higher ethanol concentration, followed by repetitive cultivations. The initial ethanol concentration was set at 5.0% (w/v) and it was changed gradually from 6.0 to 15.0%. New derivatives of \(S.\text{ cerevisiae}\) were isolated from the adapted cultures after 10 months of serial transfers into media with high concentrations of ethanol. At the end of this period, two clones were selected for further study; these clones were designated as strain ER-A and ER-M.

The adaptation of the yeast was evaluated by measuring the optical density of the culture at OD 600. Viability in ethanol was determined by maintaining yeast cells of \(S.\text{ cerevisiae}\) ER in malt medium supplemented with 5–15% ethanol for 48 h at 30°C, plating them on malt agar plate, and subsequently counting the number of colonies formed. Control cultures were maintained in the same medium without ethanol.

Inoculum preparation. For the preparation of inoculum, yeast strains were transferred from agar

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sucrose concentration (%)</th>
<th>Catalase activity (U)</th>
<th>Ethanol (% w/v)</th>
<th>Ethanol yield (% of theoretical)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>40</td>
<td>15</td>
<td>40</td>
</tr>
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<td>Candida sheataceae ATCC 22–994</td>
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<td>Kluyveromyces marxianus CCY 50–2–1</td>
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<td>4.69</td>
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<td>Saccharomyces fragilis S-24</td>
<td>9.84</td>
<td>5.91</td>
<td>3.10</td>
<td>3.20</td>
</tr>
<tr>
<td>Saccharomyces fragilis S-25</td>
<td>6.88</td>
<td>8.05</td>
<td>5.10</td>
<td>6.20</td>
</tr>
<tr>
<td>1Saccharomyces mells 1</td>
<td>3.71</td>
<td>3.78</td>
<td>0.70</td>
<td>0.80</td>
</tr>
<tr>
<td>Saccharomyces muciparus CCM 21–25–1</td>
<td>8.54</td>
<td>7.72</td>
<td>4.10</td>
<td>3.00</td>
</tr>
<tr>
<td>1Saccharomyces rouxii</td>
<td>7.53</td>
<td>8.71</td>
<td>0.60</td>
<td>0.20</td>
</tr>
</tbody>
</table>

The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium A containing 15% or 40% of sucrose during 72 h.

* Values are averages of 3 replicate determinations with standard deviations of < ± 5%

1 Wine yeasts; * Brewing yeasts; 2 Baker’s yeast; 4 Distillery yeast
slants into 50-ml Erlenmeyer flasks containing 10 ml of sterile liquid growth medium A, which were cultured at 30°C on a rotary shaker (Shaker Orbit The LAB-LINE Instruments Inc, Melrose Park, Illinois, USA) at 150 rpm for 24 h.

**Ethanol fermentations.** Fermentations with the selected strain were conducted in 300-ml Erlenmeyer flasks containing 100 ml of basal medium A (pH 5.5). The flasks were inoculated with 10% v/v seed culture. Fermentations were carried out for 72 h at 30°C (if not indicated otherwise) in a shaker at 90 rpm. Ethanol evaporation was prevented by rubber stoppers, with fermentative tubes filled with 50% H₂SO₄. The fermentation parameters were corrected by ethanol, sucrose and biomass withdrawn during sampling. Overall biomass as well as ethanol yields and sucrose consumption were calculated from end-of-batch data, where the peak ethanol concentration was recorded. Other methodological details are given in tables and figures.

**Assays.** Biomass was estimated from optical density at 600 nm. Dry mass was calculated by referring to a standard curve of cell mass versus absorbance (Hughest et al., 1984). Ethanol was quantified using the Gonchar et al. (2001) method in own modification using o-dianisidine instead of 3,3',5,5'-tetramethylbenzidine (TMB) as chromogen.

Extracellular catalase activity was measured spectrophotometrically by observing the decrease in light absorption at 525 nm during decomposition of H₂O₂ by the enzyme (Fiedurek and Gromada, 1997). One unit (U) of catalase activity was defined as the amount of enzyme catalysing the decomposition of 1 μmol hydrogen peroxide per min at 30°C. Fermentations were performed in 2 replicate cultures, and analyses were carried out in duplicate. The data given here are the averages of the measurements.

**Measurement of respiration.** Cell suspensions, prepared as described above, were used to measure the rate of yeast respiration at various medium pH, either in the absence or in the presence of ethanol. Other methodological details are given in the tables and figures. Oxygen concentration in the culture medium was measured by a polarographic dissolved oxygen sensor (Ingold, CH Industrie Nord, Urdorf, Switzerland). The readings were expressed as percentage of the initial level of saturation (100%).

**Results**

Production of ethanol during fermentation is limited by the inability of yeast to grow at high ethanol levels, which is why a great deal of effort has been devoted to creating yeast strains that would tolerate high ethanol levels, and be able to continue the fermentation to produce higher concentrations of alcohol. The development of such strains would have the major advantage of saving the energy involved in distilling and refining ethanol.

A total of 24 yeast strains were tested for their capacity to produce ethanol, and of these, 8 were characterized by the best ethanol yields (73.11–81.78%). Ethanol production, catalase activity, and ethanol yield were monitored on synthetic medium A. The effects of increasing sucrose concentration from 15 to 40% on ethanol yield, catalase activity and biomass of the yeast strains were examined. Along with the increase in sucrose concentration from 15 to 40%, a decrease in biomass and ethanol yield was observed. On the other hand, the increase enhanced extracellular catalase production, probably as an effect of stress conditions. Among the 24 strains, 13 (54.2%) were characterized by high catalase activity when grown on a medium with a high sucrose concentration (40%) (Table I). The increase in sucrose concentration in the medium (from 15 to 40%) caused a significant (1.02 to 3-fold) reduction in biomass production.

For further selection an industrial strain of *Saccharomyces cerevisiae* ER characterized by high ethanol tolerance, higher cell viability especially during “very high gravity” fermentation, and working under a wide range of temperatures (35–40°C) was used. This strain was used for preparation of inocula for adaptation with high concentrations of ethanol (5 to 15%) in order to select ethanol-tolerant yeast. New derivatives of *S. cerevisiae* were isolated from adapted cultures after 10 months of serial transfers. During this period about 120 subsequent transfers were performed. At the end of this period, a two clones were selected from enrichment for further study; those clones, designated as strain *S. cerevisiae*: ER-A and ER-M, were able to grown at 15% of ethanol in the medium (data not shown). When subjected to a stepwise increase in ethanol concentration with repetitive cultivations, the yeast cells *S. cerevisiae* ER-A adapted to the high ethanol concentration showed better biomass accumulation in the medium containing the same ethanol concentration, in comparison to the cells of the parent strain (Table II). This strain was used for further study.

The most active mutant, *S. cerevisiae* ER-A, resistant to ethanol stress, was characterized by high resistance to acidic (pH 1.0 and 2.0), oxidative (1 and 2% of H₂O₂) and high temperature (45 and 52°C) stresses. The viability of mutants during cultivation under all the mentioned stress conditions increased about 1.04 to 3.94-fold in comparison with the parent strain *S. cerevisiae* ER. It is worth noting, that mutant of *S. cerevisiae* ER-A resistant to ethanol stress, generally showed a better adaptation to higher ethanol concentration, as expressed by the increased (about 4-fold) viability at 20% of ethanol in comparison to its 10%
concentration (Table III). A similar trend was also observed for oxidative (1 and 2% of H₂O₂) and high temperature (45 and 52°C) stress, when higher numbers of cells survived in more drastic stress conditions.

Measurements of oxygen consumption at 30°C and pH 4.5 showed that ethanol inhibited the respiration rate of yeast. In the absence of added ethanol, the oxygen concentration decreased gradually over the first 15 min to the level of about 6%. When the experiment was repeated in the presence of 5% (w/v) ethanol, the respiration rate of the yeast cells was markedly inhibited, and the oxygen concentration fell more slowly, reaching 10.7% after 20 min. The higher concentration (10%) of ethanol significantly reduced oxygen consumption; in these conditions, more than half of the initial dissolved oxygen content remained.

Table II
Effect of ethanol concentration on biomass production

<table>
<thead>
<tr>
<th>Ethanol (% w/v)</th>
<th>Dry matter (g/l) after 24 h</th>
<th>Relative to the parent-type (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Parent ER</td>
</tr>
<tr>
<td>Control (none)</td>
<td>2.33</td>
<td>3.04</td>
</tr>
<tr>
<td>11</td>
<td>1.10</td>
<td>1.67</td>
</tr>
<tr>
<td>12</td>
<td>0.58</td>
<td>1.11</td>
</tr>
<tr>
<td>13</td>
<td>0.41</td>
<td>0.63</td>
</tr>
<tr>
<td>14</td>
<td>0.19</td>
<td>0.22</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae ER (parent)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium A with ethanol (11–15%) during 24–48 h. Values are averages of 6 replicate determinations with standard deviations of < ± 6%.

Table III
Effect of abiotic stresses on surviving cells of Saccharomyces cerevisiae ER

<table>
<thead>
<tr>
<th>Stress conditions</th>
<th>Surviving cells (%)</th>
<th>Relative to the wild-type (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pH (2.0)</td>
<td>81.99</td>
<td>93.70</td>
</tr>
<tr>
<td>Low pH (1.0)</td>
<td>3.85</td>
<td>7.14</td>
</tr>
<tr>
<td>Oxidative stress (1% H₂O₂)</td>
<td>80.90</td>
<td>100.0</td>
</tr>
<tr>
<td>Oxidative stress (2% H₂O₂)</td>
<td>72.0</td>
<td>96.50</td>
</tr>
<tr>
<td>High temperature (45°C)</td>
<td>47.59</td>
<td>69.85</td>
</tr>
<tr>
<td>High temperature (52°C)</td>
<td>26.34</td>
<td>43.17</td>
</tr>
<tr>
<td>Ethanol (10%)</td>
<td>89.25</td>
<td>90.37</td>
</tr>
<tr>
<td>Ethanol (15%)</td>
<td>61.02</td>
<td>72.93</td>
</tr>
<tr>
<td>Ethanol (20%)</td>
<td>10.00</td>
<td>39.40</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae ER-A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium during 24 h of stress conditions. Low pH media were obtained by adding 0.1 M HCl. Values are averages of 4 replicate determinations with standard deviations of < ± 4%.

Table IV
Effect of externally added ethanol at concentration 0–15% on CO₂ and biomass production by parent and adapted strain Saccharomyces cerevisiae MR-A

<table>
<thead>
<tr>
<th>Ethanol (% w/v)</th>
<th>Relative metabolic rate (%) a, b</th>
<th>Dry matter (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mixing (90 rpm)</td>
<td>Mixing (180 rpm)</td>
</tr>
<tr>
<td>Control (none)</td>
<td>43.48</td>
<td>100.00</td>
</tr>
<tr>
<td>5</td>
<td>30.43</td>
<td>52.17</td>
</tr>
<tr>
<td>10</td>
<td>5.43</td>
<td>13.44</td>
</tr>
<tr>
<td>11</td>
<td>4.43</td>
<td>13.00</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>4.35</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae ER-A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Relative metabolic rate (%) was calculated by counting the number of bubbles of CO₂ released from fermentative tubes during 60 min. Maximal amount of bubbles formed during culture of the adapted strain ER-A was defined as 100%. The strains were incubated in 50 ml conical flasks, each containing 20 ml of basal medium A with ethanol (5–15%) during 24 h.

b Values are averages of 6 replicate determinations with standard deviations of < ± 5%.

Table V
Effect of (NH₄)₂SO₄ concentration on ethanol production

<table>
<thead>
<tr>
<th>Medium</th>
<th>Ethanol (% w/v)</th>
<th>Ethanol yield (% of theoretical)</th>
<th>Dry matter (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae ER (parent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal medium A</td>
<td>6.30</td>
<td>78.06</td>
<td>5.38</td>
</tr>
<tr>
<td>Modified basal medium A with (NH₄)₂SO₄ = 1.0%</td>
<td>7.10</td>
<td>87.98</td>
<td>6.75</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae ER-A a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal medium A</td>
<td>7.20</td>
<td>89.22</td>
<td>5.04</td>
</tr>
<tr>
<td>Modified basal medium A with (NH₄)₂SO₄ = 1.0%</td>
<td>8.00</td>
<td>99.13</td>
<td>6.23</td>
</tr>
</tbody>
</table>

Fermentation conditions: inoculation: 2 × 10⁷ cells/ml, time fermentation 72 h at 30°C in shaker 90 rpm. The strain was incubated in 50 ml conical flasks, each containing 20 ml of basal medium A containing 0.3 and 1.0% of with (NH₄)₂SO₄.

a Values are averages of 6 replicate determinations with standard deviations of < ± 5%.
in the medium after 20 min from the beginning of the experiment (Fig. 1a). The adapted cells of *S. cerevisiae* ER-A consumed more oxygen than the parent strain in media with all the tested ethanol concentrations (Fig. 1b). After 20 min of respiration, the levels of oxygen concentration in media with 5% and 10% ethanol were lower by 10.7% and 13.2%, respectively, as compared with values for the parent strain.

The effect of 5–15% ethanol externally added to basal medium A on CO₂ production by the parent and the adapted strain of *S. cerevisiae* ER-A was examined. In comparison with the parent strain, the metabolic activity of adapted cells of *S. cerevisiae* ER-A showed higher resistance to inhibition by ethanol when the cells were grown with 5 and 10% (w/v) ethanol. The higher concentration (11%) of ethanol completely inhibited metabolic activity of the parent strain when culture was carried out in a shaker at 90 rpm, and adapted cells showed insignificant activity in these conditions. Under a higher mixing rate (180 rpm), at 11% of ethanol, adapted cells of *S. cerevisiae* ER-A were characterized by an about 3-fold higher
metabolic activity than the parent strain. A further increase in ethanol concentration in basal medium A to 12% completely inhibited metabolic activity of the parent strain, and the adapted strain showed very small this activity in these conditions (Table IV).

Table V compares ethanol production by the parental and the mutant strain of *S. cerevisiae* ER-A in basal and modified medium A. The highest ethanol yields for the two strains were obtained on modified medium A, containing 15% of sucrose, 1% of yeast extract, 1% of (NH₄)₂SO₄ and 1% of KH₂PO₄, using a very simple fermentation system (shake flask). Considerable better results were achieved for mutant *S. cerevisiae* ER-A, which was characterized by an ethanol yield of 99.13% and an ethanol concentration of 8.0%; the respective values for the parental strain were 87.98% and 7.1%.

**Discussion**

Tolerance to high ethanol and sucrose concentrations is an important property of industrial microorganisms. The accumulation of ethanol during cultivation causes stress to yeast cells, leading to a decrease in cell growth and production of target products. Thus, understanding the process of adaptation of yeast to high ethanol concentrations is important as it may lead to the construction of yeast strains able to grow well at high ethanol concentrations. Such ethanol-tolerant yeasts are highly desirable for the production of useful compounds. Improving ethanol tolerance in yeast should, therefore, reduce the impact of ethanol toxicity on fermentation performance (Dinh et al., 2008; Stanley et al., 2010).

Stanley et al. (2010) obtained ethanol-tolerant yeast mutants by subjecting mutagenised and non-mutagenised populations of *S. cerevisiae* W303-1A to adaptive evolution using ethanol stress as a selection pressure. Mutants CM1 (chemically mutagenised) and SM1 (spontaneous) had increased acclimation and growth rates when cultivated in sub-lethal ethanol concentrations, and their survivability in lethal ethanol concentrations was considerably improved compared with the parent strain. Those authors suggested that the increased ethanol tolerance of the mutants was due to their elevated glycerol production rates and the potential of these to increase the ratio of oxidised and reduced forms of nicotinamide adenine dinucleotide (NAD⁺/NADH) in an ethanol-compromised cell, stimulating glycolytic activity.

The viability of the adapted *S. cerevisiae* ER-A was always higher than for the parental strain, for all the stress conditions used (Table III). For example, the viable population (expressed as a percentage of the initial population) of the ER-culture after 24 h in 20% (w/v) ethanol was 39.40%, respectively, compared with 10.0% for the parent. Similar viability characterized SM1 and CM1 cultures obtained by Stanley et al. (2010) under lethal ethanol stress conditions (12% (w/v) ethanol, after 12 h) – 52% and 44%, respectively, compared with 5% for the parent.

Some researchers have analyzed phenomena associated with adaptation of yeast cells to high ethanol concentrations. Lloyd et al. (1993) found that yeast previously grown in the presence of 5% ethanol could grow in the medium containing 10% ethanol, whereas yeast inoculated directly into a medium containing 10% ethanol failed to grow. Ismail and Ali (1971) reported that no increase in the tolerance of yeast to a high ethanol concentration was observed after ten successive transfers to an environment containing a high ethanol concentration. Therefore, it is expected that exposing yeast cells to a stepwise increase in the level of ethanol stress should be effective for obtaining ethanol-tolerant yeast strains.

The results presented above confirmed that an adapted strain resistant to ethanol stress generally showed better adaptation to other stress conditions, as expressed by the increased survival of the mutant of *S. cerevisiae* ER-A during cultivation under acidic (pH 1.0 and 2.0), oxidative (1 and 2% of H₂O₂), and high temperature (45 and 52°C) stresses. It is worth noting that in the more drastic stress conditions, the ethanol-tolerant mutant was characterized by a higher survival rate. This is in accordance with data presented by Ogawa et al. (2000), who showed that several genes were highly expressed only in the ethanol-tolerant mutant but not in the parent strain. The ethanol-tolerant mutant also exhibited resistance to other stresses including heat, high osmolarity, and oxidative stress in addition to ethanol tolerance. These results indicate that the mutant exhibits multiple stress tolerances due to elevated expression of stress-responsive genes, resulting in accumulation of high amounts of stress protective substances such as catalase, glycerol, and trehalose (Ogawa et al., 2000). The ability of one stress condition to provide protection against other stresses is referred to as cross-protection. Several studies have shown that adaptation to acid stress confers resistance to a wide range of stress conditions including heat, salt, crystal violet, and polymyxin B (Lee et al., 1995; Bearson et al., 1997). However, adaptation to other stresses does not typically induce significant acid tolerance. This implies that acid exposure may be treated by microorganisms as a more general stress indicator, whereas salt and H₂O₂ may be more specific stress signals.

A number of specific selection schemes have been elaborated to improve the biosynthetic capacity of production strains. Thus the acid tolerance of *Leuconostoc oenos* was examined in cells surviving at pH
2.6, which is lower than the acid limit of growth (about pH 3.0). The acid-resistant mutant L. oenos, was found to be able to grow in acidic media and characterized by a high H⁺-ATPase activity at low pH. Such strains may be an important part of the technology of modern commercial wine production (Drici-Cachon et al., 1996). Accumulation of a large amounts of metabolic end-products during the fermentation period, especially in case of industrial amino acid fermentation, builds up a high osmotic strength which affects both growth and production. Enhanced l-treonine production by salt tolerant mutants of E. coli was achieved (Drici-Cachon et al., 1996). Some mutants have been described in E. coli which are more resistant to cell lysis in the presence of ethanol (Fried and Novick 1973; Ingram et al., 1980). In this respect, our results obtained in ethanol production can be compared with data provided by Ortiz-Zamora et al. (2008), who isolated and selected yeast strains from alcoholic fermentations of natural sources. These strains were exposed several times to high concentrations of glucose and ethanol in order to select ethanol- and glucose-tolerant yeast; 10 were obtained that adapted best to these conditions. Some of these strains demonstrated the highest adaptation to both ethanol (5–7% w/v) and glucose (20% w/v). The maximum yield obtained was 0.46 g/g (90% theoretical yield) in a 20-L bioreactor with cane molasses.

Araque et al. (2008) selected thermotolerant yeast strains Saccharomyces cerevisiae for bioethanol production, which were able to grow and ferment glucose in the temperature range 35–45°C. All the strains grew (in agar plates) at 35 and 40°C, only two strains grew at 42°C, and no strain grew at 45°C. Glucose-to-ethanol conversion yield was between 50% and 80% of the theoretical value. The ethanol yields by SSF using the selected strain were higher than those obtained using the control yeast.

The selected strain, S. cerevisiae ER-A, showed an ability to grow and ferment sucrose at ethanol concentrations in the medium of 15 and 12% (w/v), respectively (Tables II and IV). Its resistance to ethanol, externally added to the medium, was significantly higher than for the parent strain. An increase in the rate of mixing from 90 to 180 rpm correlated with a simultaneous increase in the relative fermentation rate (%) both for the parent and the adapted strain, probably as an effect of a higher mass transfer. The effect of ethanol on yeast growth and fermentation has been studied by Brown et al. (1981). These authors showed complex kinetics which resulted from both an inhibition of the growth rate itself and also a reduction in cell viability. The growth and viability effects had different inhibition constants. Contrary to our data, ethanol was less inhibitory toward fermentation than toward growth in sake yeast.

Some data suggest that an improvement in ethanol tolerance leads to an increase in both ethanol production rate and the total amount of ethanol produced (Jiménez and Benítez, 1988). The adapted S. cerevisiae ER-A reached an ethanol concentration of 80 g/l, an ethanol productivity of 1.1 g/l/h, and an ethanol yield (% of theoretical) 99.13. Those values were significantly higher in comparison with the parent strain (ethanol concentration of 72.9 g/l and productivity of 1.01 g/l/h).

The studies presented above seem to confirm the high effectiveness of selection of resistant yeast strains by adaptation to high ethanol concentrations for increased ethanol production. Additionally, better adaptation of these mutants to abiotic stresses can affect yeast growth and ethanol productivity. The advantage gained in direct screening is to reduce in a very specific way the number of cultures isolated from the plates, which would normally require testing of productivity via shake flask cultures. This is a significant contribution to make screening of ethanol-producing yeast more efficient. The ethanol tolerant strain was stable in the subsequent subcultures in the absence of stress during 6 months. It can be concluded from the present results that the adapted strain S. cerevisiae ER-A showed, at this stage of our studies, a moderate fermentation activity, which gave reasonable ethanol yields from sucrose. Further improvements to the isolated yeast strain and the growth conditions are necessary to utilize the strain for larger-scale fermentation.

Acknowledgements
The authors wish to thank Prof. Józef Kur (Department of Microbiology Chemical Faculty, Gdańsk University of Technology, Poland) for supplying yeast strain Saccharomyces cerevisiae ER (Ethanol Red).

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Literature


Cytotoxicity of *Aspergillus* Fungi Isolated from Hospital Environment

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Abstract

The majority of mycotoxins produced by *Aspergillus* fungi are immunosuppressive agents, and their cytotoxicity may impair defense mechanisms in humans. The objective of the study was evaluation of the cytotoxicity of fungi isolated from an environment where inpatients with impaired immunity were present. The materials comprised 57 fungal strains: *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus flavus*, *Aspergillus versicolor* and *Aspergillus ustus* isolated from hospital rooms in Cracow. The cytotoxicity of all the strains was evaluated using the MTT test (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide). To emphasize the differences in cytotoxicity among the particular strains, variance analysis (ANOVA) and Tukey’s difference test were used. Out of 57 *Aspergillus* strains tested, 48 (84%) turned out to be cytotoxic. The cytotoxicity was high (+++) in 21 strains, mainly in *A. fumigatus*. The least cytotoxic were *A. niger* fungi, this being statistically significant (p<0,05). To protect a patient from the adverse effects of mycotoxins, not only his or her immunity status should be evaluated but also the presence of fungi in hospital environment and their cytotoxicity should be monitored (possible exposure).

Keywords: *Aspergillus* sp., cytotoxicity, environment

Introduction

There is evidence that a number of *Aspergillus* fungi present in hospital environment produce toxins (Burr, 2001; Ben-Ami et al., 2009; Klich et al., 2009). Their secondary metabolites such as ochratoxin A, aflatoxins, trichotecins or sterigmatocystine are toxic for various cellular structures and interfere with key processes like RNA and DNA synthesis (Ciegler and Bennet, 1980). Depending on fungal species and/or strain, mycotoxins differ in their specificity and influence on target cells, cellular structures and processes in them (Steyn, 1995; Pitt, 2000). It should be also kept in mind that not all moulds produce mycotoxins, and their production depends on culture medium, life cycle and environmental conditions (Pitt et al., 2000; Kelman et al., 2004). To assess the ability of a given strain to produce mycotoxins, the presence of other moulds in the environment should be taken into account as well as their influence on mycotoxin production intensity. It has been documented that the fungi incubated in monocultures in laboratory setting lose their mycotoxin production potential (Fischer and Dott, 2003; Jarvis and Miller, 2005). The ability of fungi to produce mycotoxins is hard to evaluate because there is no possibility to analyze the mycotoxins produced in the tissues of living organisms. The relationship between disease (or predisposition to it) and the level of exposure (detection of pathogenic spores) as well as symptoms and signs characteristic of experimental lesions produced by mycotoxins may contribute to establishing the noxiousness of fungi to human health. The objective of the study was evaluation of the cytotoxicity of *Aspergillus* fungi isolated from hospital environment.

Experimental

Materials and Methods

The materials comprised mould strains belonging to the *Aspergillus* genus, isolated from the environment, mainly from indoor air in hospital rooms at a number of hospitals in Kraków in the years 2007–2008.
Primarily, the mycological flora was evaluated in hospital ward environment (neonatal intensive care unit, medical intensive therapy unit, the wards of chemotherapy and radiotherapy). The fungi were sampled using a MAS 100 device (Merk) from the indoor air in various hospital rooms. The species of particular strains were detected on the basis of thorough macroscopic and microscopic analysis. Out of all of the fungi isolated, 57 Aspergillus strains were randomly chosen for further examination. The chosen strains belonged to six species: A. fumigatus (21 strains), A. niger (14 strains), A. ochraceus (13 strains), A. flavus (5 strains), A. versicolor (3 strains) and A. ustus (a single strain). The fungi were divided into groups: each group comprised different species. The cytotoxicity test MTT (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide) was performed separately for each strain. To evaluate the total cytotoxicity of the fungi, swine kidney cells (SK) were used because they are sensitive to most mycotoxins. The test is based on the reduction of yellow MTT tetrazolium salt to violet formazan, insoluble in water. The reduction occurs in the presence of intact SK, not damaged by mycotoxins. The intensity of reaction is proportional to the amount of metabolically active SK. When the SK are infected with moulds producing mycotoxins, their mitochondria fail to reduce tetrazolium salt into formazan. Therefore, when the SK are damaged by mycotoxins, the reaction is less intensive or does not occur, which can be measured photometrically as more or less intensive change of colour. Thus, the reduction or the absence of the reaction gives evidence of cytotoxicity of the fungal strain tested. (Hanelt et al., 1994).

The analysis comprised the evaluation of a test sample (Petri dish with the mould on Czapek medium) and a control sample (Petri dish with Czapek medium). The SK were grown in medium containing antibiotics (penicillin and streptomycin, Sigma Aldrich) and calf fetal serum (Sigma Aldrich) in the Hera Cell incubator with carbon dioxide, manufactured by Heraeus (5% CO₂, 37°C, 98% humidity). The number of SK was 2.2 × 10^5. The ranges of testing concentrations were prepared in ratio 1:2 and amounted from 31.251 to 0.061 cm²/ml. The value was expressed in terms of cm²/ml, where the area of the Petri dish from which the moulds were extracted together with the medium, was measured in square centimetres.

The quantitative evaluation of cytotoxicity was performed using a microslide spectrophotometer (Elisa Digiscan reader, Asys Hitech GmbH, Austria) and the programme MikroWin 2000 (Mikrotek Laborsysteme GmbH, Germany). The readings were made at the wave-length of 510 nm. All of the absorption values below 50% of the threshold activity were considered as toxic. So, the borderline toxic concentration was evaluated on the basis of the dilution i.e. the mean inhibitory concentration IC50 was equal to the smallest sample (in cm²/ml) which was toxic to the SK. The cytotoxicity was considered as low (+) when the values were within the range of 31.251–15.625–7.813 [cm²/ml], intermediate (+++) for the values >3.906–1.953–0.977 [cm²/ml], and high (+++) for >0.488–0.244–0.122>0.061. The lack of cytotoxicity was reported when the absorption value exceeded 31.251 [cm²/ml] (Gareis, 1994).

The cytotoxicity was tested in the Department of Physiology and Toxicology, Institute of Experimental Biology at the Casimir the Great University in Bydgoszcz, Poland.

To evaluate differences among the particular groups of fungi, the fungi were divided into four groups. The first group – I (21 strains) comprised fungi belonging to the A. fumigatus species, the second group – II (14 strains) A. niger, the third group – III (13 strains) A. ochraceus, and the fourth group – IV (9 strains) comprised the remaining strains (A. flavus, A. versicolor and A. ustus). Descriptive statistics were used, and the mean values and standard deviations were calculated. To show the differences of cytotoxicity of the species tested ANOVA test was used. As the mean toxicities were different in particular groups, the Tuckey’s post hoc test was used to find out between which groups the differences were significant. The value of p<0.05 was assumed as the borderline of significance (Armitage, 1971).

**Results**

The amounts of fungi and fungal species varied from one room to another. The mean numbers of fungi in the particular wards varied from 5 to 2370 c.f.u.×m⁻³. The fungi were most abundant in the neonatal intensive care unit: the mean number of colonies on a single Petri dish reflected 530 c.f.u.×m⁻³ in the indoor air. The lowest number of fungi was detected in the neonatal intensive care unit: 38%.

The moulds were the most abundant in this environment. The lowest percentage of Aspergillus was isolated in the neonatal intensive care unit: 38%. The moulds were the most abundant in this environment. The lowest percentage of Aspergillus was detected in the chemotherapy ward: 29 c.f.u.m⁻³ (Table I). The dominating genera were Penicillium, Cladosporium and Aspergillus. The highest percentage of Aspergillus was isolated in the neonatal intensive care unit: 38%. The moulds were the most abundant in this environment. The lowest percentage of Aspergillus was detected in air-conditioned intensive therapy ward: 22%. The total number of Aspergillus strains isolated was too high to evaluate cytotoxicity in all of them. The strains chosen to cytotoxicity test originated from three hospital wards because it was assumed that strains from the same ward might be similar genotypically.

Out of the 57 Aspergillus strains tested, 48 (84%) turned out to be cytotoxic. They belonged to the following species: A. fumigatus 19/21, A. niger 8/14, A. ochraceus 12/13, A. flavus 5/5, A. versicolor 3/3,
Cytotoxicity of Aspergillus sp. and a single strain A. ustus. Only nine strains were not cytotoxic (16%). The absorption value >31.25 [cm²/ml] (lack of cytotoxicity) was most frequently found in A. niger (six strains). Twenty one strains tested revealed high cytotoxicity (+++), most often A. fumigatus (11 strains), and A. ochraceus (7 strains). Intermediate cytotoxicity was found in seventeen strains, mainly A. fumigatus. Ten strains revealed low cytotoxicity, mainly A. niger and A. ochraceus (Table II). The ANOVA test revealed differences of the pathogenicity within the particular fungal species (p = 0.03; p<0.05). The least cytotoxic was the Aspergillus niger species: its mean value 32.71 and SD 27.97 were the highest among the fungi tested. The Tuckey’s post hoc test, performed following ANOVA, revealed that the cytotoxicity of A. niger is significantly lower than those of A. fumigatus and A. ochraceus (p<0.05). The cytotoxicity of the other Aspergillus fungi (group IV) did not differ significantly from the three species mentioned above, which may be a result of too small number of fungi in the samples. Moreover, the confidence interval was calculated for each of the species tested; these are presented in Fig. 1.

### Discussion

Moulds can grow mainly in the environment in which humidity exceeds 45%, the temperature is within the range of 5°C–35°C (optimum 18°–27°C), and water activity above 0.8. Such environment is conducive to the production of secondary metabolites – mycotoxins. Penicillium and Aspergillus are dominating mould species in the rooms where water activity is around 0.85 (Jarviss, 2003). The Aspergillus genus is the most pathogenic to living organisms, however, it globally produces less mycotoxins than Stachybotrys even though the former dominates in the environment. This may not be true in all the Aspergillus species, because sterigmatocystein produced by A. versicolor may contribute to 1% of its biomass when water activity is equal to one (Fog Nielsen, 2003). We managed to isolate only three A. versicolor strains and to evaluate their cytotoxicity, which was intermediate in all cases. Such a small number of isolates resulted from the fact that this fungal species is rarely isolated indoor and is present mainly in colder regions such as mountains or polar areas. The species most frequently isolated from indoor air are A. fumigatus and A. niger. Just these two species were most often isolated in our study and examined for cytotoxicity.

It is reported in many papers that A. fumigatus is the most pathogenic Aspergillus species (Bennett, Radiotherapy 130 5 360 39,5 17,5 32% 9% 31% 28% 16 12 Chemotherapy 130 5 130 29 20 29% 8% 29% 34% 20 16 Neonatal ICU 30 5 2370 530 260 38% 18% 32% 12% 12 12 Intensive therapy 48 5 170 40 30 22% 17% 38% 23% 9 8 Table I

<table>
<thead>
<tr>
<th>Ward</th>
<th>Number of samples</th>
<th>Numbers of fungal colonies c.f.u.×m⁻³</th>
<th>Percentage of fungi</th>
<th>Number of Aspergillus strains examined for cytotoxicity</th>
<th>Number of strains with documented cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Mean</td>
<td>Median</td>
<td>A</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>130</td>
<td>5</td>
<td>360</td>
<td>39.5</td>
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<tr>
<td>Chemotherapy</td>
<td>130</td>
<td>5</td>
<td>130</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>Neonatal ICU</td>
<td>30</td>
<td>5</td>
<td>2370</td>
<td>530</td>
<td>260</td>
</tr>
<tr>
<td>Intensive therapy</td>
<td>48</td>
<td>5</td>
<td>170</td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>
| ICU – intensive care unit, A – Aspergillus sp., C – Cladosporium sp., P – Penicillium sp., I– other fungal

Table II

Distribution of fungal species in relation to their cytotoxicity

<table>
<thead>
<tr>
<th>No</th>
<th>Species</th>
<th>N (%)</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Aspergillus fumigatus</td>
<td>21 (37%)</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Aspergillus niger</td>
<td>14 (25%)</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Aspergillus ochraceus</td>
<td>13 (23%)</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Aspergillus flavus</td>
<td>5 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Aspergillus versicolor</td>
<td>3 (5%)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Aspergillus ustus</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>57 (100%)</td>
<td>9</td>
</tr>
</tbody>
</table>

N– Total number of fungi; (+) – low cytotoxicity; (+++) – intermediate cytotoxicity; (++++) – high cytotoxicity

Fig. 1. Intervals of confidence 95% CI and mean value of Aspergillus cytotoxicity estimated as difference between reference level equal to 50 and measured value of IC 50 cm²/ml.

Legend: the squares at the middle represent the mean value of the estimated cytotoxicity, and the vertical lines represent the size of the confidence interval of the estimated cytotoxicity.
This finding was confirmed by Kamei et al. (2002) who analyzed the virulence of *A. fumigatus, A. flavus, A. niger* and *A. terreus* and their influence on murine macrophages. The macrophages treated with 1% *A. fumigatus* filtrate were seriously damaged, up to necrobiosis while the damage caused by similar filtrates of *A. niger* was lighter and that caused by *A. flavus* and *A. terreus* was almost undetectable. Those data are consistent with our findings because *A. fumigatus* was the most frequently highly cytotoxic (+++) as compared with other species, and only two strains out of 21 were not toxic to SK. On the other hand, only eight out of fourteen *A. niger* strains were cytotoxic, but their toxicity was the lowest as compared with other species. All of the *A. flavus* strains were cytotoxic in our study. Only five strains were tested, perhaps too few to come to a conclusion.

The MTT test focused on *in vitro* influence of fungi on living cells is performed with swine, sheep or lamb cell cultures. Stec et al. (2007) investigated the influence of mycotoxins such as aflatoxin B, ochratoxin, patuline, citrinine and zelarenon on various cell cultures. They found out that SK fibroblasts were most sensitive to mycotoxins, however, the intensity of the yellow tetrazolium salt reduction to formazan depended on the kind of toxin. It appears that the choice of SK in our study was appropriate, and it could be expected that the majority of strains isolated in our study might be pathogenic to living organisms. In further trials carried out by Stec et al. (2007) cell cultures responded in different ways to treatment with various mycotoxins. Therefore, it appears essential to determine the kind of mycotoxins produced by highly mycotoxic fungi. It is not always possible. In our previous study (Gniadek and Macura, 2003) performed in 2001 with 21 *A. flavus* strains isolated from the environment at social welfare homes were examined for aflatoxins B₁, B₂, G₁ and G₂ using high performance liquid chromatography – HPLC. Aflatoxins were not detected in any of the strains tested. The strains were not tested using MTT, however we were not sure that they were not pathogenic. The isolated *A. flavus* strains could produce the so-called “masked mycotoxins”, e.g. aflatrem, aspergillic acid, cyclopiazic acid, kojic acid, maltoryzin, paspalicin or highly toxic sterigmatocystine. It should be kept in mind that fungi are able to produce mycotoxins in presence of other fungi (toxic metabolites are released only in a cross-reaction with toxins secreted by other fungi). (Fischer et al., 2000).

Such a conclusion may be confirmed by the findings of Murtoniemi et al. (2005), who examined the influence of cytotoxicity of various fungi (*Stachybotrys chartarum, Streptomyces californicus, A. versicolor* and *Penicillium spinulosum*) isolated from wet plaster bars on murine macrophages. They have shown that some microcultures, after prolonged growth on wet cardboard sheets, were mutually stimulated or caused a synergic growth of cytotoxic fungi (especially *S. chartarum* and *A. versicolor*), but they did not cause inflammatory reaction in the cells tested.

Variable environmental conditions may stimulate fungi to produce mycotoxins, e.g. gliotoxin produced by *A. fumigatus* (Ben-Ami et al., 2009; Kwon-Chung and Sugui, 2009). This substance inhibits T-cell activity, stimulates macrophage apoptosis and is included into pathogenesis of severe allergic rhinitis, bronchial asthma and allergic alveolitis in form of farmer’s lung, baker’s lung as well as allergic broncho-pulmonary alveolitis (ABPA). The study carried out by Watanabe et al. (2004) gives evidence that a good access to oxygen stimulated *A. fumigatus* to produce toxic gliotoxins and increased the general cytotoxicity of the fungi. The conditions in the rooms in our study were conducive to fungal growth: the mean temperature was 24°C, and humidity around 40%.

The indoor environment is an active ecosystem that changes in the course of time and as a result of changing temperature and humidity, and in the presence of other microorganisms. The toxin production by moulds is influenced by those factors and depends also on the fungal culture age, the stage of sporulation and the access to nutrients (Kelman et al., 2004). The examination of the environment of a patient diagnosed with a mycotoxin infection is most often performed after the initial phase of the disease and may not reflect the exposure to harmful substances present in the course of the disease (Gniadek et al., 2005; Hardin et al., 2003). On the basis of the measurements of environmental factors which appeared after the infection, the real exposure at the onset of the disease may be only estimated. Therefore, it is a reasonable prophylactic measure to monitor mycological cleanliness of the environment where immunocompromised patients are present and to evaluate the cytotoxicity of the fungi known as pathogenic.

**Conclusions.** 1. The majority of the fungi *Aspergillus* in the environment of the rooms tested were cytotoxic and most often (p<0.05) were non- *A. niger*. 2. To protect patients from harmful influence of mycotoxins, the immunity status of the patients should be evaluated and the presence of fungi in the environment should be monitored, including their cytotoxicity testing (possible exposure).

**Literature**

Cytotoxicity of Aspergillus sp.


INTRODUCTION

Enzymes produced by thermophilic bacteria have been highlighted for their potential as biocatalysts in biotechnology. The importance of this was referred to the general relationship between enzyme thermostability and the thermophilicity of the host bacterium (Herbert, 1992).

The α-amylase (EC 3.2.1.1) is a well-known endoamylase that hydrolyzes starch by randomly cleaving internal α-1,4-α-glucosidic linkages. The spectrum of α-amylase applications has widely used in many fields, such as starch saccharification, textile, food, brewing, distilling industries, medical and analytical chemistries (Pandey et al., 2000). Despite this, interest in new and improved α-amylase is growing vastly. Therefore, the search for thermostable Ca²⁺ independent α-amylase (Tonkova, 2006) is continuous. Enzymes of the thermophilic actinomycetes Thermoactinomyces species, especially their α-amylase (Ito et al., 2007), have attracted much interest because of their activity at high temperature. The advantages for using thermostable α-amylases in industrial processes include the decreased risk of contamination, the increased diffusion rate and the decreased cost of external cooling. In addition, the stability of biocatalysts is often a limiting factor in the selection of enzymes for industrial applications due to the elevated temperature or extreme pH of many biotechnological processes.

The aim of the investigations was to determine the optimum conditions for production of highly active and industrial stable α-amylase by an Egyptian isolate of the thermophilic actinomycete Thermoactinomyces vulgaris and to investigate the enzyme properties.

EXPERIMENTAL

MATERIALS AND METHODS

T. vulgaris strain. T. vulgaris strain was isolated from fertile soil samples collected from Egypt at 50°C and identified according to Bergey’s Manual of Systematic Bacteriology (Lacey and Cross, 1989). The culture was maintained on Czapek-yeast-casein (CYC) agar slants.

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Abstract

Optimizing production of α-amylase production by Thermoactinomyces vulgaris isolated from Egyptian soil was studied. The optimum incubation period, temperature and initial pH of medium for organism growth and enzyme yield were around 24 h, 55°C and 7.0, respectively. Maximum α-amylase activity was observed in a medium containing starch as carbon source. The other tested carbohydrates (cellulose, glucose, galactose, xylose, arabinose, lactose and maltose) inhibited the enzyme production. Adding tryptone as a nitrogen source exhibited a maximum activity of α-amylase. Bactopeptone and yeast extract gave also high activity comparing to the other nitrogen sources (NH₄Cl, NH₄NO₃, NaNO₃, KNO₃, CH₃CO₂NH₄). Electrophoresis profile of the produced two α-amylase isozymes indicated that the same pattern at about 135–145 kDa under different conditions. The optimum pH and temperature of the enzyme activity were 8.0 and 60°C, respectively and enzyme was stable at 50°C over 6 hours. The enzyme was significantly inhibited by the addition of metal ions (Na⁺, Co²⁺ and Ca²⁺) whereas Cl⁻ seemed to act as activator. The enzyme was not affected by 0.1 mM EDTA while higher concentration (10 mM EDTA) totally inactivated the enzyme.

Key words: Thermoactinomyces vulgaris, high molecular weight α-amylase

Production and Partial Characterization of High Molecular Weight Extracellular α-amylase from Thermoactinomyces vulgaris Isolated from Egyptian Soil

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Production of $\alpha$-amylase. The organism was grown in conical flasks containing starch-nitrate medium (Waksman, 1959) as a basal medium with pH adjusted to 7.5. The medium (20 ml taken in 250 ml Erlenmeyer flasks) was inoculated with 1 ml of spore suspension of pure colonies of the organism and incubated at 50°C with shaking (150 rpm) for 72 hours. The initial pH of the medium (4.0–10.0), temperature of incubation (30–60°C), and incubation period (12–96 h) were tested for $\alpha$-amylase production. Different carbon sources (1%) and nitrogen sources (equimolecular nitrogen amounts equivalent to the nitrogen content of the basal medium) were used for optimization of nutritional factors. The various tested carbon sources were: starch, cellulose, glucose, galactose, xylose, arabinose, lactose and maltose. The nitrogen sources were included NH$_4$Cl, NH$_4$NO$_3$, NaNO$_3$, KNO$_3$, ammonium acetate, bactopeptone, yeast extract and tryptone were tested.

On the compilation of the previous experiments, the cell-free enzyme supernatant was obtained by centrifugation at 8000×g for 20 min. Experiments were carried out in triplicate and the results were treated statistically and standard errors are shown.

$\alpha$-Amylase assay. The amylase assay was based on the reduction in blue colour intensity resulting from starch hydrolysis (Palanivelu, 2001). The reaction mixture consisted of 0.4 ml of diluted enzyme, 0.5 ml of 0.1% soluble starch and 1 ml of phosphate buffer (0.1 M, pH 7) was incubated at 50°C for 10 minutes. The reaction was stopped by adding 0.5 ml of 0.1 N HCl and the colour was developed by adding 0.5 ml of (2% KI in 0.2% I$_2$) solution. The optical density (OD) of the blue colour solution was determined using a Unico 7200 SERIES spectrophotometer at 690 nm. One unit of enzyme activity is defined as the quantity of enzyme that caused 20% reduction of blue colour intensity of starch iodine solution at reaction incubation temperature in 1 min per ml.

Intracellular protein content of $T$. vulgaris. The harvested mycelia of $T$. vulgaris strain were used for determination of intracellular protein. Washed pellets were dissolved in 20 ml of NaOH (1 M), and boiled for 20 minutes. Dilution of clarified solution was used to determine the intracellular protein concentration using Bradford method (1976). Bovine serum albumin was used as standard.

Physicochemical properties of $\alpha$-amylase. Enzyme activity at various temperatures and pH was studied by incubating reaction mixtures at different temperatures (30–80°C) and Tris-HCl buffer (pH 4.0–9.0). Enzyme stability at various temperatures was also studied by pre-incubating cell-free supernatants for different time (1–6 h) at various temperatures (50°C–80°C). The effect of metal salts (NaCl, CoCl$_2$ and CaCl$_2$) and EDTA on activity was determined by adding of different concentrations of each salt to the standard assay. Activities were expressed as a percentage of the maximal activity.

Ammonium sulphate precipitation of the enzyme. The supernatant of culture was brought to 70% ammonium sulphate saturation in an ice bath. The precipitated protein was collected by centrifugation at 3000×g at 4°C and dissolved in 1–2 pellet volumes of phosphate buffer (0.1 M, pH 7.0). The enzyme solution was dialyzed overnight at 4°C against the same buffer then concentrated over sucrose bed. The final enzyme solution was taken for polyacrylamide gel electrophoresis (PAGE).

Electrophoresis and molecular weight determination. Nondenaturing PAGE was carried out by omitting SDS from the method of Laemmli (1970) with 10% polyacrylamide. The reference pre-stained protein marker (Molecular weights 10 to 170 kDa, SM 0671, Fermentas) was used. Amylase activity of proteins was determined according to Garcia-Gonzalez et al., (1991) by incubating the gels at 50°C for 20 min in 0.2 M phosphate buffer (pH 7.0) containing 2% starch and then immersing in staining solution (KI 13 g/l and I$_2$, 6 g/l). The gel was destained with distilled water. The stain was stable for only a few minutes.

Statistical analysis. Data were statistically analyzed for variance and the least significant difference (LSD at 0.01 level) using one-way analysis of variance (ANOVA). A software system SPSS version 15 was used.

Results

The production of $\alpha$-amylase by $T$. vulgaris increased significantly during the growth of the organism, with the maximum production after 24 hours (1.03 ± 0.09 U/ml). After 36 hours the activity was reduced rapidly by 72.8% (Fig. 1). Although there was
Extracellular α-amylase from *Thermoactinomyces vulgaris*

There was a significant increase in the production after 72 hours, the α-amylase profile; on active PAGE didn’t differ from that at 24 hours (Fig. 2) as they produce two α-amylase isozymes with approximate molecular weight 135–145 kDa.

The optimum temperatures for the production of α-amylase were in the range 45°C to 55°C with non-significant difference in this range. Below 45°C, the organism could grow weakly but no activity could be detected (Fig. 3).

Maximum production occurred in the pH range of 6.0 to 7.0 (3.5 U/ml), increasing the pH above 7.0 induced a significant decrease in the yield. At pH 10.0 and below pH 6, the production of the enzyme was completely inhibited (Fig. 4).

*T. vulgaris* was able to grow well using different carbon sources (1% w/v), namely, starch, cellulose, glucose, galactose, xylose, arabinose, lactose and maltose. However, the used carbon sources other than starch induced an extremely significant decrease in the enzyme yield (Table I). Although different carbon sources affected the quantity of the enzyme production, it didn’t affect its isozymal pattern as they produced the same two α-amylase isozymes of approximate molecular weight 135–145 kDa (data not shown).

Tryptone, bactopeptone, yeast extract and NH₄Cl caused a highly significant increasing in enzyme production comparing with KNO₃ of the basal medium (Table II). The highest production of α-amylase (93.81 ± 0.20 U/ml) was recorded with tryptone and the lowest (1.86–0.16) was recorded with NaNO₃. The different used nitrogen sources (NH₄Cl, NH₄NO₃, NaNO₃, KNO₃, ammonium acetate, bactopeptone, yeast

<table>
<thead>
<tr>
<th>Carbon sources (1.0%) (w/v)</th>
<th>Amylase (U/ml)</th>
<th>Intracellular protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>3.43 ± 0.02</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.04 ± 0.02</td>
<td>0.78 ± 0.02</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.02 ± 0.01</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.10 ± 0.06</td>
<td>0.76 ± 0.02</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.07 ± 0.02</td>
<td>0.76 ± 0.01</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.11 ± 0.02</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.36 ± 0.05</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.05 ± 0.03</td>
<td>0.52 ± 0.01</td>
</tr>
</tbody>
</table>

± standard error
extract and tryptone) didn’t have any effect on the enzyme electrophoretic profile. The \( \alpha \)-amylase profile had the same previous pattern of two \( \alpha \)-amylase isozymes at about 135–145 kDa (data not shown).

**Physicochemical properties of \( \alpha \)-amylase.** The \( \alpha \)-amylase activity couldn’t be detected at pH 4.0 but it increased significantly with the increase of the pH until it reaches its optimum point at pH 8.0 (Fig. 5). The optimum activity occurred at temperature range between 50°C and 60°C, with an optimum point of 60°C (Fig. 5). The minimum level of the activity (54.4 ± 2.3 U/ml) was occurred at 80°C. The enzyme was stable at 50°C retaining about 80% of its activity over 6 hours incubation period, but at higher temperature the activity of the enzyme declined (Fig. 6). At 60°C and 70°C, the enzyme has a half-life of about one hour. However, at 80°C it retained more than 20% of its activity up to 4 hours. At 90°C the enzyme lost its activity rapidly.

### Table II

<table>
<thead>
<tr>
<th>Nitrogen sources</th>
<th>Final PH</th>
<th>Amylase (U/ml)</th>
<th>Intracellular protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium acetate</td>
<td>6.71</td>
<td>1.06 ± 0.15</td>
<td>6.46 ± 0.11</td>
</tr>
<tr>
<td>Tryptone</td>
<td>6.75</td>
<td>1.10 ± 0.12</td>
<td>93.81 ± 0.20</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>6.87</td>
<td>0.72 ± 0.03</td>
<td>45.29 ± 0.08</td>
</tr>
<tr>
<td>Bactopeptone</td>
<td>6.64</td>
<td>0.93 ± 0.07</td>
<td>63.05 ± 0.63</td>
</tr>
<tr>
<td>NH(_4)Cl</td>
<td>6.46</td>
<td>0.66 ± 0.06</td>
<td>21.56 ± 3.64</td>
</tr>
<tr>
<td>NH(_4)NO(_3)</td>
<td>6.40</td>
<td>0.95 ± 0.03</td>
<td>6.34 ± 0.17</td>
</tr>
<tr>
<td>NaNO(_3)</td>
<td>6.81</td>
<td>0.80 ± 0.01</td>
<td>1.86 ± 0.16</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>6.73</td>
<td>0.53 ± 0.02</td>
<td>3.54 ± 0.01</td>
</tr>
</tbody>
</table>

± standard error

The enzyme was significantly influenced by the different metal salts including NaCl, CoCl\(_2\), and CaCl\(_2\) (Fig. 7). CoCl\(_2\) induced significant inhibition of the enzyme activity. An amount of 1 mM of NaCl and CaCl\(_2\) caused extremely significant decrease in enzyme activity.

### Table III

<table>
<thead>
<tr>
<th>Concentration of EDTA (mM)</th>
<th>Amylase relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>99.62 ± 4.46</td>
</tr>
<tr>
<td>0.01</td>
<td>104.36 ± 1.70</td>
</tr>
<tr>
<td>0.1</td>
<td>108.85 ± 0.10</td>
</tr>
<tr>
<td>0.5</td>
<td>53.59 ± 0.52</td>
</tr>
<tr>
<td>1</td>
<td>18.81 ± 5.78</td>
</tr>
<tr>
<td>5</td>
<td>1.12 ± 0.70</td>
</tr>
<tr>
<td>10</td>
<td>0.00 ± 0</td>
</tr>
</tbody>
</table>

± standard error

The enzyme activities are represented relative to the control activity.

---

**Fig. 5.** Effect of temperature and pH on \( \alpha \)-amylase activity. The enzyme activities are represented relative to the maximal value.

**Fig. 6.** Effect of temperature on the stability of \( \alpha \)-amylase. The enzyme activities are represented relative to the maximal value.

**Fig. 7.** Effect of various metal salts concentrations on \( \alpha \)-amylase activity. The enzyme activities are represented relative to the control activity.
activity by about 80% and 70% respectively. However, increasing of their concentration up to 10 mM of NaCl and CaCl₂ resulted in retaining 60.8% and 63.4% of its activity respectively. The enzyme activity was not affected by 0.001, 0.01 and 0.1 mM EDTA but it was totally inactivated by 10 mM EDTA (Table III). On the other hand, 0.5 mM EDTA was found to decrease the activity to 53.6% comparing with the control (96.6 U/ml⁻¹).

Discussion

α-Amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of α-amylase, microbial sources are used for the industrial production. This is due to their advantages such as cost effectiveness, consistency, less time and space required for production, and ease of process modification and optimization (Lonsane and Ramesh, 1990). Growth expressed as intracellular protein and α-amylase production by the thermophilic actinomycete *Thermoactinomyces vulgaris* reached maximum values after 24 hrs, after which, the activity was reduced rapidly by 72.8%. This might be corresponding to the rapid autolysis of *Thermoactinomyces* species (Lacey, 1971). However, it increased significantly at 60 and 72 hours. After 72 hrs, the production decreased gradually and reached its minimum recorded level at 96 hours. As the isozymal pattern was the same at 24 hrs and 72 hrs, the observed peaking and troughing of the production can be attributed to differential inhibition by products of substrate hydrolysis rather than the isozymes down or over expression. In support, studies on both gluco-amylase and α-amylase indicate that inhibition does not occur below a critical concentration of product (Wang et al., 2006). These results indicated that the production of extracellular α-amylase by *T. vulgaris* was growth associated and this is in agreement with other investigators (Kuo and Hartman, 1966; Shimizu et al., 1978; Murthy et al., 2009; Asoodeh et al., 2010).

The influence of temperature on amylase production is related to the growth of the organism. The present results revealed an optimum yield of α-amylase at 45°C to 55°C. In spite of the relative good growth of the organism at 40°C, α-amylase activity couldn’t be detected. This referred to the action of protease; that is rapidly inactivated at higher temperature (Behnke et al., 1982), which suppresses the amylolytic activity.

The pH change of the growth medium not only affected the growth and α-amylase secretion of *T. vulgaris* but also influence the enzyme stability in the medium. Therefore, the difference of the final pH of the medium from pH 10.0 to pH 6.0 could explain the complete inhibition of the enzyme activity at pH 10.0 in contrast to the optimum production at pH 6.0 in spite of the same ability of the organism to grow.

The results confirms the inducibility nature of the α-amylase when different carbon sources were used and compared. In support, starch is known to induce amylase production in different bacterial strains (Aiyer, 2004; Ryan et al., 2006; Asoodeh et al., 2010). The α-amylase production is also appeared to be subjected to catabolite repression by maltose and glucose, like most other inducible enzymes that are affected by substrate hydrolytic products (Bhella and Altosaar, 1985; Morkeberg et al., 1995). Other carbon sources have been found to be strongly repressive although they supported good growth.

Among nitrogen sources, organic nitrogen sources have been preferred for the production of α-amylase, with ammonium acetate as an exception. It was recorded that organic nitrogen sources supported maximum α-amylase production by various bacteria and fungi due to their high nutritional amino acids and vitamins content (Gupta et al., 2003). The role of amino acids and vitamins in enhancing the α-amylase production in different microorganisms have been reported to be highly variable (Gupta et al., 2003). Tryptone was the best nitrogen source that increased the productivity of α-amylase by 26.5 fold comparing to KNO₃. Ammonium chloride induced a significant increase in the enzyme yield that is higher than that of the ammonium acetate. Similar effect of ammonium chloride was obtained for *B. subtilis* DM-03 (Das et al., 2004). Although the different nitrogen sources had a variable effect on α-amylase activity, they did not produce different isozymal profile. This indicate that they have no effect on the isozymal α-amylase over expression.

Most raw starch degrading enzymes had optimum pH in the acidic to neutral range (Pandey et al., 2000; Sun et al., 2010). In the present work, α-amylase from *T. vulgaris* showed a pH activity profile with a flat top which retaining more than 75% of the enzyme activity in the pH range 5.0–9.0, despite it was completely inhibited at pH 4.0. This pH profile could be attributed to the limitation of the enzyme catalysis by protonation of the nucleophile at low pH and by deprotonation of the hydrogen donor at high pH values (Nielsen et al., 2001). At reaction temperature of 60°C, the enzyme expresses maximum activity whereas it showed less than 50% of its activity at 30°C. This reduced activity might be attributed to the reduced molecular flexibility of the thermophilic protein under mesophilic conditions. Although the enzyme showed only 4% of its activity at 80°C, it showed more than 20% of its activity at 50°C after being kept at 80°C for 4 hrs indicating inhibition of the enzyme catalysis.
at 80°C rather than inactivation of the enzyme. In general, α-amylase from *T. vulgaris*, in the present work, is fairly stable at 50°C over 6 hrs and with a half-life of about one hour at both 60°C and 70°C.

It is proposed that α-amylases belong to a new class of metallo-enzymes characterized by a prosthetic group, i.e., an alkaline-earth metal rather than a transition element, and which plays primarily a structural role (Prakash and Jaiswal, 2010). At different concentrations of CaCl₂ and NaCl, a significant inhibition in the enzyme activity was recorded. The Co²⁺ effect was explained by Leveque *et al.* (2000) to be a result of competition between the exogenous cations and the protein-associated cation. In spite of the important role of both calcium and sodium ions to retain the structure and function of α-amylases (Prakash and Jaiswal, 2010), α-amylase in the present study was strongly inhibited by both 1 mM CaCl₂ and NaCl. Similar inhibitory effect of CaCl₂ was reported to amylase of *Aspergillus oryzae* El 212 (Kundu *et al.*, 1973). Calcium independent α-amylase is suitable for the manufacture of fructose syrup, where Ca²⁺ is an inhibitor of glucose isomerase (Tonkova, 2006).

Increasing the concentration of CaCl₂ and NaCl was found to retain the enzyme activity, assuming that Cl⁻ ion has a stabilizing role. Chloride ions have been found mainly in the active site of mammalian α-amylases, which have been shown to enhance the catalytic efficiency of the enzyme (Prakash and Jaiswal, 2010). In accordance with retaining the enzyme activity at high concentration of CaCl₂ and NaCl, the enzyme yield when using ammonium chloride was found to be 3.3 fold of that using other sources of ammonium i.e., NH₄NO₃ and ammonium acetate. Regarding to the effect of EDTA, the enzyme retained almost 100% activity when 0.1 mM EDTA was added to the reaction mixture. But 0.5 mM EDTA inhibited the enzyme activity retaining only 53% of its activity. The inhibitory effect of EDTA was also documented by many studies (Gupta *et al.*, 2003). Although the molecular weights of microbial α-amylases are usually range between 50 to 60 kDa (Vihinen and Mantсалa, 1989), the present result revealed a highly molecular weight of two α-amylase isozymes (135–145 kDa). In support, *Thermoactinomyces vulgaris* R-47 was reported to produce two α-amylases, TVAI and TVAII with molecular weights of 71 kDa and 67.5 kDa respectively (Ohtaki *et al.*, 2003). However, molecular weight of some α-amylases was found to rise owing to carbohydrate moieties (Gupta *et al.*, 2003). In conclusion, the present results indicated a new active highly molecular weight, thermostable and calcium independent α-amylase of *T. vulgaris* which could be of importance for the starch-processing industries. Further work is in progress to purify the α-amylase of *T. vulgaris* and characterize the purified enzyme.

**Literature**


Extracellular α-amylase from *Thermoactinomyces vulgaris*


**Halomonas sp. nov., an EPA-Producing Mesophilic Marine Isolate from the Indian Ocean**

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**Abstract**

Marine samples from the Indian Ocean were used to isolate and characterize the organisms with respect to their fatty acid profiles. Six mesophilic isolates (MBRI 6, MBRI 8, MBRI 9, MBRI 10, MBRI 12 and MBRI 13) were obtained from three different water samples. They were i) Gram-negative, ii) catalase positive, iii) produced acid from glucose and maltose, iv) tolerated 5 to 15% NaCl v) except MBRI 9, showed pH tolerance in the range of 5.0 to 9.0 with optimum pH 7.0 to 8.0 v) grew well at 30°C and were able to grow in the range of 15 to 45°C. EPA, an essential omega-3 fatty acid, was produced by these isolates in the range of 12 to 60% at 30°C. MBRI 12 was found to be a potential source as it produced 60% EPA. This isolate was further identified by partial 16S rDNA sequencing and phylogenetic analysis revealed that the strain belonged to *Gammaproteobacteria* and was closely related to *Halomonas boliviensis* (96% sequence similarity, 570 bp). Thus a new genus of *Halomonas* may be included in earlier reported EPA- producing prokaryotic genera affiliated to the *Gammaproteobacteria*.

**Key words:** Gammaproteobacteria, Halomonas boliviensis, Alpha-linoleic acid, nutraceuticals, polyunsaturated fatty acid

**Introduction**

Long chain polyunsaturated fatty acids (LC-PUFAs) are essential components of membrane lipids of various organisms (Berge and Barnathan, 2005). They are well documented for their beneficial physiological effects in human health including i) lowering of plasma cholesterol and triglycerols ii) prevention of certain cardiovascular diseases (atherosclerosis and thrombosis) and iii) reducing the risk of breast, colon and pancreatic cancer (Robert et al., 2009). Alpha-linoleic acid (ALA), ecosapenthaenoic acid (EPA) and docosahexaenoic acid (DHA) are the major LC-PUFAs of general nutritional importance, suggested by numerous nutritional bodies (Simopoulos et al., 1999). Although the recommended ratio of dietary omega-6 and omega-3 fatty acids is 5:1 (Sargent, 1997), it has shifted heavily towards omega-6 acids in the current western diet and by some estimate, is up to 30-fold too high (Simopoulos, 1999). The main reason being an increase in the consumption of vegetable oils rich in omega-6 fatty acids. Since omega-6 and omega-3 fatty acids are not interconvertible in the human body, the ratio of linoleic acid (LA)/ALA in our diet influences the ratio of omega-6/omega-3 fatty acids LC-PUFAs. To correct this imbalance, the needed omega-3 LC-PUFAs, can be obtained in our diet from external sources such as fish oil.

The current commercial sources of EPA and DHA are restricted to fish and algal-derived oils. Problems exist with both of these sources. Commercial fish stocks are likely to decline in the future, since the demand for fish oil in the aquaculture industry alone is estimated to exceed the supply by 2010 due to increase in global population (Meyers and Worm, 2003). Besides the concern for the presence of contaminants, such as mercury and polychlorinated biphenyls, in some fish oils, makes it evident that alternative sources of LC-PUFAs must be found (Jacobs et al., 2004). Algal-derived oils require a relatively high investment in technology compared to bacterial fermentation, although bacteria contain a lower proportion of lipid (Nichols et al., 1996). A key advantage of bacterial PUFA production is that only a single PUFA is produced, rather than the complex mixture yielded from fish or algal oils (Russell and Nichols, 1999). Thus bacterial sources of PUFA remove the expense * Corresponding author: R. Bhadekar, phone: 020-24365713; fax: 91-20-24379013; e-mail: neeta.bhadekar@gmail.com
of preparative purification in the production of high-purity PUFA oils. In addition to their potential use as “cell factories” bacteria in particular offer the biotechnological opportunity to study the structure and regulation of the genes and enzymes responsible for PUFA production.

Numerous bacterial species of marine origin producing PUFAs are particularly prevalent in high-pressure, low temperature and deep-sea habitats (Yano et al., 1997). This is an important adaptation for countering the effects of hydrostatic pressure and low temperature on fluidity or phase of membrane lipid (Allen and Bartlett, 2002). Halophilic organisms are also known to be good sources of different bioactive compounds (Margesin and Schinner, 2001). Recently we have reported halotolerant organisms from different food samples (Jadhav et al., 2010a) as well as from wall scrapings of historical building in India (Jadhav et al., 2010b). They were novel owing to their abilities to fix atmospheric nitrogen and produce industrially important enzymes.

Considering the significance of PUFA in human health and limitations in using fish oils, microorganisms prove the best suitable alternative. However use of psychrophilic or piezophilic organisms producing EPA, necessitates the need of low temperature and high pressure facilities (Gentile et al., 2003). Hence use of organisms producing these nutraceuticals at room temperature will be efficient and reasonably priced. The present work was aimed at isolation of marine organisms from the Indian Ocean and characterizing them with respect to their fatty acid profiles. Being mesophilic in nature, high PUFA producing isolates could be conveniently used for pilot scale production and for further scale-up. To our knowledge this is the first report of a high EPA-producing marine isolate from the Indian Ocean.

**Experimental**

**Material and Methods**

**Sampling.** Water samples were collected during summer 2008 from different locations in the Indian Ocean (Table I). All chemicals were procured from Himedia and Merck, India. All were of A.R. grade.

<table>
<thead>
<tr>
<th>Location</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Depth (m)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Salinity</th>
<th>Conductivity (ms)</th>
<th>D.O. (mg/l)</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>6NT/LT/BOT</td>
<td>18o48”52”N</td>
<td>72o46’30”E</td>
<td>13</td>
<td>8.02</td>
<td>30.4</td>
<td>71.61</td>
<td>974.7</td>
<td>3.98</td>
<td>MBRI 9, MBRI 10 and MBRI 12</td>
</tr>
<tr>
<td>2NT/LT/BOT</td>
<td>15o33.01”6”N</td>
<td>73o56.58”E</td>
<td>9.5</td>
<td>7.62</td>
<td>31.8</td>
<td>27.05</td>
<td>412.4</td>
<td>4.72</td>
<td>MBRI 8</td>
</tr>
<tr>
<td>2ST/LT/BOT</td>
<td>15o33.01”6”N</td>
<td>73o56.58”E</td>
<td>11</td>
<td>7.62</td>
<td>31.8</td>
<td>27.05</td>
<td>412.4</td>
<td>4.72</td>
<td>MBRI 6 and MBRI 13</td>
</tr>
</tbody>
</table>

**Isolation and characterization of microorganisms.** 10 ml of each individual sample were inoculated in 100 ml Marine Salt Medium (MSM) (Composition per litre: 81.0 g NaCl, 10.0 g yeast extract, 9.6 g MgSO_4_, 7.0 g MgCl_2, 5.0 g protease peptone no 3, 2.0 g KCl, 1.0 g glucose, 0.36 g CaCl_2, 0.06 g NaHCO_3 and 0.026 g NaBr with pH adjusted to 7.0±0.2) and incubated at 30°C, 120 rpm for 48 hrs. After 48 hrs, 0.1 ml of each sample was spread on MSM agar plates and incubated for another 48 hrs. The isolated colonies were maintained on MSM agar slants.

The isolates were characterized morphologically, physiologically and biochemically. Acid production was studied by using different sugars (glucose, fructose, sucrose, lactose, mannitol and maltose). They were also studied qualitatively for their ability to secrete extracellular enzymes (amylase, catalase, urease, protease, and gelatinase) (Collee et al., 1989).

MSM broth supplemented with various concentrations of NaCl (ranging from 1–25%) was used to examine salt-tolerance of the isolates. The cultures were incubated at 30°C, 120 rpm for 24 hrs and cell growth was determined by measuring the absorbance at 660 nm. Temperature tolerance was examined by incubating the cultures at temperatures 15°C, 30°C and 45°C for 24 hrs. Further these isolates were screened for pH tolerance in MSM broth adjusted to pH 5.0–11.0. The media at different pH were inoculated with overnight grown inoculum (10^7 cells/ml). Cell growth was determined by measuring the absorbance at 660 nm.

**Preparation of fatty acid methyl esters (FAMES).** 10% inoculum of all the isolates was used to inoculate MSM and incubated at 30°C in an orbital shaker (Remi, India) at 120 rpm. Cells were harvested after 24 hrs by centrifugation (Plastocraft, India) at 10,000 rpm for 12 mins. Following centrifugation, the supernatant was discarded, the cell pellet resuspended in 1.0% NaCl (w/v), and recentrifuged. Each bacterial culture tube was capped and stored at 4°C. Cells were reweighed, to which a fresh solution of the transesterification reaction mix (methanolic HCl (0.6 N) 4 ml) was added in the tubes (Carrapiso and Garcia, 2000). The tubes were capped tightly and the solutions were vortexed for 5 s to 10 s and heated in an 80°C ± 2°C water bath for 2 hrs. The tubes were then cooled quickly in ice.
The resultant FAMES were extracted twice by adding 2 volumes of hexane and then 1 volume of hexane by centrifugation at 5000 rpm for 15 mins. The upper phase of hexane layer was separated and stored for gas chromatography analysis.

**Gas chromatography analysis of bacterial extracts.** Analyses of the FAMES were performed with a Chemito GC 1000 equipped with a 50 m × 0.25 mm internal diameter cross-linked methyl silicone fused-silica CP – SIL 88 capillary column and flame ionization detector. Samples were injected at 100°C in the split mode. After 5 mins the oven was temperature-programmed from 100°C to 198°C at the rate of 1.5°C min⁻¹ and hold for 9 mins. Nitrogen was used as a carrier gas, and the injector and detector were maintained at 225 and 250°C respectively. Peak areas were quantified using chromatography software (IRIS 32, India).

**16S rDNA sequencing.** The isolate producing high amount of PUFA was used for identification by 16S rDNA sequencing. The genomic DNA was isolated as described by Ausubel *et al.* (1987). The PCR assay was performed using Applied Biosystems, model 9800 with 1.5 µl of DNA extract in a total volume of 25 µl. The PCR master mixture contained 2.5 µl of 10X PCR reaction buffer (with 1.5 M MgCl₂), 2.5 µl of 2 mM dNTPs, 1.25 µl of 10 pm/µl of each oligonucleotide primers 27f (5’CCAGAGTTTGATCGTGGCTCAG3’), 1488r (5’CGGTTACCTTGTTACGA), 10X PCR reaction buffer (with 1.5 M MgCl₂), 2.5 µl of 2 mM dNTPs, 1.25 µl of 10 pm/µl of each oligonucleotide primers 27f (5’CCAGAGTTTGATCGTGGCTCAG3’), 1488r (5’CGGTTACCTTGTTACGA). It began with an initial denaturation at 98°C for 1 min, followed by 35 cycles of PCR consisting of denaturation at 94°C for 10 sec, annealing at 50°C for 10 sec and extension at 68°C for 4 min.

The samples were purified using standard protocols described by Applied Biosystems, Foster City, USA. To this, 10 µl of Hi-Di formamide was added and vortexed briefly. The DNA was denatured by incubating at 95°C for 3 min, kept on ice for 5–10 min and was sequenced in a 3730 DNA analyzer (Applied Biosystems) following manufacturer’s instructions.

**Nucleotide sequence accession number.** The partial 16S rDNA sequence generated in this study was deposited in Gen Bank + EMBL + DDBJ + PDB under the accession number GU593323. Sequence was compared with the compilation of 16S rDNA genes available in the Gen Bank + EMBL + DDBJ + PDB library by BLASTN 2.2.17 searching.

**Results and Discussion**

**Isolation and characterization.** Total six bacterial isolates were isolated from the three samples of Indian Ocean (Table I). The isolates were grown and maintained on MSM. They were named as MBRI 6, MBRI 8, MBRI 9, MBRI 10, MBRI 12 and MBRI 13. All 6 MBRI isolates were characterized morphologically, biochemically and physiologically. They i) were Gram-negative ii) produced catalase, iii) produced acid from glucose and maltose, iv) tolerated 5 to 15% of salt, v) except MBRI 9, showed pH tolerance in the range of 5.0–9.0 with optimum pH of 7.0–8.0, vi) grew well at 35°C and vii) were able to grow in the range of 15 to 45°C. MBRI 6, MBRI 8, MBRI 9 and MBRI 10 produced acid from fructose while MBRI 6, MBRI 8 and MBRI 13 used lactose. Only MBRI 11 was able to utilize sucrose as carbon source. MBRI 10 and MBRI 12 could grow on citrate as carbon source. MBRI 9 tolerated a wide pH range of 6.0–9.0.

**Fatty acid profile.** All six isolates were analysed for their fatty acids profiles (Table II). The results indicated that total saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids were in the range of 21 to 100%, 11 to 30% and 1 to 62% respectively. Interestingly, MBRI 10 did not show presence of unsaturated fatty acids. Linoleic acid was the only omega-6 fatty acid detected in MBRI 8, MBRI 9 and MBRI 12. It was detected in the range of 1 to 6%, while omega-3 fatty acids were ALA and EPA (0.5 to 16% and 12.0 to 60%, respectively). Arachidonic acid and docosahexaenoic acid were not detected in any of the isolates. Trans-fatty acids were also not detected in these isolates. So far, EPA has mainly been reported to occur in eukaryotes and some pezizophilic or psychrophilic microorganisms (Freese *et al.*, 2009). Bacteria are known to alter the composition of their phospholipid fatty acids side-chains in order to maintain appropriate membrane organization and function (Suutari and Laakso, 1994). At low temperatures or high pressures, the content of unsaturated fatty acids often increases with a concomitant decline in saturated fatty acids (Allen *et al.*, 1999). Polyunsaturated fatty acids (PUFAs), such as EPA and DHA, are particularly effective in the adjustment of membrane fluidity due to their low melting points (Hazel, 1995). Hence several EPA producing marine organisms isolated
were psychophilic and peizophilic from polar regions and deep sea, as mentioned earlier. However the recent isolation of mesophilic EPA-producing *Shewanella* species from a temperate estuary (Skerratt et al., 2002) and from shallow seawater samples (Frolova et al., 2005; Freese et al., 2008) suggested that EPA may not be restricted to psychrophiles and piezophiles.

Figure 1 shows the comparison of % EPA produced by our isolates with that of microalgae and mesophilic bacterial cultures. Vazhappilly and Chen (1998) have reported up to 34.2% EPA in various microalgae at 25°C. However recovery of EPA from these sources is difficult along with poor yields. Freese et al. (2009) have documented up to 1.2% EPA in various organisms at 30°C (Fig. 1). Different sp. of *Shewanella* were reported to produce EPA up to 6.5% at 28°C (Ivanova et al., 2003; Ivanova et al., 2004; Hirota et al., 2005), probably indicating that temperature sensitive enzymes were involved in biosynthesis. Thus our isolates appear novel owing to their ability to produce high EPA at 30°C. Also these studies contradict the notion that only barophilic or cold-adapted species are able to produce significant levels of PUFAs such as EPA.

Oleaginous fungi are also known to store large amounts of lipid, mainly triglycerols, and *Mortierella* spp. are noteworthy for the n-3 and n-6 PUFA contents of their stored lipid. Typically up to 40% of the fungal dry weight may be triacylglycerol in which the acyl chains are up to 15% EPA or 55% AA (Singh and Ward, 1997). Our results are comparable to these observations besides the advantage of rapid cultivation and easy recovery.

Branched chain fatty acids (BCFAs) have repeatedly been reported to promote cold adaptations (Chattopadhyay and Jagannadham, 2001). Our results showing comparatively low levels (1 to 34%) of BCFAs in all the isolates except MBRI 10 support these observations. However in some other bacteria no clear changes in BCFAs with varying growth temperature were observed (Nichols et al., 2002).

**Phylogeny.** MBRI 12, the highest EPA producing isolate was identified using partial 16S rDNA sequencing. The sequence was deposited in EMBL+Genbank under accession number GU593323. BLAST analysis of partial 16S rDNA sequence revealed that the strain belonged to *Gammaproteobacteria* and was closely related to *Halomonas boliviensis* (96% sequence similarity, 570 bp). All so far known EPA-producing prokaryotes affiliate with only a few genera within two bacterial phyla: The *Gammaproteobacteria* (e.g. genera *Shewanella*, *Mortiella*, *Colwellia*, *Alteromonas*,

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**Table II**

Fatty acid profile of MBRI isolates

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>MBRI 6</th>
<th>MBRI 8</th>
<th>MBRI 9</th>
<th>MBRI 10</th>
<th>MBRI 12</th>
<th>MBRI 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 4:0</td>
<td>0.2</td>
<td>7.7</td>
<td>11.6</td>
<td>21.9</td>
<td>6.8</td>
<td>19.3</td>
</tr>
<tr>
<td>C 6:0</td>
<td>0.4</td>
<td>2.2</td>
<td>2.7</td>
<td>8.4</td>
<td>2.0</td>
<td>7.3</td>
</tr>
<tr>
<td>C 8:0</td>
<td>2.2</td>
<td>4.5</td>
<td>8.3</td>
<td>2.5</td>
<td>N.D.</td>
<td>2.4</td>
</tr>
<tr>
<td>C 10:0</td>
<td>0.5</td>
<td>0.7</td>
<td>3.8</td>
<td>5.1</td>
<td>1.2</td>
<td>N.D.</td>
</tr>
<tr>
<td>C 11:0</td>
<td>0.7</td>
<td>0.8</td>
<td>1.2</td>
<td>6.1</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>C 12:0</td>
<td>0.8</td>
<td>1.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.8</td>
</tr>
<tr>
<td>C 13:0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>C 14:0</td>
<td>18.1</td>
<td>34.3</td>
<td>8.7</td>
<td>14.1</td>
<td>1.7</td>
<td>4.1</td>
</tr>
<tr>
<td>C 15:0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.9</td>
</tr>
<tr>
<td>C 16:0</td>
<td>25.1</td>
<td>19.7</td>
<td>22.0</td>
<td>42.1</td>
<td>8.5</td>
<td>8.1</td>
</tr>
<tr>
<td>C 17:0</td>
<td>N.D.</td>
<td>0.9</td>
<td>0.9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>4.8</td>
</tr>
<tr>
<td>C 18:0</td>
<td>7.2</td>
<td>11.7</td>
<td>0.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.5</td>
</tr>
<tr>
<td>Total Saturated Fatty acids</td>
<td>55.3</td>
<td>83.5</td>
<td>61.4</td>
<td>100</td>
<td>21.6</td>
<td>52.3</td>
</tr>
<tr>
<td>C 16:1</td>
<td>1.6</td>
<td>2.7</td>
<td>25.7</td>
<td>N.D.</td>
<td>12.1</td>
<td>10.7</td>
</tr>
<tr>
<td>C 18:1</td>
<td>6.9</td>
<td>0.9</td>
<td>2.7</td>
<td>N.D.</td>
<td>4.7</td>
<td>2.5</td>
</tr>
<tr>
<td>C 22:1</td>
<td>0.6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>C 17:1</td>
<td>2.3</td>
<td>11.7</td>
<td>1.1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Total Unsaturated Fatty Acids</td>
<td>11.4</td>
<td>15.3</td>
<td>29.5</td>
<td>N.D.</td>
<td>16.8</td>
<td>13.2</td>
</tr>
<tr>
<td>C 18:2 n6t</td>
<td>N.D.</td>
<td>1.1</td>
<td>5.9</td>
<td>N.D.</td>
<td>1.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>C 18:2 n6c</td>
<td>4.7</td>
<td>N.D.</td>
<td>0.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>C 18:3 n6</td>
<td>15.9</td>
<td>N.D.</td>
<td>2.5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>C 20:5 n3</td>
<td>12.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>59.5</td>
<td>34.4</td>
</tr>
<tr>
<td>Total Polyunsaturated Fatty Acids</td>
<td>33.8</td>
<td>1.1</td>
<td>9.2</td>
<td>N.D.</td>
<td>61.7</td>
<td>34.4</td>
</tr>
</tbody>
</table>

N.D. – Not detected
and Photobacterium) and the Bacteroidetes (e.g. Flexibacter and Psychroserpens). Therefore, EPA may be a potentially useful marker for these genera in environmental microbial communities (Freese et al., 2009). Our results suggest that a new genus of Halomonas may be added to earlier reported EPA producing prokaryotic genera belonging to Gammaproteobacteria.

Conclusions. 1. The occurrence of high levels of EPA in MBRI isolates (present work) with MBRI 12 being highest producer of EPA which could be commercially exploited. 2. This is probably the first report of Halomonas sp. producing EPA at 30°C in the presence of high salt-concentration.

Acknowledgements
This work is financially supported by the Department of Biotechnology, New Delhi, Government of India. We are thankful to Honorable Dr. Shivajirao Kadam, V.C., Bharati Vidyapeeth Deemed University and Dr. R.M. Kothari, Principal, Rajiv Gandhi Institute of IT and Biotechnology, (BVDU) for providing facilities to undertake this work. We are also thankful to Dr. D.P. Nerkar and Ms. V. V. Jadhav for their assistance.

Literature


Fig. 1. Comparison of % EPA produced by MBRI isolates (this work) with other mesophilic marine bacteria and microalgae. 1. MBRI 12; 2. MBRI 13; 3. Monodons subterraneus UTEX 151; 4. Chlorella minutissima UTEX 2341, 5. Phaeodactylum tricornutum UTEX 642 (Vazhappilly and Chen, 1998); 6. MBRI 6; 7. Shewanella pneumatophori (Hirota et al., 2005); 8. Shewanella waksmanii (Ivanova et al., 2003); 9. Shewanella pacifica (Ivanova et al., 2004); 10. Photobacterium sp. SAMA2, 11. Vibrio sp. NB73, 12. Shewanella sp. NB72 (Freese et al., 2009).
The genus *Yersinia* is a highly heterogeneous group of the family *Enterobacteriaceae* comprising acknowledged pathogens and a variety of strains that are widely distributed in nature in both aquatic and terrestrial ecosystems (Cover and Aber, 1989). Many species such as *Yersinia enterocolitica* are psychrophilic bacteria capable of persisting and growing in cold environments (Kato *et al.*, 1985; Gill and Reichel, 1989).

The wild-living birds, due to their great mobility, may play a significant role as effective spreaders of *Yersinia* through faecal contamination of pastures and surface waters (Kaneuchi *et al.*, 1989; Niskanen *et al.*, 2003). There are only a few studies on bacteria living in wild birds in the extreme environments of the subalpine and alpine vegetation levels. Janiga *et al.* (2007) identified the composition of microflora in the digestive tract of the alpine accentor (*Prunella collaris*) as a typical species of alpine environments. Novotný *et al.* (2007) specifically discussed the ecology of *Yersinia* spp. in relation to alpine accentors considering that the genus *Yersinia* may play an important role in the ecology of this bird species.

The objective of this study was to continue research of yersiniosis in lower sub-alpine habitats in the West Carpathians. The dunnock (*Prunella modularis*) is a dominate bird species of the dwarf-pine ecosystem (950–1700 m a.s.l) and as the alpine accentor is the member of the family *Prunellidae*, therefore it was chosen as experimental host species for this microbiological study.

From April 2007 to July 2009, 97 free-living dunnocks were captured with mist nets in the local mountains of the Slovakian part of the Western Carpathians – The West, High, Low and Belianske Tatras, Great and Small Fatra, Babia hora, and Choč (from 1000 to 1750 m a.s.l.). Captured birds were identified as adults or juveniles (age ≤ 2 months). Adult birds were sexed by the presence of a cloacal protuberance in males (Nakamura, 1990). They were weighed with 0.2 g accuracy using a Pesola spring scale and standard morphometric measurements were taken. Two types of samples were collected from each bird – pharyngeal and cloacal swabs. Samples were taken using sterile transport swabs suitable for both aerobes and anaerobes (DispoLab, Copan Italia, Brescia, Italy).

Isolated bacterial cultures from cloacal and pharyngeal swabs were enriched in Tryptone-soya broth at 26–28°C for 48 hours (Nikolova *et al.*, 2001). Enriched cultures were detected from cloacal and pharyngeal swabs from 97 specimens using PCR assay. *Yersinia enterocolitica* showed the highest prevalence (47.4%) from among the determined *Yersinia* species. *Yersinia* species (except *Y. frederiksenii*) were detected more frequently in pharyngeal than cloacal samples. The highest prevalence of yersiniosis was detected in April (*Yersinia* spp. – 80%, *Y. enterocolitica* – 70%). No statistically differences were observed in the prevalence of *Yersinia* spp. between males and females and between juveniles and adult birds. Bacterial contamination did not affect body weight or tarsus length.

**Key words**: *Yersinia* spp. *Yersinia enterocolitica*, Dunnock *Prunella modularis*
cultures were plated on CIN agar presented as highly selective medium for *Yersinia* spp. (Schiemann, 1979) and cultivated at 26–28°C for 48 hours (Neubauer et al., 2000; Hussein et al., 2001).

Bacterial DNA was extracted and then *Yersinia* species were identified among isolated bacterial cultures using the PCR method developed by Neubauer et al. (2000). The authors designed primers Y1 and Y2 for amplification of the specific region of the 16S rRNA gene of the genus *Yersinia*. Primers A1 and A2 were used to amplify a 430 bp fragment of the *ail* gene, found exclusively in pathogenic *Yersinia* spp. strains (Wannet et al., 2001). For the identification of *Yersinia* spp., a reference strain (CCM 5671) of *Y. enterocolitica* subsp. *enterocolitica*, serovar 0:3, biobar 4 was obtained from the Czech collection of microorganisms, Masaryk University, Brno. The PCR products were independently sequenced in both directions on the Genome Lab GeXP Single genetic analyzer (Beckman Coulter Inc.) The obtained sequences were subjected to BLAST searches in sequence database GenBank for species identification.

*Y. pseudotuberculosis* was also independently examined in all samples. The AmpliSens *Yersinia pseudotuberculosis* kit (Russia) was used for the identification.

A $\chi^2$ statistics (STATISTICA 8.0) was used to test the hypothesis of independence of frequencies of selected factors (prevalence, sex, age and season) which may influence the occurrence of bacteria. Morphometric data were compared by one-way ANOVA.

From 97 examined birds, a total of 47.4% individuals were *Yersinia* positive (28.9% of pharyngeal and 27.8% of cloacal samples). Seven *Yersinia* species were detected by comparison of PCR-product’s to the nucleotide sequences in BLAST (GenBank, Table I). *Y. enterocolitica* showed the highest total incidence (34.0%) of the genus *Yersinia*, therefore it is considered in this study as separate species. The higher prevalence of *Y. enterocolitica* tended to be in cloacal (20.6%) than in pharyngeal (16.5%) samples. Also other *Yersinia* species (except *Y. frederiksenii*) were detected more frequently in pharyngeal than cloacal swabs. The differences were not statistically significant.

A seasonal variation in the distribution of *Yersinia* spp. was observed. The highest prevalence of *Yersinia* species was detected in April (80%). Even, the contamination of *Y. enterocolitica* reached a statistically significant value compared to other months ($P \leq 0.05$), in which the prevalence of bacteria decreased significantly (Table II).

Comparison of the total prevalence of *Yersinia* spp. between males and females did not show significant differences but in females was nearly 10% higher than in males. A much lower frequency of infection in juveniles than in adult individuals was found but the difference didn’t reach the statistical significance value ($P \geq 0.05$, Table II).

Table III shows the potential relation between the occurrence of *Yersinia* spp., resp. *Y. enterocolitica* and morphological features of the adult birds. Measurements of body weight and tarsus length were

### Table I

<table>
<thead>
<tr>
<th>Bacteria species</th>
<th>Strain according GenBank</th>
<th>Prevalence (n = 97)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total N (%)</td>
</tr>
<tr>
<td><em>Yersinia</em> spp.</td>
<td></td>
<td>46 (47.4)</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>8081 FE 80735 KM 1 PO/Y/1-3 ARCTIC-P11</td>
<td>33 (34.0)</td>
</tr>
<tr>
<td><em>Y. kristensenii</em></td>
<td>ATCC 33638 N1SF35 Y 160</td>
<td>10 (10.3)</td>
</tr>
<tr>
<td><em>Y. molareti</em></td>
<td>H279-36/86</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td><em>Y. intermedia</em></td>
<td>H9-36/83 253</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td><em>Y. aleksici</em></td>
<td>991 Y 388</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td><em>Y. bercovieri</em></td>
<td>H632-36/85 WS 52/02</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td><em>Y. frederiksenii</em></td>
<td></td>
<td>2 (2.1)</td>
</tr>
</tbody>
</table>

n – number of examined birds
N – number of *Yersinia* positive samples with relative value in the brackets
considered. No significant relationships were found between the *Yersinia* spp., resp. *Y. enterocolitica* infection and tarsus length or body weight of hosts.

When the dunnock sex and infestation by yersiniosis were considered as independent variables, no statistically significant difference was found in prevalence of *Yersinia* between males and females (Table II). Therefore, both sexes were pooled together and tested against *Yersinia* as one group.

*Y. pseudotuberculosis* was not detected in any of pharyngeal and cloacal swabs. The presence of the *ail* gene associated with the pathogenic strains of *Yersinia* was not confirmed in any of the examined samples.

Recently, PCR method is widely used to identify specimens and studying the relationship of bacteria. However, the target sequences of both primers Y1 and Y2 using in this study are presented in more members of the genus *Yersinia*; it was possible to determinate more *Yersinia* species by using only one PCR array (Neubauer et al., 2000).

In this study, a lower occurrence of yersiniosis was found in subalpine habitats compared to the alpine zone in previous study (Novotný et al., 2007). The authors showed that in alpine accentors there is an unusually high prevalence of yersiniosis in comparison to many other bird species. The authors attribute this to high adaptability of *Yersinia* species to cold environment. For example, Gill and Reichel (1989) referred to the ability of *Y. enterocolitica* to grow at −2°C. This might be the reason for its successful

<table>
<thead>
<tr>
<th>Variable (n)</th>
<th><em>Yersinia</em> positive N (%)</th>
<th><em>Yersinia</em> negative N (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. enterocolitica</em> April (10)</td>
<td>7 (70)</td>
<td>3 (30)</td>
<td>0.03</td>
</tr>
<tr>
<td>May (32)</td>
<td>13 (40.6)</td>
<td>19 (59.4)</td>
<td></td>
</tr>
<tr>
<td>June (25)</td>
<td>6 (24)</td>
<td>19 (76)</td>
<td></td>
</tr>
<tr>
<td>July (30)</td>
<td>7 (23.3)</td>
<td>23 (76.7)</td>
<td></td>
</tr>
<tr>
<td><em>Yersinia</em> spp. April (10)</td>
<td>8 (80)</td>
<td>2 (20)</td>
<td>NS</td>
</tr>
<tr>
<td>May (32)</td>
<td>15 (46.9)</td>
<td>17 (53.1)</td>
<td></td>
</tr>
<tr>
<td>June (25)</td>
<td>10 (40)</td>
<td>15 (60)</td>
<td></td>
</tr>
<tr>
<td>July (30)</td>
<td>13 (43.3)</td>
<td>17 (56.7)</td>
<td></td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> Females (45)</td>
<td>17 (37.8)</td>
<td>28 (62.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Males (35)</td>
<td>13 (37.1)</td>
<td>22 (62.9)</td>
<td></td>
</tr>
<tr>
<td><em>Yersinia</em> spp. Females (45)</td>
<td>25 (55.6)</td>
<td>20 (44.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Males (35)</td>
<td>16 (45.7)</td>
<td>19 (54.3)</td>
<td></td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> Adults (80)</td>
<td>30 (37.5)</td>
<td>50 (62.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Juveniles (17)</td>
<td>3 (17.7)</td>
<td>14 (82.4)</td>
<td></td>
</tr>
<tr>
<td><em>Yersinia</em> spp. Adults (80)</td>
<td>41 (51.3)</td>
<td>5 (29.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Juveniles (17)</td>
<td>39 (48.8)</td>
<td>12 (70.6)</td>
<td></td>
</tr>
</tbody>
</table>

n – number of examined birds
N – number of *Yersinia* positive (+) or negative (−) samples with relative value in the brackets
NS – not significant P > 0.05 (Chi-square test)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Morphological variables</th>
<th>Yersinia + / −</th>
<th>Number of adult individuals</th>
<th>Mean ± SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>Body weight (g)</td>
<td>+</td>
<td>25</td>
<td>19.7 ± 0.8</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>46</td>
<td>19.1 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tarsus length (mm)</td>
<td>+</td>
<td>29</td>
<td>24.9 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>48</td>
<td>24.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Yersinia</em> spp.</td>
<td>Body weight (g)</td>
<td>+</td>
<td>34</td>
<td>19.6 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>37</td>
<td>19.1 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tarsus length (mm)</td>
<td>+</td>
<td>39</td>
<td>24.8 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>38</td>
<td>24.9 ± 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS – not significant, P > 0.05 (one – way ANOVA test)
expansion in high mountain environments and for its higher prevalence in alpine accentors than in dunnocks. In this study we have confirmed that Y. enterocolitica is the most common yersinia in dunnocks in comparison with other Yersinia species. Similar findings were reported in other species of birds (Niskanen et al., 2003; Novotný et al., 2007). Also other species of Yersinia (no pathogenic environmental species e.g. Y. intermedia, Y. frederiksenii or Y. kristensenii ect.) were frequently found in other species of birds (Niskanen et al., 2003; Janiga et al., 2007).

As our results show that distribution of yersiniosis in dunnock is seasonally dependent. Prevalence of avian bacterial infections is known to have seasonal dynamics where social and other behaviours have been hypothesized to be a factor shaping these dynamics (Faustino et al., 2004). The high occurrence of Y. enterocolitica in cold months was confirmed in many studies. For example, Kato et al. (1985) reported significantly higher incidence of Y. enterocolitica in birds in the spring than in the summer or autumn months. The highest prevalence of all Yersinia species in the dunnock was detected in April. In this month, the dunnock breeding season begins and birds begin to mate. Avian copulation involves cloacal contact, which has been documented to host rich bacterial communities (Lombardo, 1998; Lucas and Heeb, 2005). So, the risks of bacterial transmission during copulations in birds are expected to be high. Dunnocks have a variable mating system from monogamy through polyandry to polygynandry (Davies, 1985) and copulation with multiple partners further increases the risk of avian infection. It seems bird diet may also influence the occurrence of bacterial infection. The dunnock is an insectivorous species and in April, when the occurrence of insects increases in the mountains, the risk of infection is higher. In the next months (outside the mating period) the prevalence of bacterial infection declines gradually. In July, when the juveniles have left the nests, the bacterial infection may be slightly increased again. This phenomenon is probably associated with reduced body condition of birds. The birds are exhausted after breeding and subsequent moult and may also have a significant impact on the physiological status of the individuals.

Colonisation of nestlings by environmental microbes begins soon after hatching. Mills et al. (1999) detected cloacal microorganisms in nestlings of the tree swallow (Tachycineta bicolor) two days after hatching. The inoculation of nestlings by microbes may occur from the environment via ingestion of adult saliva, from food provided by the parents or from nest materials (Mills et al., 1999; Berger et al., 2003). In our study, we detected about 20% fewer contamination of Yersinia in juveniles than in adult birds. The low infection of juveniles during whole research period indicates that infection of juveniles by Yersinia occurs later. We therefore suggest that young birds are infected by bacteria mainly during the breeding season in the following year.

To conclude, we confirmed that the prevalence of bacterial infections in dunnocks shows seasonal dynamics. In early spring, the high occurrence of Yersinia contamination may be associated with changes in diet, but is most likely a consequence of the increased risk of contamination during mating. We observed that the sex of birds did not influence the distribution of Yersinia spp. in the dunnock and that bacterial occurrence in birds increases with nestling age and stabilizes in adults. We didn’t found relationship between morphological parameters (tarsus length and body weight) and Yersinia infestation, therefore we assume that bacteria do not affect health and body condition of individuals. Our findings support the idea that the Yersinia species are surely a common member of bacterial microflora in dunnocks.

**Literature**


First Report of *Serratia plymuthica* Causing Onion Bulb Rot in Poland

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Institute of Horticulture, Skierniewice, Poland

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Abstract

Specific bacterial disease symptoms were observed on onion bulbs in almost all regions in Poland. For the purpose of identification of agents causing disease, bacteria were isolated from the symptomatic plants. Their pathogenicity was confirmed by using pathogenicity test on onion scales. These bacteria were identified biochemically and molecularly as *Serratia plymuthica*.

Key words: *Serratia plymuthica*, bacterial disease, onion

Onion (*Allium cepa* L.) is one of the major vegetable crops grown in Poland. Since the onion harvesting period often coincides with rainy weather and during cultivation it may hail, complex bacterial and fungal diseases often occur. In the last years especially bacterial diseases of onion cause very serious problems in Poland. These diseases may cause significant economic losses because they are difficult to control. Bacterial soft rot of onion bulbs is the most frequent and it can appear during cultivation, storage or transportation. It is common knowledge that in Poland, bacterial diseases of onion are caused by *Burkholderia gladioli*, *Burkholderia cepacia*, *Pectobacterium carotovorum* subsp. *carotovorum* (Sobiczewski and Schollenberger, 2002). Foreign reports conclude that bacteriosis of onion can also be caused by *Pseudomonas marginalis* (Kim et al., 2002; El-Hendawy, 2004), *Pseudomonas syringae*, *Pseudomonas viridiflava* (Gitaïtis et al., 1998), *Pantoea ananatis* (Gitaïtis et al., 2002), *Enterobacter cloacae* (Schroeder et al., 2009; Schwartz and Mohan, 2008), *Burkholderia ambifaria* and *Burkholderia pyrocinia* (Jacobs et al., 2008). Also bacterium *Serratia* spp. was noted as an onion pathogen in Brasil (Beriam, 2007). The liberalization of policies concerning border protection and intense trade favor transmission of pathogens from foreign countries.

During the summer and autumn of 2003, 2006 and 2007, disease symptoms of unknown origin were observed on onion (*Allium cepa* L.) bulbs in the field and storage houses in different places in Poland. These symptoms were typical for bacterial disease – water soaked and pale brown lesions appeared on the internal scales, they enlarged and extended to external scales with an associated sour smell. Bacteria from the infected bulb tissues were isolated and purified on nutrient agar. About forty isolates were obtained and these isolates were examined for the ability to macerate onion tissue. The pathogenicity test was conducted on healthy onion bulbs cv. Grabowska. The bulbs were peeled, washed with running water and sterilized in 70% ethanol for 30 sec and in 0.5% NaOCl for 5 min. The bulbs were washed in sterile water and cut lengthwise into two parts. Three onion pieces were placed, cut side down, into a Petri dish (180 mm in diameter). The outer scale of each piece was wounded with the microbiological needle and inoculated by 20-µl aliquot of bacterial suspension of density 1.0–2.5×10⁸ cfu/ml. For each isolate three Petri dishes were included. Control treatment remained uninoculated. The Petri dishes were incubated 4 days at 28°C. Nine of forty isolates expressed rot symptoms on the scales. These isolates were studied by using biochemical and molecular methods.

Biochemical identification of the isolates were performed by using the API 20E system (bioMerieux) which gave a bacterial code of 1207763 (Table I)
Table I
Biochemical profile of tested isolates conducted using identification system API 20E

| ONPG | ADH | LDC | ODC | CIT | H+S | URE | TDA | INO | VP | GEL | GLU | MAN | INO | SOR | RHA | SAC | MEL | AMY | ARA | OX |
|------|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|----|----|----|----|----|----|
| +    | –   | –   | +   | +   | –   | –   | +   | +   | +  | –   | –   | –   | +   | +   | –   | –  | +  | +  | +  | –  |

Test reactions / enzymes
- ONPG: β-galactosidase
- ADH: arginine dihydrolase
- LDC: lysine decarboxylase
- ODC: ornithine decarboxylase
- CIT: citrate utilization
- H+S: H+S production
- URE: urease
- TDA: tryptophane deaminase
- IND: indole production
- VP: acetoin production
- GEL: gelatinase
- GLU: fermentation / oxidation (glucose)
- MAN: fermentation / oxidation (mannitol)
- INO: fermentation / oxidation (inositol)
- SOR: fermentation / oxidation (sorbitol)
- RHA: fermentation / oxidation (rhamnose)
- SAC: fermentation / oxidation (saccharose)
- MEL: fermentation / oxidation (melibiose)
- AMY: fermentation / oxidation (amygdalin)
- ARA: fermentation / oxidation (arabinose)
- OX: cytochrome oxidase

(% identity = 59.6; T = 1.0). The numerical profile showed a correct identification of all examined strains as *Serratia plymuthica*. Additionally, some biochemical tests were conducted. The microorganisms were Gram-negative, catalase positive, oxidase negative and grew in anaerobic condition.
The identity of the isolates was confirmed by molecular techniques – 16S rRNA sequence analysis. DNA was isolated by “Genomic mini” kit according to producer’s clues (A & A Biotechnology). The 16S rDNAs were amplified by using universal primers fD1 (5’AGAGTTGTGATCMTGGCTC3’) and rP2 (5’ACGGCTACCTTGTTACGACTT3’) (Weisburg et al., 1991). Additional sequencing primers were used: 800f (5’ATTAGATACCCTGGTAG3’) and 800r (5’CTACCAGGGTATCTAAT3’) (Fouad et al., 2002).

The amplified PCR products, length 1500 bp, were separated by 1.5% agarose gel electrophoresis, then extracted and purified from gel with the DNA Fragment Purification Kit (A & A Biotechnology). DNA sequences were compared to NCBI database (www.ncbi.nlm.nih.gov) using BLAST program (Basic Local Alignment Search Tool), demonstrated that 16S rRNA gene of the studied isolates of bacteria shared high identities (99.7%) with *Serratia plymuthica* RVH1, NCBI GenBank database accession no. AY394724.1 (Fig. 1, Table II). The 16S rRNA sequence of the one representative isolate (466) has been deposited in the GenBank database under the accession number HM596429.1.

The biochemical test data along with sequence analysis of a portion of the 16S rRNA gene confirmed all the isolates to be *Serratia plymuthica*. According to our knowledge, this is the first report of *S. plymuthica* causing a bulb rot of onion in Poland.

**Table II**

<table>
<thead>
<tr>
<th>S. plymuthica isolate</th>
<th>Accession no</th>
<th>Country</th>
<th>Identity</th>
<th>Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM 4540 – type strain</td>
<td>AJ233433.1</td>
<td>Germany</td>
<td>98.9%</td>
<td>1449/1465</td>
</tr>
<tr>
<td>466 – tested isolate</td>
<td>HM596429.1</td>
<td>Poland</td>
<td>100%</td>
<td>1460/1460</td>
</tr>
<tr>
<td>RVH1</td>
<td>AY394724.1</td>
<td>Belgium</td>
<td>99.7%</td>
<td>1456/1461</td>
</tr>
</tbody>
</table>

**Literature**


**Acknowledgments**

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Instruction to Authors
(modified, effective Vol. 60, 2011)

Scope

*Polish Journal of Microbiology* publishes descriptions of all aspects of basic and applied research that focuses on topics of basic research and practical value in microbiology. Topics that are considered include microbiology in the area of basic biological properties of bacteria and archaea, viruses, and simple eukaryotic microorganisms, genetics and molecular biology, microbial ecology, medical bacteriology, public health as well as food microbiology, industrial microbiology and bacterial biotechnology.

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The title should describe the contents of the paper in a concise but attractive for readers way. Below the title of the manuscript include: full name (including first name and middle initial) of each author, name of the institution(s) at which the work was performed, or each co-author’s affiliation if different.

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Limit the abstract to 250 words or fewer. Because the abstract will be published separately by abstracting services, it must be complete and understandable without reference to the text. It should be written in an impersonal form. Abbreviations, diagrams and references are not allowed.

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Acknowledgements

Acknowledgements for financial support and for a personal assistance (with the permission of person named) are given in two separate paragraphs below the main text.

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